HALLAZGOS COMPLEMENTARIOS SOBRE LA ACTIVIDAD ANTIMALÁRICA DEL AZUL DE METILENO Y SU TOXICIDAD

COMPLEMENTARY FINDINGS ON THE ANTIMALARIAL ACTIVITY AND TOXICITY OF METHYLENE BLUE.

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RESUMEN

En 1881, Ehrlich reportó el azul de metileno como el primer antimalárico sintético, pero dejo de ser empleado con este propósito; el debe ser reconsiderado pues son necesarias con urgencia nuevas alternativas de bajo costo, en el arsenal de medicamentos antimaláricos. En este trabajo la actividad antimalárica del azul de metileno es evaluada *in vivo* frente a parásitos de malaria murina. Una dosis de 15 mg/kg/día inhibe el 50% del crecimiento eritrocítico parasitario de *Plasmodium berghei* y *P. yoelii nigeriensis*, en tanto que es prácticamente inactivo frente a los estadíos hepáticos de *P. yoelii yoelii*. Sobre un cultivo de la línea celular linfoblástica BJAB, el azul de metileno es 20 veces más citotóxico que la cloroquina, no obstante el azul de metileno presenta valores de índice de selectividad del mismo nivel que la cloroquina, frente a varias cepas de *P. falciparum* de diferentes niveles de sensibilidad y provenientes de diferentes lugares geográficos.

Palabras clave: Antipalúdicos – *Plasmodium* – Malaria - Azul de metileno

ABSTRACT

Methilene blue was reported as the first synthetic antimalarial by Ehrlich in 1881. It is currently no longer used for that purpose but it should be reconsidered since new economic alternatives are urgently needed in the arsenal of antimalarial drugs. The antimalarial activity of methylene blue is investigated here *in vivo* against rodent malaria parasites. 15 mg/kg daily dose of methylene blue inhibits 50% of the erythrocytic parasite growth of *Plasmodium berghei* and *P. yoelii nigeriensis*, while on hepatic stages of *P. yoelii yoelii* is almost inactive. In cell culture experiments with the lymphoblast-like BJAB cells line, it is 20 times more cytotoxic than chloroquine. Nevertheless, methylene blue shows a similar selectivity index as chloroquine against strains of different level of sensitivity of *P. falciparum* original from different geographical areas.

Keywords: Antimalarials, *Plasmodium*, Malaria, Methylene blue.

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INTRODUCTION

About 3 billion people live in endemic areas where malaria is one of the major health problems. 500 million cases are registered annually, leading to the death of 1,5 million people, most of them children (1). Control measures are largely based on vector eradication programs and clinical treatment, but their efficacy is dramatically reduced by increasing the resistance of vector and parasite. New drugs at low cost are urgently needed. Unfortunately, although the World Health Organization negotiates with pharmaceutical companies in order to reduce the prices of antimalarial drugs, these remain unattainable for most of the affected populations (2). Therefore new and affordable treatments are a major priority for affected countries. In this context, Methylene blue (MB), which has been proven to be active (3, 4, 5) and inexpensive, could be a suitable alternative.

Methylene blue is the oldest and cheapest synthetic drug ever used in antimalarial therapy (Figure 1). In 1891 Guttmann and Ehrlich published the first report of its antimalarial activity (4). Two years later, Ferreira (5) recommended the use of MB by oral route in children with malaria. At the beginning of the 20th century, MB served as structural starting point for the development of 8-aminoquinolines (3, 6, 7) and disappeared thereafter from the antimalarial arsenal. Afterwards, few works on the antimalarial properties of MB have been reported because it was thought to enhance haemolysis in glucose-6-phosphate-deshydrogenase (G6PD) deficiency (8) and could stain the tissues of MB-treated people (9). Recently Mandi *et al.* (10) and Meissner *et al.* (11) showed that G6PD deficiency is not commited by the use of MB for malaria treatment, even in class III G6PD deficient young children. These observations renewed the interest of MB as a cost-effective alternative in the treatment of malaria.

