

***In vitro* evaluation of verapamil and other modulating agents in Brazilian chloroquine-resistant *Plasmodium falciparum* isolates**

Avaliação *in vitro* do verapamil e de outros agentes moduladores em isolados de *Plasmodium falciparum* resistentes à cloroquina

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Abstract Verapamil, was assayed to record its modulating effect upon Brazilian *Plasmodium falciparum* isolates resistant to chloroquine. Other cardiovascular drugs known to be modulating agents in resistant malaria and/or multidrug-resistant neoplasias, including nifedipine, nitrendipine, diltiazem and propranolol, were also evaluated. Concentrations similar to those for cardiovascular therapy were used in the *in vitro* microtechnique for antimalarial drug susceptibility. Intrinsic antiplasmodial activity was observed from the lowest concentrations without a significant modulating action. Other reported modulating agents, such as the antipsychotic drug trifluoperazine and the antidepressants desipramine and imipramine, demonstrated similar responses under the same experimental conditions. Results suggest a much higher susceptibility of Brazilian strains, as well as an indifferent behaviour in relation to modulating agents.

Key-words: Chloroquine. Multidrug-resistance. Verapamil. Modulating agents. Reversal agents.

Resumo Verapamil foi ensaiado quanto ao efeito modulador em isolados brasileiros de *Plasmodium falciparum* resistentes à cloroquina. Outros agentes cardiovasculares, considerados como moduladores da resistência em malária e/ou em neoplasias multiresistentes a fármacos, como nifedipino, nitrendipino, diltiazem e propranolol foram ensaiados quanto ao mesmo efeito. Concentrações semelhantes às da terapia cardiovascular foram empregadas no ensaio de microtécnica de sensibilidade para fármacos antimaláricos. Atividade antiplasmodica intrínseca foi observada desde as menores concentrações, sem, entretanto, ocorrência de modulação significativa da resistência. Sob as mesmas condições experimentais, respostas semelhantes foram observadas para outros agentes moduladores conhecidos como o antipsicótico trifluoperazina e os antidepressivos desipramina e imipramina. Em conjunto, estes resultados sugerem alta sensibilidade e comportamento indiferente de cepas brasileiras ao efeito de agentes moduladores da resistência.

Palavras-chaves: Cloroquina. Resistência múltipla a fármacos. Verapamil. Agentes moduladores da resistência. Agentes reversores da resistência.

Malaria is a parasitic disease that has affected man since primeval times. Nowadays, it is considered to be the most pernicious infectious disease transmissible by mosquitoes. Millions of people are infected in tropical and sub-tropical areas and over one million deaths per year are estimated to occur. Insecticide and drug resistance are major problems for the control of the disease, particularly the resistance of most *Plasmodium falciparum* strains to chloroquine¹¹.

In 1987, Martin *et al*¹⁵ suggested that multidrug resistance (MDR) may be a possible mechanism to explain the resistance of *P. falciparum* to chloroquine, since the combination of verapamil with chloroquine caused reversal of *in vitro* antimalarial resistance.

With the object of evaluating the *in vitro* modulation of chloroquine resistance in fresh Brazilian isolates of *P. falciparum*, a series of compounds were assayed reportedly to be modulating agents in resistant malaria

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and/or multidrug-resistant neoplasias. This paper reports the results observed for the cardiovascular drugs verapamil (*R,S*-verapamil), nifedipine, nitrendipine, diltiazem and propranolol. In addition, the results observed for other modulating agents, such as the phenothiazine antipsychotic drug trifluoperazine¹⁹

and the tricyclic antidepressants desipramine and imipramine¹⁷, were also recorded to characterize the behavior of Brazilian strains. The chemical structures of all studied drugs, including the antidepressant agents imipramine and desipramine and the antipsychotic trifluoperazine are depicted in Figure 1.

