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In vitro antimalarial activity of methylene blue against field isolates of *Plasmodium falciparum* from children in Southwest Nigeria

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***Background & objectives*: Methylene blue (MB), a thiazine dye is used in the treatment of various methemoglobinaemias. However, sporadic reports have shown some antimalarial therapeutic effect when administered to patients with clinical manifestations of malaria. The inhibitory concentration of schizont maturation and antimalarial activity of MB have not been fully elucidated. The present study therefore aimed at determining the antimalarial activity of MB in *Plasmodium falciparum* isolates obtained from children with malaria using standard *in vitro* drug susceptibility test.**

***Methods*: Twenty children (8 boys and 12 girls) within the age range 4.5-11.5 yr were enrolled into the study and 2 ml of blood withdrawn aseptically. The standard microtest technique of schizont inhibition assay was used to culture fresh isolates obtained from *P. falciparum* infected patients. Chloroquine (CQ) and quinine (QN) were used as reference standards for *in vitro* drug susceptibility tests.**

***Results*: The mean 50 per cent inhibitory concentration (IC_{50}) values were 9.59 ± 3.25nM, 196 ± 21.11nM and 607 ± 27.41nM for MB, CQ and QN respectively. Ten of the 14 isolates were sensitive to MB, 11 were sensitive to CQ while nine were sensitive to QN. Three isolates were resistant to CQ, and of these, two were sensitive to MB and one was sensitive to QN.**

***Interpretation & conclusion*: This preliminary study showed that MB has high antimalarial activity comparable with CQ and QN and may be used as a potent schizonticidal drug against CQ-resistant isolates.**

Key words Antimalarial - chloroquine - *in vitro* activity - methylene blue - *Plasmodium falciparum* - quinine

Plasmodium falciparum malaria is one of the most virulent and complex diseases that varies widely in epidemiology and clinical manifestations in different endemic areas worldwide[1]. Approximately, 1.5 –2.7 million deaths occur each year, most of which are children under 5 yr[2] of age. In the absence of effective malaria vaccine, chemotherapy and chemoprophylaxis remain the major control strategies for reducing the morbidity and mortality of malaria. However, the increased prevalence of *P. falciparum* resistant to chloroquine (CQ) and other antimalarial drugs in Sub Saharan Africa poses a serious threat to malaria control[3,4]. Therefore, there is a need to closely monitor the changing patterns of antimalarial drug susceptibility, cross-resistance and also investigate new potent antimalarial drugs. *In vitro* drug sensitivity assay by inhibition of parasite multiplication at specific stage of development remains essential in this quest[5,6]. The current rates of CQ and sulphadoxine-pyrimethamine (SP) resistance in *P. falciparum* in Southwest Nigeria are on the increase[7], while available alternatives and combination therapy are more expensive and associated with significant adverse effects[8, 9].

Methylene blue (MB), a thiazine dye is basically used in the treatment of various methemoglobinaemias. However, there are reports of its antimalarial efficacy in rodents[10] and in patients with clinical manifestations of malaria[11-14]. Some toxicological effects of MB have also been noted but not extensively studied[15]. *In vitro* antimalarial effects of its analogues on *P. falciparum* suggested that MB might inhibit the growth of parasite in culture[15]. The present study was carried out is to determine the *In vitro* antimalarial activity of MB in *P. falciparum* isolated obtained from children with symptoms of malaria, and to establish its threshold concentration value in comparison with standard antimalarial drugs.