This report presents data on the *in vivo* antimalarial activity of MB against blood and liver forms of rodent malaria parasites and the *in vitro* cytotoxicity on BJAB cells, thereby completing other studies published by our research team. (12, 13).

MATERIALS AND METHODS

In vivo antimalarial activity

In vivo studies were conducted according to the French and Colombian legislations on laboratory animal use and care (N°2001-464 and N° 008430, respectively).

Effect on the intraerythrocytic cycle

The classical 4-day suppressive test (14) was conducted with *P. yoelii nigeriensis* or *P. berghei* ANKA. Swiss female mice (*Bioterio DFUNC* and Charles River France) weighing 20 ± 2 g, were infected with 10^7 parasitized cells in 0,9% saline solution (day 0). Batches of five mices were orally treated with MB at doses of 50, 25, 12.5 and 6.35 mg/Kg/day, two hours after infection and at the same hour during 4 consecutive days. A control group received 0,9% saline solution while a reference group was administered chloroquine diphosphate (CQ) at 3 mg/kg/day (oral route). The survival of mice was checked daily and the percentage of parasitized erythrocytes was determined on day 4, by Giemsa-stained thin blood smears from peripheral blood. The percentage of inhibition of parasitaemia was calculated, and the Effective Dose 50 (ED_{50}) was determined with a linear least square regression analysis.

Effect on the hepatic stage

The *in vivo* activity on the pre-erythrocytic stage was determined according to Peters and Robinson (15): Batches of three mice were intravenously inoculated with 10^8 sporozoites of *P. yoelii yoelii* (17X strain; clone 1,1) in 0,9% saline. The treatment (around twice the *in vivo* determined ED_{50}) was given to mice by oral route, 2 h after inoculation; Primaquine at 60 mg/kg was administered to a reference group. The suppressive effect was estimated on 48, 72, 96, 120, 144, and 168 hours after inoculation by Giemsa-stained thin blood smears inspection. Animals which showing no signs of infection on day 14 were considered as cured.

In vitro studies on BJAB cells

Culture of lymphoblast-like line BJAB cells was carried out on 2 mM L-glutamine-enriched RPMI supplemented with 1ml/L of 10mg/ml gentamycin and 10% heat-inactivated foetal bovine serum at 37°C in 5% CO_2 environment. BJAB cells at 2×10^5 were exposed to serial dilutions of methylene blue for 24 h in culture conditions (test in triplicate). 24 h after initial cell-plating, the cell growth was estimated by [^{3}H]-thymidine incorporation (2,5 μCi/ml in culture medium) for 16 h. Incorporated [^{3}H]-thymidine was then determined with a beta-counter (1450-Microbeta Trilux, Wallac-

PerkinElmer). The CC_{50} (Cytotoxic Concentration 50 i.e. concentration that kills 50% of cultured cells), was determined with a linear least square regression analysis. A control of viability was carried out in parallel.

Reagents

All chemicals reagents were provided by Sigma-Aldrich. Radiolabels were provided by PerkinElmer (Courtaboeuf, France).

RESULTS AND DISCUSSION

Against erythrocytic forms in rodent malaria parasites, MB was more active on *P. berghei* than on *P. yoelii* (ED_{50} of 12,8 $\pm$ 8 mg/kg/day and 19,4 $\pm$ 4 mg/kg/day, respectively) with no signs of acute toxicity after a 4 days treatment (Table 1, Figure 2). MB antimalarial activities were ten times lower than those observed with CQ, that showed ED_{50} of 0,53 and 1,21 mg/kg/day against *P. berghei* and *P. yoelii nigeriensis*, respectively. A previous study reported that MB had ED_{50} of 11,03 and 1,21 mg/kg/day against *P. yoelii nigeriensis* and *P. vinckei petteri*, respectively (3). The low ED_{50} found with *P. vinckei* can be explained by the degree of synchronicity of the infection, being *P. vinckei* more sensitive to antimalarial agents than other rodent malaria species (16). On the contrary, any activity against hepatic stages of *P. yoelii yoelii* (pre-erythrocytic stage) with a dose of up to 100 mg/kg was not observed (figure 3). This was the expected result since the main action of MB is based on the inhibition of heme-GSH degradation (13), a process not occurring during the pre-erythrocytic cycle of *Plasmodium yoelii*.

