IN VITRO EVALUATION OF ERYTHROMYCIN IN CHLOROQUINE RESISTANT BRAZILIAN *P. falciparum* FRESHLY ISOLATES: MODULATING EFFECT AND ANTIMALARIAL ACTIVITY EVIDENCE

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SUMMARY

Erythromycin, a reversal agent in multidrug-resistant cancer, was assayed in chloroquine resistance modulation. The *in vitro* microtechnique for drug susceptibility was employed using two freshly isolates of *Plasmodium falciparum* from North of Brazil. The antimalarial effect of the drug was confirmed, with an IC_{50} estimates near the usual antimicrobial therapy concentration, and a significant statistical modulating action was observed for one isolate.

KEYWORDS: Erythromycin; Modulating effect; Antimalarial activity; Chloroquine *P. falciparum* resistance

INTRODUCTION

The multidrug resistance (MDR) is a phenomenon observed in neoplastic cells which have been shown to be resistant to structurally and functionally unrelated cytostatic agents. According to this mechanism, the resistance would be associated to the efflux of the drug from the cell, hindering the display of the therapeutic function. This efflux would result from the amplification of two genes, *mdr1* and *mdr2*, and the consequent overexpression of an ATP-energy dependent protein, the P-glycoprotein, Pgp. Nevertheless, different hypotheses have been advanced based on the lower drug accumulation in resistant cells compared to sensitive ones, independently on the mechanism involved[6,13].

In addition, calcium channel blockers, as verapamil, and other drugs as calmodulin antagonists, steroids and hormone analogues, and cyclosporins, in combination to an antineoplastic agent were shown to restore the sensibility level in resistant cells. These anti-MDR drugs were called reversal agents, chemosensitizers, or modulating agents, and have as a characteristic the ability of potentiating the antiproliferative activity of the chemoterapeutic agent in the resistant cells, having little or no influence on the sensitive ones[13]. This approach was further applied to human cancer therapy, leading to the search for the ideal modulating agent that should be deprived of intrinsic effect and toxicity[4,13,26].

In 1987, MARTIN *et al.*[22] advanced the MDR mechanism hypothesis to explain the reversal of chloroquine *P. falciparum* resistance by verapamil. Although *in vitro, in vivo* and human assays have shown the usefulness of the combination of a modulating agent with chloroquine, there are many controversies about the MDR biochemical and genetic similarity in malaria. The antimalarial cross-resistance, the amplification and function of the plasmodio multidrug resistance genes, and the overexpression of a homologue protein to Pgp have been discussed. Also, the differences in chemosensitization mechanism and in the kinetics and mechanism of drug accumulation and release between the infected erythrocytes and mammalian multidrug-resistant cells have been considered[6,12].

The resistance reversal degree in malaria reports has been associated to the doses of the combined agent and to the resistance level of the strain. Higher doses of chlorpromazine and prochlorperazine were needed to reverse the *in vitro* chloroquine resistance in the highly resistant *P. falciparum* freshly isolates, although only a potentiation effect was observed in the most resistant isolate[2].

Aiming the evaluation of the modulating effect in Brazilian chloroquine resistant *P. falciparum* strains, this paper reports the erythromycin behavior in two freshly isolates from the North of the country. Erythromycin was classified as a reversal agent in multidrug-resistant cancer, specially for its lipophilicity[16,17].

MATERIALS AND METHODS

The drugs used were chloroquine diphosphate (Fundação para o Remédio Popular) and erythromycin. For the microtechnique for drug susceptibility[21,27], two freshly isolates of *P. falciparum* were employed: Isolate1 (SUCEN 198/94), from a woman with 6,600 asexual forms of

parasites/mm^{3} in her third malaria, and Isolate 2 (SUCEN 206/94), from a man in the second infection with 7,500 parasites/mm^{3}. These people were infected in the North of Brazil and had not been submitted to antimalarial treatment in the 14 or 28 days before the tests, as stated by WHO[8].

Microtechnique assay

Twofold serial dilution of the drugs were used to titrate the plates. Chloroquine was employed in concentrations from 3.75 to 240.00 µg/L and erythromycin from 7.81 to 500.00 µg/L, the average values corresponding to the respective therapeutic concentrations[9,11]. In a third plate, a fixed concentration of chloroquine (30.00 µg/L) was combined to the erythromycin series. The later is the plasma concentration effective against sensitive *falciparum* malaria[3].

