In Vitro Chloroquine Resistance Modulation Study on Fresh Isolates of Brazilian *Plasmodium falciparum*: Intrinsic Antimalarial Activity of Phenothiazine Drugs

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Phenothiazine drugs – fluphenazine, chlorpromazine, methotrimeprazine and trifluoperazine – were evaluated as modulating agents against Brazilian chloroquine-resistant fresh isolates of Plasmodium falciparum. *Aiming to simulate therapeutic schedules, chloroquine was employed at the concentration used for sensitive* falciparum *malaria treatment and anti-psychotic therapeutic concentrations of the phenothiazine drugs were adopted in two-fold serial dilutions. The in vitro microtechnique for drug susceptibility was employed. Unlike earlier reported data, the phenothiazine modulating effect was not observed. However, all the drugs demonstrated intrinsic antiplasmodial activity in concentrations lower than those described in the literature. In addition, IC_{50} estimates have been shown to be inferior to the usual anti-psychotic therapeutic concentrations. Statistical analysis also suggested an increase in the parasitaemia rate or, even, a predominant antiparasitic effect of phenothiazine over chloroquine when used in combination.*

Key words: antimalarial - chloroquine - multidrug resistance - phenothiazines - modulating agents - chemosensitizer agents

The global situation of malaria is claiming attention more than ever. It has been estimated that about 300 million acute clinical cases are reported each year, with at least one million deaths (WHO 2000). Antimalarial drug resistance is among the principal factors responsible for this serious public health problem. Resistance has been associated with the parasite's natural biological mechanisms in response to uncontrolled and unregulated drug distribution programs, resulting in genetic mutations (Wellems & Plowe 2001).

Many efforts have been made in the search for a new and effective antimalarial agent. However, little success has been achieved and, therefore, the restoration of current available drugs becomes an important alternative. Combination therapy is particularly studied concerning chloroquine (Fig. 1), although the major *Plasmodium falciparum* strains and the emergent *P. vivax* are resistant, this drug remains the most important antimalarial agent. Good pharmacokinectic features, easy use and low cost are its principal properties (Ward & Bray 2001).

With the purpose of restoring chloroquine efficacy and considering the reported reversal effect of tricyclic drugs on resistant malaria (Bitonti et al. 1988, Basco & Le Bras 1990, 1991, Kyle et al. 1990, 1993, Peters et al. 1990, Basco & Le Bras 1992, Miki et al. 1992, Oduola et al. 1998),

we evaluated the potential effect of the phenothiazine drugs – fluphenazine, chlorpromazine, methotrimeprazine and trifluoperazine (Fig. 1) –, on modulating the chloroquine resistance of Brazilian *P. falciparum* fresh isolates.

MATERIALS AND METHODS

The drugs used were chloroquine diphosphate (Fundação para o Remédio Popular), chlorpromazine hydrochloride, fluphenazine dihydrochloride and methotrimeprazine maleate (Cristália Produtos Químicos e Farmacêuticos Ltda.), and trifluoperazine dihydrochloride (SmithKline Beecham).

Two fresh isolates of *P. falciparum* were used: Isolate 1 (Sucen 198/94) and Isolate 2 (Sucen 206/94). Isolate 1 was collected from a 37-year-old woman in her third infection (6,600 asexual parasites per mm^3) and Isolate 2 from a 21 year-old-man in his second infection (7,500 asexual parasites per mm^3). The infections occurred in the North of Brazil and the individuals had not been submitted to antimalarial treatment in the previous 28 days (Bruce-Chwatt 1986). Blood samples were collected after formal consent from the patients.

Assays were performed in microplates with 96 flat-bottomed wells. The culture medium was RPMI-1640, HEPES buffer, gentamicine sulphate, glucose, hypoxanthine, sodium bicarbonate and human type A serum.

The statistical analysis was carried out using the software S-Plus, version 4.5, and the Microsoft Excel for Windows, version 5.0.