In a previous paper was shown that MB was very active *in vitro* against laboratory-reared strains from various geographic regions (concentration of MB required to inhibit 50% of the parasite growth IC_{50}: 5 nM) and that late rings and early trophozoites were the most sensitive stages; while early rings, late trophozoites and schizonts were less sensitive (12). Furthermore, combination of MB with commercially available antimalarials resulted in: antagonism with amodiaquine; antagonism/additivity with atovaquone, doxycycline, pyrimethamine; additivity/lack interaction with artemether, chloroquine, mefloquine, primaquine; and synergy with quinine (12). The model described in this study (on BJAB cells) showed MB with CC_{50} of 577 $\pm$ 12 nM (Figure 4) while doxorubicin and CQ, used as controls, had CC_{50} of 416 $\pm$ 8 and 11563 $\pm$ 1647 nM respectively. Thus, MB was as toxic against BJAB cells as doxorubicin, and almost 20 times more toxic than CQ. Results were surprising since previous studies reported that MB had CC_{50}s of 1610 and 26700 nM on KB and J111 cells (3, 17). Nevertheless, Kirszberg *et al.* (18) showed that MB was more toxic on leukaemia cells, than on normal cells. This could explain our results, because BJAB cells are derived from lymphoblast-like lines. A previous study measured the IC_{50} on *P. falciparum* chloroquine sensitive (F32, HB3) and resistant strains (FcB1 and FcM29) (12). With the data on BJAB cells found in this study and those previously described, the Selectivity Index (SI) was calculated. SI is defined as the ratio between concentration which produces a cytotoxic effect (CC_{50}) and the antimalarial concentration which causes the desired effects (IC_{50}) (Table 1). The values of SI for CQ were higher than those obtained with MB on sensitive strains (on HB3 the CC_{50} was 321 times higher than the CI_{50}). On the contrary, MB had highest SI values on resistant strains (Figure 5).

Table 1. General Results.

| *In vivo* activity	MB		CQ	
Effect on the intraerythro-cytic cycle	ED50 (mg/kg/day)		ED50 (mg/kg/day)	
P. bergei	12,8		0,53	
P. yoelii nigeriensis	19,4		1,21	
Effect on the hepatic stage				
P. yoelii yoelii	Inactive		Not tested	
In vitro activity	CI50 (nM)*	SI	CI50 (nM)*	SI
P. falciparum F32	5	115	70	165
P. falciparum HB3	4	144	36	321
P. falciparum FcB1	8	72	186	62
P. falciparum FCM29	5	115	600	19
In vitro citotoxicity	CC50 (nM)		CC50 nM	
BJAB cells	577		11563	

⋆ Previously reported data (12)