Material & Methods

The study was carried out between April and October 2003 and included patients attending the out-patient clinic of the University College Hospital in Ibadan, Southwest Nigeria. Twenty fresh isolates of *P. falciparum* were obtained from symptomatic 20 Nigerian children (8 boys 12 girls, 4.5-11.5 yr) prior to drug administration. The following inclusion criteria were set for this study: presence of signs and symptoms of acute uncomplicated malaria, mono-infection with *P. falciparum*, verified microsopically using thin films initial parasitaemia >4000 asexual parasites/µl of blood and no history of recent antimalarial drug intake in the preceding two weeks. The University of Ibadan-University College Hospital ethical review committee approved the study protocol. Informed consent was obtained from the parents of the children. Two milliliters of venous blood was collected aseptically from each patient and transferred into sterile acid citrate dextrose (ACD) bottles. The patients were then appropriately treated with standard doses of chloroquine, sulphadoxine-pyrimethamine or coartemether.

Drugs : Chloroquine diphosphate was obtained from Sigma Chemical Company, USA; Quinine (QN) sulphate from BDH Chemicals Ltd Poole, England; Methylene blue (anhydrous) from SERVA Heidelberg, Germany. The drugs were dissolved in 70 per cent ethanol to a concentration of 1mg/ml and diluted subsequently with culture medium. CQ was first dissolved in distilled water before the addition of absolute ethanol to give the same concentration. The preparation of drugs and the design of the test plates were based on the procedures of Rieckmann *et al*[16]. The drugs were serially diluted ten-fold from rows A to G of the 96-well microtiter plates using multichannel micropipette. Row H served as the control without drug. Each drug was tested in duplicate columns.

Preparation of culture media : Culture media was prepared by dissolving 10.4g of powdered RPMI 1640 (Gibco Grand Island, New York) and 5.94g of HEPES (N-2 hydroxyethyl piperazine-N-2-ethane sulphonic acid) (Sigma Chemical Company, USA) in 1l of sterile distilled water. The medium was filtered using 0.22µm millipore filter, stored at 4°C and used within 4 wk. Wash medium was prepared by adding 1.6 ml of 7.5 per cent $NaHCO_3$ (Sigma Chemical Company, USA) to 43.4 ml of the medium above (*i.e.*, RPMI 1640 with HEPES) in 50 ml centrifuge tubes for use.

In vitro assay : The procedures for the short-term culture technique was based on the previously described schizont inhibition assay[16].

Parasite suspension (200µl) was transferred in each well of the 96 well tissue culture plates that were either drug-free or inoculated with test compounds to a final volume of 225µl per well.

The plates were incubated at 37ºC in a candle jar (5% CO_2, 17% O_2, 78% N_2) according to the method of Trager & Jensen[17] for 18-48 h depending on the time taken by the parasite to develop to schizonts as monitored by microscopically observing the parasites in the control wells. Cells were harvested on clean microscope slides, prepared as thick films, and stained with 10 per cent Giemsa for 15 min when 60 per cent of parasites in control well had matured to the schizont stage. The slides were viewed under the light microscope (X100 objective) and results expressed as number of mature schizonts (with 3 or more nuclei) per 200 white blood cells. The minimum inhibitory concentration (MIC) defined as the lowest concentration that inhibited >95 per cent of the parasite development from rings to schizonts (based on the average of the untreated controls on each plate) was determined using the method of Childs *et al*[18]. The parasite density was estimated as the number of mature schizonts per 200 white blood cells. Isolates having parasite density of <40,000/µl of blood were diluted using a 1:10 dilution factor while those having >40,000/µl of blood were diluted using a higher dilution factor (1:20). This variation in the dilution ratios allowed the parasites a larger surface area to grow, which enhanced their susceptibility to the drugs.

Data analysis: The data were fitted to a dose-response curve (using the Graphpad prism software 3.0). The 50 per cent inhibitory concentration (IC_{50}) defined as the drug concentration corresponding to 50 per cent inhibition of schizonts development in the control wells, was determined by nonlinear regression analysis of log dose/response curves.