In vitro assays - The biological assays were carried out based on the in vitro microtechnique for drug susceptibility (Rieckmann et al. 1978). The microplates were titrated with two-fold serial dilutions of fluphenazine (0.06-4 µg/l), chlorpromazine (3.75-240 µg/l), methotrimeprazine

Fluphenazine

Chlorpromazine

Methotrimeprazine

Trifluoperazine

Chloroquine
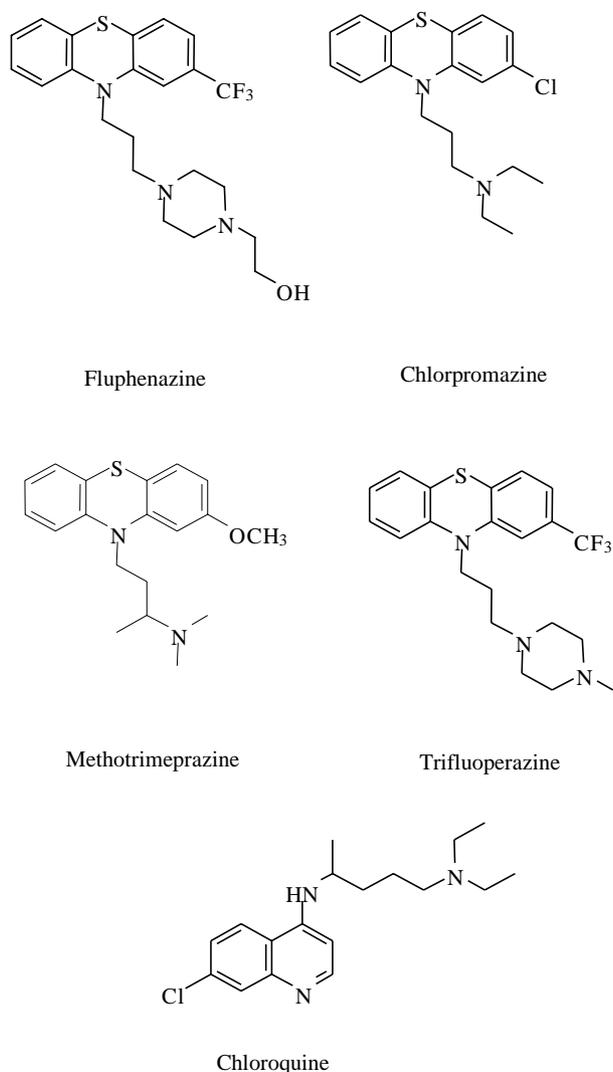
Fig. 1: chemical structures of the phenothiazines and chloroquine.

(5-320 µg/l), and trifluoperazine (0.31-20 µg/l). In each series, the intermediate value corresponded to the usual antipsychotic therapeutic concentration (Clarke 1986, Benet et al. 1996). Chloroquine (30 µg/l) was added along the series (Tracy & Webster 1996). The phenothiazine series were also assayed alone with the purpose of evaluating the intrinsic antiplasmodial effect. The chloroquine susceptibility was tested in a range from 3.75 to 240 µg/l.

A 10% haematocrit solution of infected blood was added to the plates. These were incubated according to the candle jar method (Trager & Jensen 1976) at 37°C for 40 h (Isolate 1) and for 46 h (Isolate 2). Schizonts with three or more nuclei in 200 parasites were counted.

Statistical analysis - Lines for the parasitaemia rate – number of parasites in each concentration/number of parasites in the control – as a function of the chloroquine and phenothiazine drug concentrations and the respective combinations with chloroquine were constructed and submitted to descriptive and inferential analyses (Breslow &

Day 1980, Collett 1991). Linear and quadratic logistic models and the log-log complement model were considered. According to these, coincident and separated, parallel and concurrent (one or two intercepts) lines were fitted. The 10% significance level was adopted for the likelihood ratio statistic which, in this case, corresponds to the difference between two goodness-of-fit statistics. In addition, the 50% inhibitory concentration, IC_{50}, of each drug and its combination with chloroquine was estimated.

RESULTS

The susceptibility of the isolates to chloroquine, phenothiazine drugs and their combinations can be seen in the descriptive analysis lines (Figs 2 to 6 show the lines calculated for Isolate 1). A decrease in the parasitaemia rate can be observed along the drug concentrations, markedly for phenothiazines.