These results complete those obtained in previous works (12, 13) and confirm the interest of MB as an ancient effective molecule that could be integrated into a new low cost antimalarial therapy, after confirmation of the benefit/risk ratio by further clinical trials.
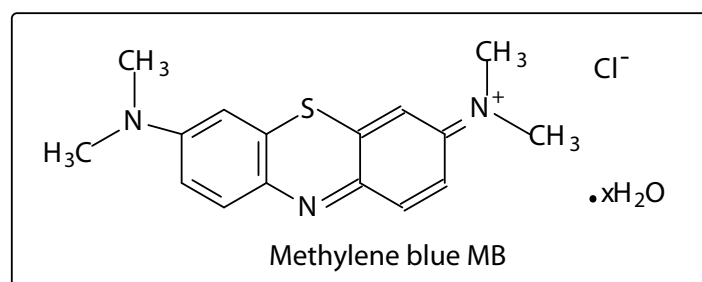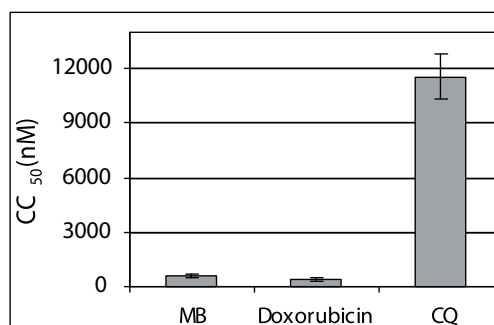
Figure 1. Methylene blue: 3,7-Bis(dimethylamino)phenothiazin-5-ium chloride; $C_{16}H_{18}ClN_3S \cdot xH_2O$; m.w. 319.85 (anhydrous base).
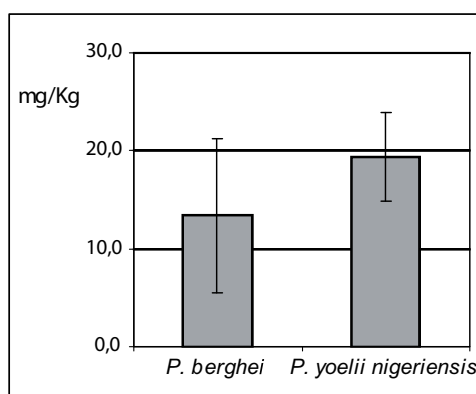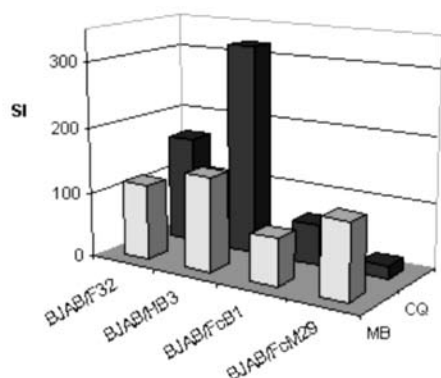

Figure 2. Effective Dose 50 (mg/kg/day) of MB on *P. berghei* ANKA and *P. yoelii nigeriensis* rodent malaria.
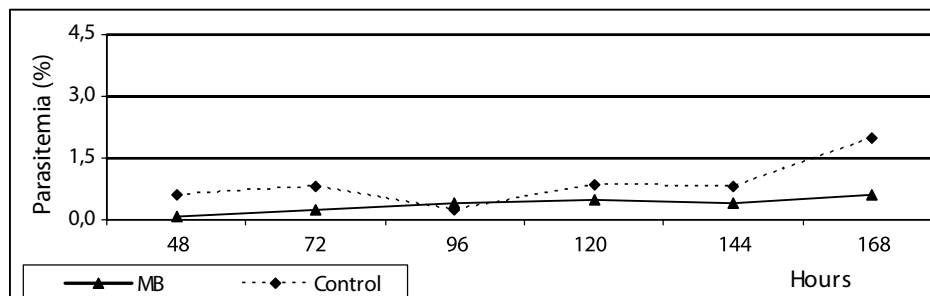
Figure 3. Effect of MB (100 mg/Kg) on hepatic stage of *P. yoelii yoelii* infected mice.

Figure 4. Cytotoxic Concentration 50 (CC_{50}) of methylene blue (MB), doxorubicin and chloroquine (CQ) on BJAB cells.

Figure 5. Selectivity Index of MB and CQ on different *P. falciparum* strains (chloroquine sensitive strains: F32, HB3 chloroquine resistant strains: FcB1, FcM29) in culture.

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