A 10% hematocrit solution of the infected blood was added to the plates. These were incubated according to the candle jar method[31] at 37 °C for 40 hours for Isolate 1, and for 46 hours for Isolate 2. Schizonts with three or more nuclei were counted in 200 parasites.

Statistical analysis

The statistical analysis comprehended the descriptive and the inferential studies of the data obtained[7,10] and was carried out using the software S-Plus, version 4.5, and the Microsoft Excel for Windows, version 5.0.

In the descriptive analysis, the behavior of the parasitaemia rate as a function of the concentration was considered. The parasitaemia rate is the number of parasites in the different wells in relation to the controls. The fitting of statistical models to these lines was carried out in the inferential analysis. The logistic and log-log complement models were employed with the possibilities of coincident, parallel, and concurrent lines, the later with one or two intercepts. The 10% significance level was adopted for the likelihood ratio statistic, that in this case corresponds to the difference between 2 goodness-of-fit statistics. Then, the 50% inhibitory concentration, IC_{50}, of chloroquine, erythromycin, and their combination was estimated.

RESULTS

The parasitaemia rate decreased as the concentration increased, as observed in the individual drugs and their combination in both isolates (Figures 1 and 2). These figures also illustrate the standard errors for the parasitaemia rate. The antiplasmodial effect of chloroquine only occurred in higher concentrations. The isolates could be classified as resistant, since the parasite growth was observed in concentrations higher or equal to 5.7 pmol/well (36.47 µg/L)[8]. Erythromycin displayed antimalarial activity even in the first concentration assayed, which is eight times lower than the usual antimicrobial therapy[9,11]. The respective IC_{50} estimates, in the natural logarithm form, are presented in Table 1.

The combination of erythromycin with chloroquine showed a lower parasitaemia for Isolate 1 whilst quite similar rates to those of erythromycin alone were observed in the case of Isolate 2 (Figure 2). In the inferential analysis, the logistic model allowed us to consider the parallel and coincident lines as the best fitted models for Isolate 1 and 2, respectively (Table 2).
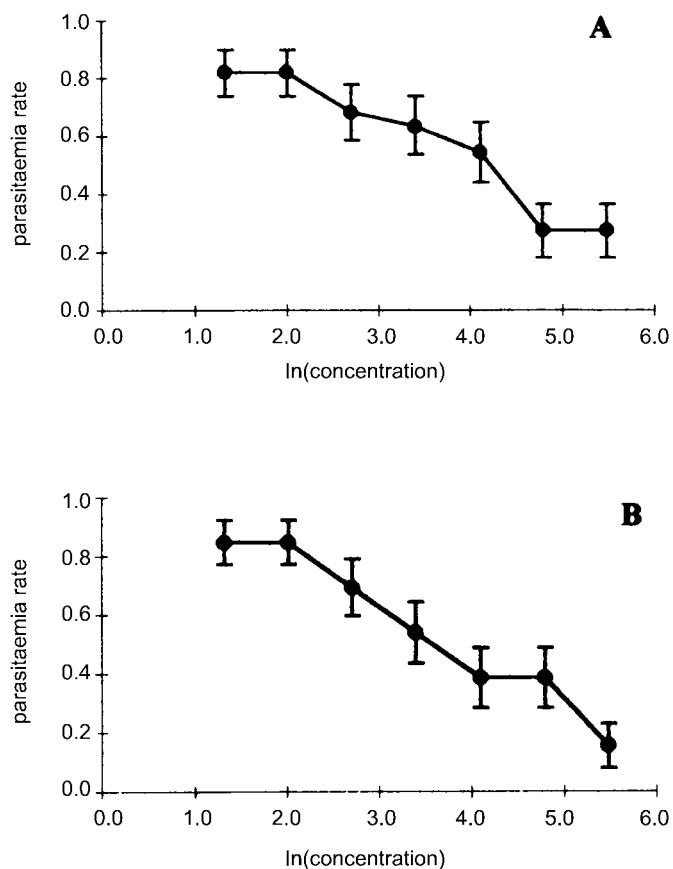

Fig. 1 - Ratios of the number of *P. falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to chloroquine (A, Isolate 1 and B, Isolate 2).