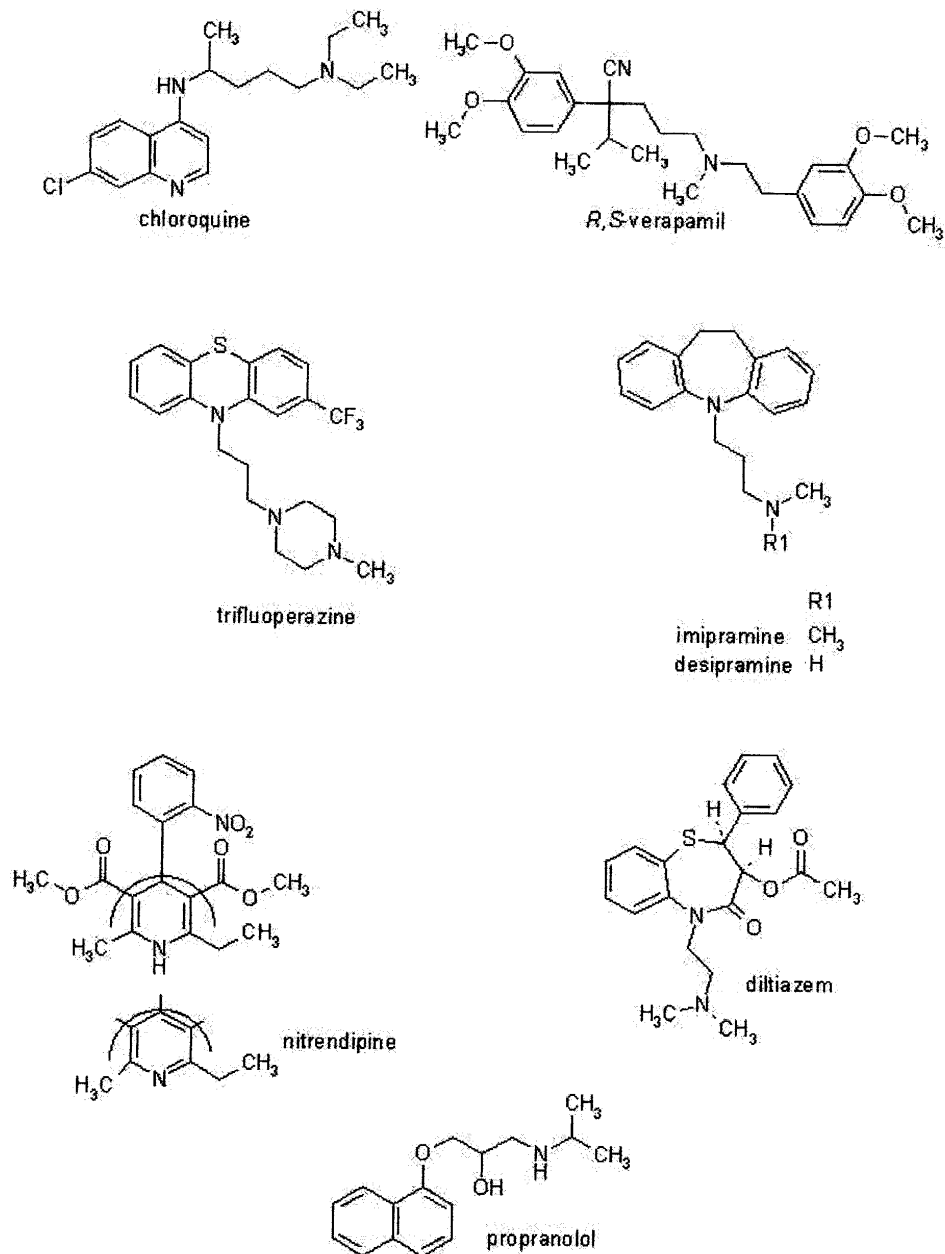


Figure 1 - Chemical structures of chloroquine, and of cardiovascular, antidepressant and antipsychotic drugs studied as modulating agents in Brazilian chloroquine-resistant *P. falciparum* isolates.

MATERIAL AND METHODS

Drugs employed were *R,S*-verapamil hydrochloride (Knoll S/A Produtos Químicos e Farmacêuticos, Brazil), nifedipine and nitrendipine (Laboratórios Biosintética Ltda, Brazil), diltiazem hydrochloride (Boehringer De Angeli Química e Farmacêutica Ltda, Brazil) and propranolol hydrochloride. The two chloroquine-resistant strains were obtained from patients who had been infected in the northern region of Brazil. Isolate 1 (SUCEN 198/94) was collected from a 37-year-old woman in her third infection (6,600 asexual parasites per mm³). Isolate 2 (SUCEN 206/94) was from a 21 year-old-man in his second infection (7,500 asexual parasites per mm³). The patients had not received any antimalarial treatment during the previous 28 days⁶. Blood samples were collected after formal consent from the patients. The biological assay was performed on microplates with 96 flat-bottomed wells and the culture medium used was RPMI-1640 supplemented with HEPES buffer, gentamicin sulfate, glucose, hypoxanthine, sodium bicarbonate, and human type A serum. The S-Plus software, version 4.5, and Microsoft Excel for Windows, version 5.0, were used for statistical analysis.