The inferential analysis suggested the concurrent lines (two intercepts) as the best fitted model for fluphenazine, chlorpromazine (at the 5% significance level), and methotrimeprazine for Isolate 1. The coincident lines were fitted to trifluoperazine in the same isolate (Table I). For Isolate 2 the coincident lines model was the best fit for all the phenothiazines, with the exception of methotrimeprazine. The concurrent lines model (two intercepts) was the best fit for this drug (Table II).

The IC_{50} estimates are presented in the natural logarithm form in Table III.

Fig. 2: ratios of the number of *Plasmodium falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to chloroquine (A: Isolate 1; B: Isolate 2).
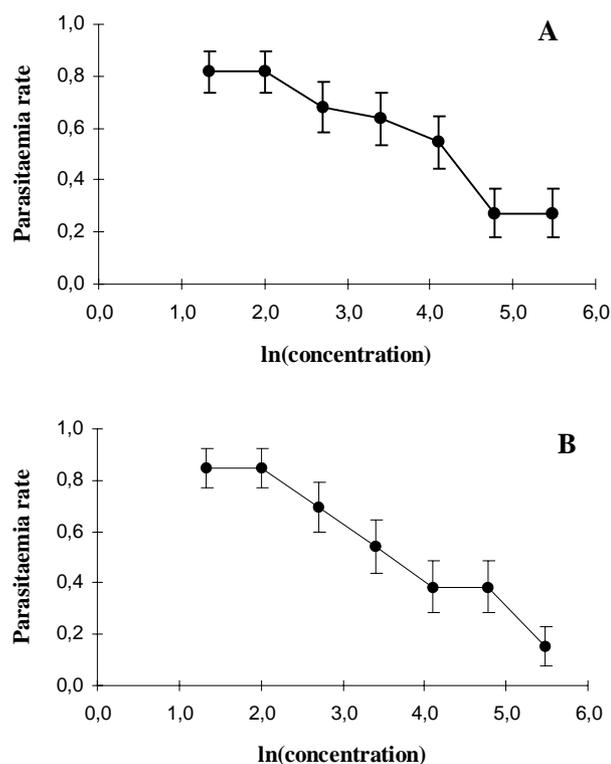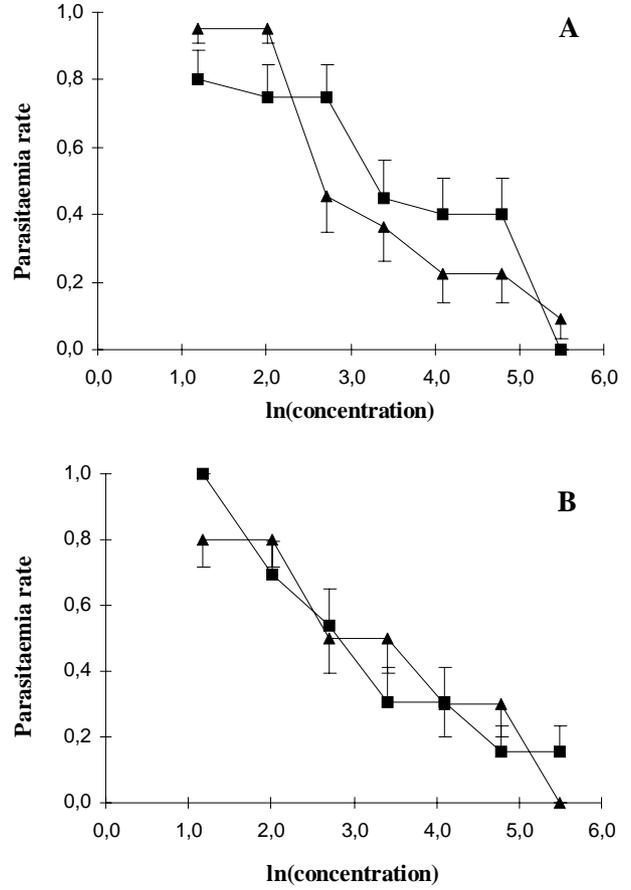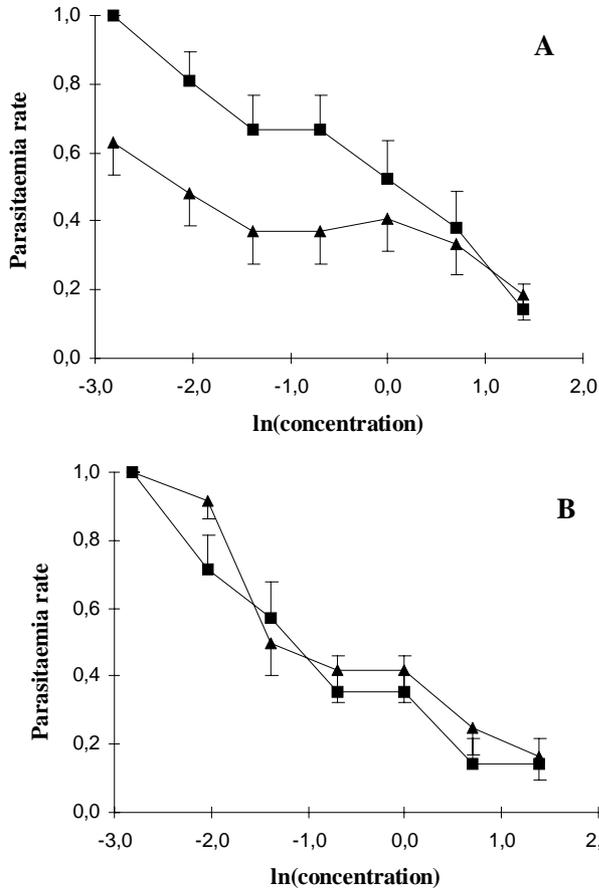
Fig. 3: ratios of the number of *Plasmodium falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to fluphenazine (▲) and fluphenazine plus chloroquine (■) (A: Isolate 1; B: Isolate 2).