Results

Fourteen of the 20 isolates (70%) were successfully cultivated to mature schizonts within 48 h. The MIC values were in the range of 96.9 – 969nM for CQ; 10 – 1000nM for MB and >638.6nM for QN (Table). The mean IC_{50} values were 9.59 ± 3.25nM for MB (range 0.01 – 28.02; at 95 per cent confidence interval (CI) 0.35 – 231.38nM); 196 ± 21.11nM for CQ (range 1.47 – 910nM, at 95% C.I 0.124 – 92564nM); and 607± 27.41nM for QN (range 25.17 – 2551.86nM, at 95% C.I 7.25×10^{-4} – 5.05×10^{8}nM).

Ten of the 14 isolates were sensitive to CQ (IC_{50} <100nM). Eight of the twelve (75%) successful isolates in the plate were sensitive to QN (IC_{50}<500nM). Ten of the 14 successful isolates were sensitive to MB (IC_{50}<9.59nM) *in vitro*. On the other hand, four of the 14 isolates were resistant to CQ (IC_{50} >100nM), four of the twelve isolates were resistant to QN (IC_{50} > 500nM), and four of the fourteen isolates were resistant to MB (IC_{50} > 9.59 nM).

Three of the Four CQ-resistant isolates were sensitive to MB while only one (25%) was resistant to MB (IC_{50} = 26.56nM). Two of the Four QN-resistant isolates was also sensitive to MB. Three of the Four CQ-resistant isolates were also resistant to QN meaning that only one was sensitive to QN. Two of the three isolates showing cross-resistance between CQ and QN were sensitive to MB *in vitro*. One isolate (024) was resistant to all the three drugs.

Discussion

MB inhibited the maturation of the parasite to schizont in a similar manner to CQ and QN. Its schizonticidal activity appeared to be effective against both CQ-sensitive and resistant *P. falciparum* at low concentration, and suggests its high potency against schizont stage of falciparum malaria.

Table. Values of 50 per cent inhibitory concentration (IC_{50}) of each drug on isolates of *P. falciparum*

Isolate code	IC_{50} CQ		MIC CQ	Status	IC_{50} MB		MIC MB	Status	IC_{50} QN		MIC QN	Status
	ng/ml	nM	nM	R/S	ng/ml	nM	nM	R/S	ng/ml	nM	nM	R/S
009	50.27	97.44	969	S	2.30	7.20	100	S	122.80	156.84	638.6	S
011	16.05	31.11	96.9	S	6.36	19.88	100	R	ND	ND	638.6	-
012	36.56	70.87	969	S	0.48	1.50	10	S	19.71	25.17	≥ 638.6	S
014	18.32	35.51	96.9	S	7.65	23.92	100	R	225.90	288.52	638.6	S
016	67.35	130.55	969	R	2.03	6.34	100	S	473.40	604.60	638.6	R
017	5.75	11.14	969	S	0.002	0.01	100	S	147.30	188.13	638.6	S
018	25.08	48.61	969	S	0.2316	0.72	10	S	233.00	297.59	638.6	S
019	8.94	17.33	96.9	S	3.47	10.85	100	R	ND	ND	638.6	-
021	469.90	910.84	969	R	0.53	1.66	10	S	827.80	1057.27	638.6	R
023	0.76	1.47	969	S	0.17	0.53	10	S	373.70	477.29	638.6	S
024	377.80	732.31	969	R	8.50	26.56	100	R	1998.00	2551.86	638.6	R
028	12.97	25.14	96.9	S	8.97	28.02	100	R	900.30	1149.87	638.6	R
033	308.30	597.02	969	R	1.78	5.58	1000	S	238.10	304.10	638.6	S
036	18.14	35.16	96.9	S	0.50	1.55	10	S	148.70	189.92	638.6	S

ND, not determined; R, resistant; S, sensitive; CR, chloroquine; QN, quinine sulphate; MB, methylene blue