Table 1
Estimated median inhibitory concentration of chloroquine, erythromycin, and the respective combination on *P. falciparum in vitro*

Drug	Isolate	ln IC_{50} (µg/L)	Confidence interval (95%)
Chloroquine	1	3.96	3.42 ; 4.50
	2	3.72	3.13 ; 4.31
Erythromycin	1	4.30	- 0.30 ; 8.91
	2*	3.31	- 2.70 ; 3.90
Chloroquine + Erythromycin	1	3.60	- 1.01 ; 8.20
	2*	3.31	- 2.70 ; 3.90

* same values (coincident lines)

Table 2
Fitted linear logistic model for erythromycin and its combination with chloroquine

| Model	Isolate	Goodness-of-fit statistic	Degrees of freedom	p-value	Likelihood ratio statistic	Degrees of freedom	p-value
Coincident lines	1	19.447	12	0.078			
	2	4.173	12	0.980			
Parallel lines	1	13.836	11	0.242	5.611	1	0.018
	2	3.990	11	0.970	0.183	1	0.510
Concurrent lines (1 intercept)	1	16.035	11	0.140	3.412	1	0.065
	2	3.739	11	0.977	0.434	1	0.669
Concurrent lines (2 intercepts)	1	11.396	10	0.327	8.051	2	0.018
	2	3.678	10	0.961	0.495	2	0.781
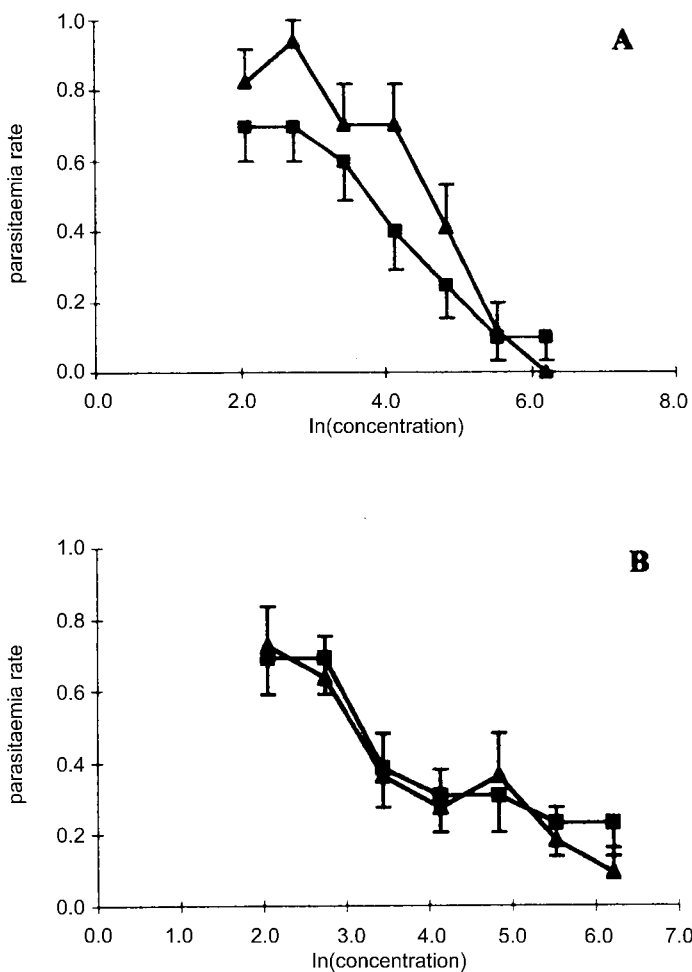
Fig. 2 - Ratios of the number of *P. falciparum* parasites in drug-treated cultures to the number in control cultures (parasitaemia rate) after exposure to erythromycin (▲) and erythromycin plus chloroquine (■) (A, Isolate 1 and B, Isolate 2).

DISCUSSION

In the last decades the global malaria situation has become serious, showing a tendency to get worse. It is estimated that about 1.5 to 2.7 million people, from which 1 million children under five years old, die annually. The rate of clinical cases/year is of 300 to 500 million. *Plasmodium* resistance to current antimalarials, mainly the *P. falciparum* resistance to chloroquine, and *Anopheles* insecticide resistance are the major responsibles for this dark picture[30,33]. Nevertheless, chloroquine is still considered a useful drug in sensitive malaria, and this fact has been the driving force that keeps the studies on modulating resistance going on[30].

In our work a series of drugs reported as modulating agents in the literature, including erythromycin, was assayed in chloroquine resistant malaria[23].