Fig. 4: ratios of the number of *Plasmodium falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to chlorpromazine (▲) and chlorpromazine plus chloroquine (■) (A: Isolate 1; B: Isolate 2).

TABLE I

Fitted linear logistic model for the phenothiazine drugs and their combinations with chloroquine (Isolate 1, Sucen 198/94)

Drug	Fitted model	Goodness-of-fit statistic	Degrees of freedom	P-value	Likelihood ratio statistic	Degrees of freedom	P-value
Fluphenazine	Coincident lines	33.182	12	0.001			
	Parallel lines	17.612	11	0.091	15.570	1	< 0.001
	Concurrent lines (1 intercept)	12.932	11	0.298	20.250	1	< 0.001
	Concurrent lines (2 intercepts)	8.036	10	0.625	25.145	2	0.001
Chlorpromazine	Coincident lines	24.346	11	0.011			
	Parallel lines	23.679	10	0.008	0.667	1	0.414
	Concurrent lines (1 intercept)	22.742	9	0.007	1.604	2	0.448
	Concurrent lines (2 intercepts)	14.768	8	0.064	9.579	3	0.023
Methotrimeprazine	Coincident lines	17.798	12	0.122			
	Parallel lines	7.597	11	0.748	10.201	1	0.001
	Concurrent lines (1 intercept)	12.117	11	0.355	5.681	1	0.017
	Concurrent lines (2 intercepts)	4.290	10	0.933	13.508	2	0.001
Trifluoperazine	Coincident lines	15.846	11	0.147			
	Parallel lines	15.787	10	0.106	0.059	1	0.808
	Concurrent lines (1 intercept)	13.138	9	0.156	2.708	2	0.258
	Concurrent lines (2 intercepts)	12.189	8	0.143	3.657	3	0.301