Biological assay. The modulating effect was evaluated by the *in vitro* microtechnique for antimalarial drug susceptibility²⁴. Microplates were titrated with two-fold serial dilutions so that the intermediate value

corresponded to the current cardiovascular therapeutic concentration^{3 7}. Verapamil has intermediate concentrations of 125µg/L. Similarly, nifedipine and nitrendipine were assayed at 40µg/L, and diltiazem and propranolol at 50µg/L. Two plates were prepared and chloroquine was added to one at a fixed concentration of 30µg/L³. Chloroquine antimalarial activity was assayed in the range of 3.75 to 240µg/L. A 10% hematocrit solution of infected blood was added to the plates, which were incubated according to the candle jar method²⁶ at 37°C for 46 (Isolate 1) and 48 (Isolate 2) hours. Schizonts with three or more nuclei in 200 parasites were counted.

Statistical analysis. A descriptive study was performed to analyze the parasitaemia rate as a function of drug concentration. The parasitaemia rate corresponded to the number of parasites in each drug concentration in relation to that in the respective control. Two lines were constructed, one for the modulating agent and the other for the combination with chloroquine. Coincident and separate (parallel and concurrent with one or two intercepts) lines were further fitted using the logistic and log-log complement models in the inferential analysis^{5 8}. A 10% significance level was adopted for the likelihood ratio statistic, which, in this case, corresponded to the difference between two goodness-of-fit statistics.

RESULTS

With both isolates and with the different cardiovascular drugs, a decrease in parasitaemia rate as a consequence of increasing drug concentration was observed whether or not the drug was combined with chloroquine. A similar decrease was observed with chloroquine (Figure 2). Using inferential analysis, a coincident lines model fitted the best for the lines of verapamil and its combination with

chloroquine. Coincident lines were the most suitable fitted model for the other cardiovascular compounds assayed, with the exception of nitrendipine for which parallel lines was the best fit for Isolate 1 (data not shown). In order to illustrate these findings, Figure 2 and Table 1 show the verapamil results against Isolate 1. Similar results were obtained for Isolate 2.

DISCUSSION

Verapamil may be considered to be the first modulating agent in multidrug-resistant neoplasias²⁸. Martin *et al*¹⁵ observed that this drug reversed *in vitro* *P. falciparum* resistance to chloroquine and the MDR mechanism has been suggested to malaria. Furthermore, other cardiovascular agents such as diltiazem^{13 14 26}, nicardipine²⁶, amlodipine² and different therapeutic drugs such as desipramine⁴, cyproheptadine²³ and promethazine²⁰, were shown to modulate antimalarial drug resistance. From these, the anti-histaminic chlorpheniramine has been successfully employed in combination with chloroquine to treat Nigerian children with *falciparum* malaria²¹.

In our work, a particular procedure was adopted with the aim of simulating the modulating effect of drugs at therapeutic schedules. The current therapeutic concentration was employed as the intermediate concentration in the two-fold serial dilutions of the

modulating agents (ca. 125µg/L for verapamil) and a fixed concentration of chloroquine (30µg/L) was combined. This chloroquine concentration is responsible for the clearance of parasitaemia in sensitive *P. falciparum* infections³. Thus, if a modulating effect was observed, chloroquine was considered to be therapeutically efficient. An analogous drug combination was used to determine the response modification index (RMI), with the exception that the modulating effect was evaluated with respect to the median inhibitory concentration (IC₅₀) of the antimalarial agent^{14 20}.