Antibiotics have been known for their antiplasmodial activity since 1940. Although these agents are less effective and slow acting when compared to chloroquine or quinine, they are alternative drugs for resistant malaria. However, considering the inherent counterindications, adverse effects and, specially, the possibility of antimicrobial resistance dissemination, public health authorities have been trying to restrict the use of antibiotics in combinations with classical antimalarials, particurlay in areas of resistant or multi-resistant *P. falciparum* prevalence. Nowadays, WHO recommends the administration of tetracycline in combination with quinine in the treatment of resistant malaria, and doxycycline for short-term prophylaxis[34]. Other antiplasmodial antibiotics are erythromycin, clindamycin, rifampicin and chloramphenicol[14,19,28,29,36]. Also, azithromycin, a semisynthetic derivative of erythromycin, has been showing chemoprophylatic action and may be used as an option[1,20] or, yet, as a substitute for doxycycline in pregnant women and in children[34].

ROBINSON & WARHURST first observed the *in vitro* and *in vivo* antimalarial activity of erythromycin, a macrolide antibiotic, in 1972[28]. In a dosage inferior to its toxicity limit the drug was effective against chloroquine sensitive and resistant strains of *P. berghei*. Besides, the

coadministration with chloroquine was positive only against resistant strains. The potentiation was confirmed in mice infected with chloroquine resistant strains of *P. berghei*[32] and in *P. falciparum* multidrug-resistant strains *in vitro*[14,15]. Thus, erythromycin could be an excellent choice for the treatment of chloroquine *P. falciparum* resistance, due to its lower cost and use in children and pregnant women. However, failures were observed when the combination chloroquine/erythromycin was employed *in vitro* in isolates of *P. falciparum* with different chloroquine sensitivities[18] and in human *falciparum* malaria[5,24,25]. Meanwhile, in 1994 the possibility of antimalarial use for erythromycin was restored with the observation of *in vitro* potentiation effect over artemisinin in chloroquine sensitive and resistant *P. falciparum* strains. If these results persist after clinical trials, the combination could be valuable in preventing the recrudescence commonly observed with this sesquiterpene[35].

In our study, erythromycin confirmed its antimalarial effect, the IC_{50} estimates (73.70 µg/L and 27.39 µg/L for Isolate 1 and 2, respectively) being near its usual concentration as an antimicrobial agent (62.50 µg/L). However, distinct effects were obtained from the inferential analysis when erythromycin was combined with chloroquine. The fitting of parallel lines for Isolate 1 suggests a positive result for the combination. In the analysis of the parasitaemia rate of erythromycin plus chloroquine (Figure 2, A), it can be observed that, in spite of the intrinsic antimalarial effect of erythromycin, the combination showed to be more effective than erythromycin or chloroquine (Figure 1, A). In fact, the combination was 2.020 times more potent than the antibiotic alone. This allow us to consider a modulating effect of erythromycin in chloroquine resistance. Unlikely, this effect was not verified for Isolate 2, coincident lines being the best fitted model. No significant statistical differences were observed in the parasitaemia rates for the combination in relation to those of erythromycin alone (Figure 2, B). This would indicate the predominance of the erythromycin antiplasmodial effect over that for chloroquine. Also, those rates seem to be lower than the observed for chloroquine (Figure 1, B).

It is worth noting that our results are the first report about modulating agents in Brazilian *P. falciparum* isolates resistant to chloroquine. Erythromycin was shown to promote the modulation of one of the two isolates and, in addition, its antiplasmodial activity was confirmed for both isolates. Nevertheless, after a QSAR (quantitative structure-activity relationships) analysis developed with several drug classes, including erythromycin, the influence of the lipophilic parameter on modulating effect, as reported in the literature for this antibiotic, was not confirmed[23]. This deserves further studies.

RESUMO

Avaliação *in vitro* da eritromicina em isolados brasileiros frescos do *P. falciparum* resistente à cloroquina: efeito da modulação e prova de atividade do antimalárico

A eritromicina, agente reversor em neoplasias multi-resistentes, foi ensaiada na modulação de malária resistente à cloroquina. Empregou-se a técnica *in vitro* do microteste de sensibilidade em dois isolados sanguíneos frescos de *Plasmodium falciparum* oriundos da região Norte do Brasil. Confirmou-se o efeito antimalárico do antibiótico, o qual apresentou valores estimados de IC_{50} próximos à concentração usualmente empregada na terapia antimicrobiana. No entanto, efeito modulador estatisticamente significativo foi observado em apenas um dos isolados ensaiados.

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