Fig. 5: ratios of the number of *Plasmodium falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to methotrimeprazine (▲) and metho-trimeprazine plus chloroquine (■) (A: Isolate 1; B: Isolate 2).
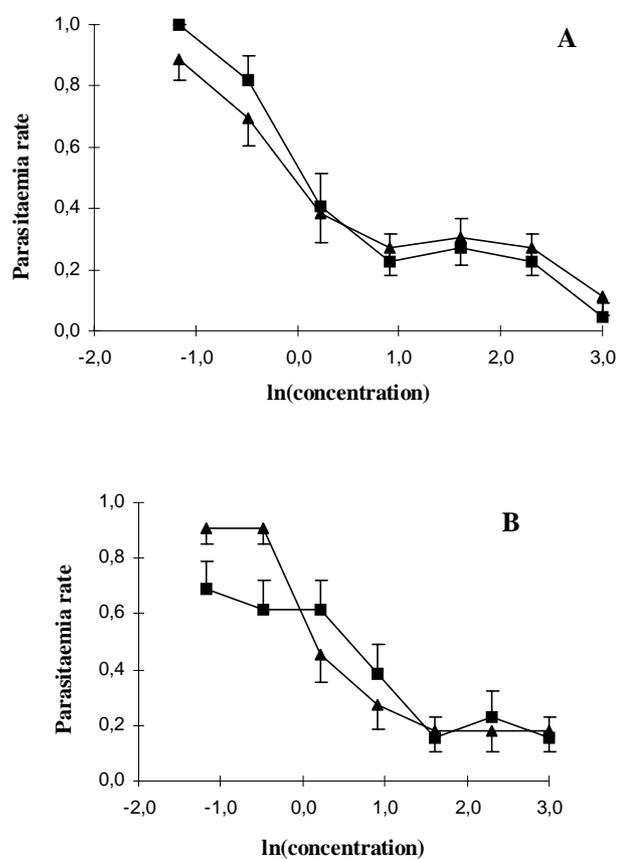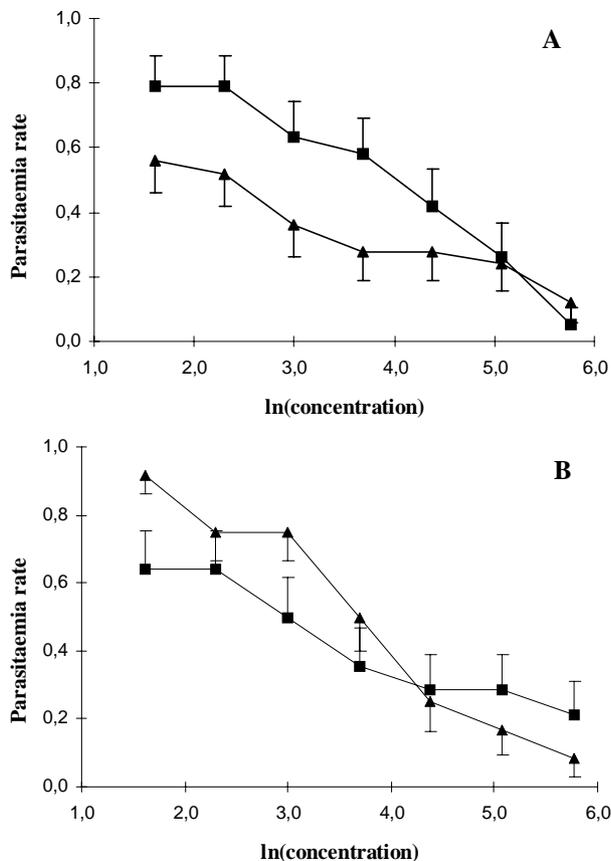
Fig. 6: ratios of the number of *Plasmodium falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to trifluoperazine (▲) and trifluoperazine plus chloroquine (■) (A: Isolate 1; B: Isolate 2).

TABLE II

Fitted linear logistic model for the phenothiazine drugs and their combinations with chloroquine (Isolate 2, Sucen 206/94)