In this study, the descriptive statistical analysis showed a decrease in the parasitaemia rate as a function of concentration for all the compounds and combinations assayed (Figure 2). Chloroquine sensitivity was observed at the highest concentrations classifying the strains as resistant to the antimalarial⁶. IC₅₀ values and the 95% confidence interval (in parentheses) were

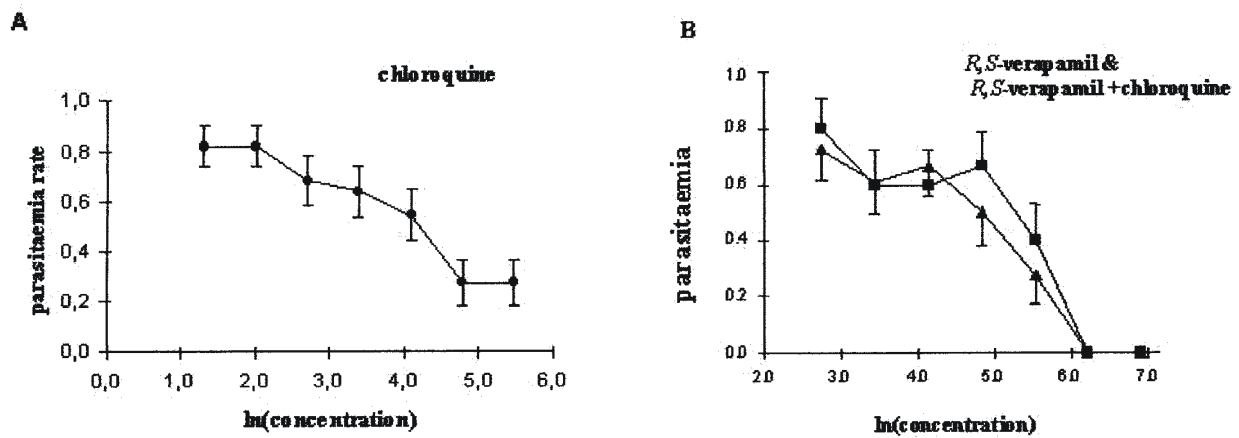


Figure 2 - Ratios of the number of *P. falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after 46h exposure to chloroquine (A) and to verapamil (R,S-verapamil) (\blacktriangle) and verapamil plus chloroquine (\blacksquare) (B) for Isolate 1.

Table 1 - Fitted linear logistic model for verapamil (R,S-verapamil).

Lines models	Goodness-of-fit statistic	Degrees of freedom	Likelihood ratio statistic	Differences in degrees of freedom	P
Coincident	26.775	12	-	-	-
Parallels	26.195	11	0.579	1	0.446
Concurrent (1 intercept)	26.177	11	0.598	1	0.439
Concurrent (2 intercepts)	26.174	10	0.601	2	0.741

52.46 μ g/L (30.57 - 74.41) for Isolate 1 and 41.26 μ g/L (22.87 - 74.40) for Isolate 2.

However, it is important to note that under the adopted experimental conditions, the intrinsic antiparasitic activity observed for modulating agents was not expected. For example, 1 μ M verapamil was considered ineffective against parasite growth in antimalarial resistance reversal studies¹⁵, this effect being only reported at 10 μ M¹. In this study, verapamil was evaluated at serial dilutions ranging from 15.63 to 1,000.00 μ g/L (0.034 - 2.2 μ M) and showed antimalarial activity from the initial concentration. Similar considerations can be established in respect to the other compounds. Moreover, the fitting of coincident lines is indicative of a non-significant change in parasitaemia rate when chloroquine was combined. Nitrendipine demonstrated contrasting results to those of the other cardiovascular drugs,

and in the total series studied with erythromycin¹⁸, which showed a weak modulating effect only for Isolate 1.

In general, intrinsic antiplasmodial activity and lack of chloroquine resistance modulation were observed for most of the modulating agents assayed, such as the antipsychotic drug trifluoperazine¹⁹ and the antidepressants desipramine and imipramine¹⁷.

Independently of the mechanism of action responsible for the intrinsic antiplasmodial effect^{9 10 12 21 24}, our final results (as far as we know, this is the first study performed with fresh isolates), suggest a much higher susceptibility of Brazilian strains, as well as an indifferent behaviour, in response to modulating agents as reported by Mehlotra¹⁶. They associated this finding to genetic polymorphisms among chloroquine-resistant strains from different endemic areas.

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