Drug	Fitted model	Goodness-of-fit statistic	Degrees of freedom	P-value	Likelihood ratio statistic	Degrees of freedom	P-value
Fluphenazine	Coincident lines	12.491	12	0.407			
	Parallel lines	11.705	11	0.386	0.786	1	0.375
	Concurrent lines (1intercept)	12.311	11	0.341	0.180	1	0.671
	Concurrent lines (2 intercepts)	11.703	10	0.305	0.788	2	0.674
Chlorpromazine	Coincident lines	9.617	12	0.650			
	Parallel lines	9.607	11	0.566	0.010	1	0.920
	Concurrent lines (1 intercept)	9.568	11	0.570	0.049	1	0.825
	Concurrent lines (2 intercepts)	9.474	10	0.488	0.143	2	0.931
Methotrimeprazine	Coincident lines	7.723	12	0.806			
	Parallel lines	6.609	11	0.830	1.114	1	0.291
	Concurrent lines (1 intercept)	7.657	11	0.744	0.066	1	0.797
	Concurrent lines (2 intercepts)	1.471	10	0.999	6.252	2	0.044
Trifluoperazine	Coincident lines	9.446	12	0.664			
	Parallel lines	9.173	11	0.606	0.273	1	0.601
	Concurrent lines (1 intercept)	9.406	11	0.584	0.040	1	0.841
	Concurrent lines (2 intercepts)	7.735	10	0.655	1.711	2	0.425

TABLE III

Estimated median inhibitory concentrations (IC_{50}) of chloroquine, the phenothiazine drugs and the respective combinations on
Plasmodium falciparum in vitro

Drug/Combination	Isolate 1 (Sucen 198/94)		Isolate 2 (Sucen 206/94)	
	IC_{50} [a] (μg/l)	Confidence interval (95%)	IC_{50} [a] (μg/l)	Confidence interval (95%)
Chloroquine	3.96	3.42; 4.31	3.72	3.13; 4.31
Fluphenazine	- 1.94	- 3.03; - 0.85	- 0.72 [b]	- 1.06; - 0.38
Chloroquine + Fluphenazine	- 0.10	- 0.52; 0.32		
Chlorpromazine	2.96	1.33; 4.59	3.11 [b]	2.72; 3.50
Chloroquine + Chlorpromazine	3.75	0.68; 6.81		
Methotrimeprazine	2.14	1.19; 3.08	3.62	1.30; 5.94
Chloroquine + Methotrimeprazine	3.71	3.25; 4.17	2.95	0.64; 5.27
Trifluoperazine	0.19 [b]	- 0.062; 0.44	0.42 [b]	- 0.009; 0.85
Chloroquine + Trifluoperazine				

a: $\log_n$ 50% inhibitory concentration; *b*: same values (coincident lines model)

DISCUSSION

Many hypotheses have been advanced to explain the *P. falciparum* resistance to chloroquine. The reversal of chloroquine resistance by verapamil suggested a similar mammalian tumor cells multidrug-resistant phenotype in malaria. Furthermore, many different drugs and compounds have been assayed for their modulating effect in restoring antimalarial drugs effectiveness (Ward & Bray 2001).

In our study, a series of drugs reported as modulating agents in resistant malaria and/or neoplastic multidrug-resistant cell lines were evaluated in Brazilian chloroquine-resistant fresh isolates of *P. falciparum*. The in vitro microtechnique (Rieckmann et al. 1978) was chosen as it matches closely with scintillometric measurements (Le Bras et al. 1984), and it presents great feasibility in field studies (Yang et al. 1997, Philipps et al. 1998, Arez et al. 1999, Warsame et al. 1999). The chloroquine IC_{50} estimates indicated that the isolates were resistant, according to WHO statements (Bruce-Chwatt 1986). Moreover, these concentrations are among those reported in previous studies on chloroquine *P. falciparum* resistance reversal (Krogstad et al. 1987, Martin et al. 1987, Bitonti & Mc Cann 1989, Basco & Le Bras 1990, Kyle et al. 1990).

With the purpose of simulating therapeutic schedules, a particular drug combination was adopted. The intermediate concentration in the two-fold serial dilutions corresponded to the usual therapeutic concentration of the assessed modulating agent. A fixed concentration of chloroquine (30 μg/l) was employed in the combinations. This concentration is capable of clearance of parasitaemia in sensitive *P. falciparum* infections (Tracy & Webster 1996). Once the modulating effect was observed, chloroquine would return its effectiveness. A fixed concentration of chloroquine was also used to determine the antimalarial response modification index throughout the combination of modulating agents (Kyle et al. 1990, Oduola et al. 1998).

This study reports the evaluation of the phenothiazine drugs: fluphenazine, chlorpromazine, metho-

trimeprazine, and trifluoperazine. Other tricyclic compounds, e.g., desipramine, cyproheptadine, and their analogues (Bitonti et al. 1988, Basco & Le Bras 1990, Peters et al. 1990, Basco et al. 1991) were early reported as modulating agents on resistant malaria. Previous studies with chlorpromazine, trifluoperazine and promethazine have also demonstrated the in vitro and in vivo reversal of chloroquine resistance (Kyle et al. 1990, 1993, Basco & Le Bras 1992, Miki et al. 1992, Oduola et al. 1998).

However, different results were observed in our study. The phenothiazines drugs did not modulate chloroquine resistance. Similar behavior was observed for verapamil (Menezes et al. unpublished data), imipramine (Menezes et al. 1997) and most of the other modulating agents assessed. These results corroborated recent observations when Brazilian chloroquine-resistant strains demonstrated a lesser degree of susceptibility to verapamil when compared to strains from Africa and Southeast Asia (Mehlotra et al. 2001).

It is also important to note that, in our study, all the phenothiazine drugs demonstrated intrinsic antiplasmodial effects at concentrations inferior to those described as sub-inhibitory in modulating the antimalarial resistance (as example, 625 nM, that corresponds to 199.30 μg/l, for chlorpromazine) (Kyle et al. 1990, Basco & Le Bras 1992). However, in spite of the common phenothiazine chemical structure (Fig. 1), distinct behaviors were observed in the combinations with chloroquine. For Isolate 1, the concurrent lines model (two intercepts) was the best fit for methotrimeprazine, fluphenazine and chlorpromazine while the coincident lines model was the best model for trifluoperazine. The latter model was also the best fit for all drugs except methotrimeprazine, in the case of Isolate 2. The distinct fitted statistical lines models may be related to peculiarities of the isolates. To date, these results are most interesting.

Inhibition of falcipain and the interaction with heme moiety (ferriprotoporfyrin IX) have been associated to the antiplasmodial activity of phenothiazine compounds (Panijpan & Kantakanit 1983, Domínguez et al. 1997). Dur-

ing the intraerythrocytic stage, inside the food vacuole, specific parasite proteases, such as falcipain, degrade the host hemoglobin in order to generate amino acids for protein synthesis. The resulting heme is detoxified by a polymerization process to form the malarial pigment, hemozoin. Thus, inhibition of falcipain would precede the interference in the heme polymerization process, the most accepted hypothesis regarding the mechanism of chloroquine action (Padmanaban & Rangarajan 2000).

Our results should be contemplated taking into account the phases in which chloroquine and phenothiazine compounds are believed to interfere with the *Plasmodium* hemoglobin metabolism. Inhibition of falcipain and/or a stronger interaction of the phenothiazine drugs with heme moiety compared to that of chloroquine could be responsible for the predominant antiplasmodial effects of these compounds over chloroquine. This hypothesis may be supported by the coincident lines model. Conversely, the competition for the heme moiety could explain the concurrent lines model. In this situation, the addition of chloroquine caused an increase in the parasitaemia rate for most phenothiazine concentrations.

Although further assays are needed to check the proposed mechanistic hypothesis, we believe our findings are important to demonstrate the antiplasmodial effect of phenothiazine drugs at concentrations lower than those employed in anti-psychotic therapy, as observed by the estimated IC_{50} values. In addition, those concentrations showed to be lower than those previously related to antimalarial activity. Falcipain inhibition as well as the interaction with heme moiety (Panijpan & Kantakanit 1983, Domínguez et al. 1997) and other antiplasmodial activities of phenothiazines, such as calmodulin antagonism (Scheibel et al. 1987), membrane stabilization (Kristiansen & Jepsen 1985), disturbance in mitochondrial function (Geary et al. 1986) and the inhibition of glutathione reductase (Luond et al. 1998) were all observed in higher and, in general, toxic concentrations.

In resume, the results here presented corroborate previous findings in considering phenothiazine derivatives as interesting lead compounds in the design of new antimalarial agents (Vennerstrom et al.1995, Atamna et al. 1996, Domínguez et al. 1997).

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