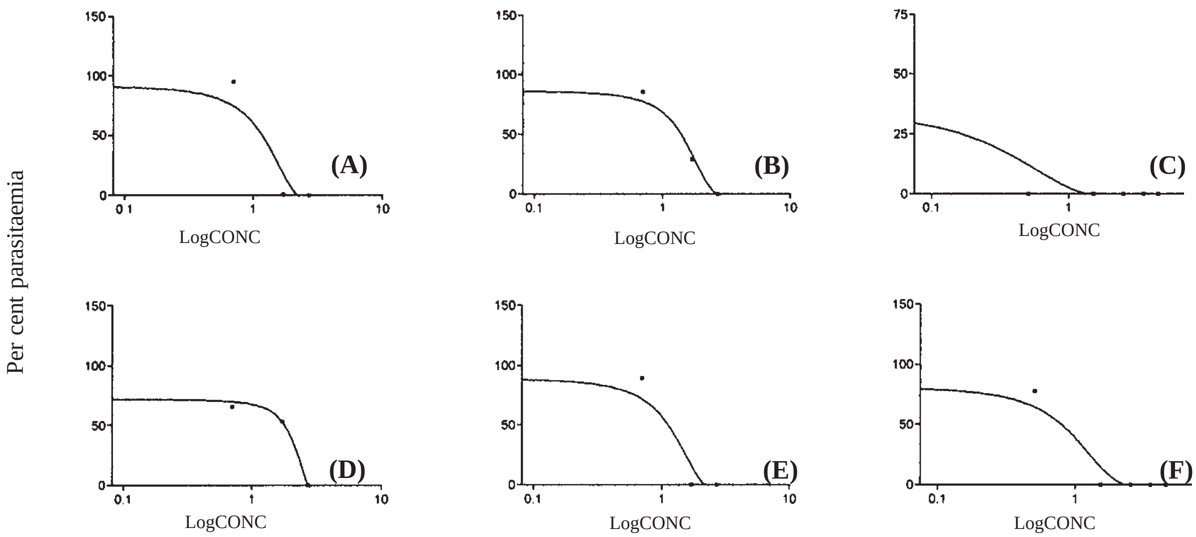

Fig. *In vitro* activity of quinine (QN), chloroquine (CQ) and methylene blue (MB) on two selected isolates (012 and 014). **A.** LAB012[QN] IC50=19.71µg/ml, **B.** LAB012[CQ] IC50=36.56µg/ml, **C.** LAB012[MB] IC50=0.48µg/ml, **D.** LAB014[QN] IC50=225.9µg/ml, **E.** LAB014[CQ] IC50=18.32µg/ml, **F.** LAB014[MB] IC50=7.65µg/ml.

The mean IC_{50} for the effect of MB on viability of *P. falciparum* was in agreement with previous reports[12,15]. The mean IC_{50} values for CQ and QN were also comparable with those of previous studies carried out in other malaria endemic areas[6,19].

MB was shown to be active against both CQ and QN resistant isolates. It also showed a high activity as a potent schizonticidal agent at low concentration. The ratio of IC_{50} between CQ and MB was 20:1. Based on statistical calculations using the mean IC_{50}, the threshold value of MB was 9.59nM. However, this cut-off value of MB needs to be validated in further studies using larger populations in different settings of malaria endemicity.

All CQ-sensitive isolates had an IC_{50} value for MB <28.02nM. The IC_{50} value of MB for the moderately CQ-resistant isolate of *P. falciparum* (arbitrarily defined as those isolates with IC_{50} of CQ between 100 - 300nM[20] was 6.34nM. Of the three CQ-resistant isolates, two displayed high susceptibility or low IC_{50} values while one displayed an elevated IC_{50} for MB. This isolate was also resistant to QN *in vitro*. This may be interpreted as cross-resistance between CQ and QN. This is in agreement with the fact that CQ resistance is fast spreading and cross-resistance with other antimalarial drugs such as mefloquine (MFQ), halofantrine (HF) and QN has been documented in previous studies carried out in this area[16]. However, in Thailand where *P. falciparum* is highly resistant to CQ, increasing resistance to QN and even mefloquine has been reported[21].

The result from the log dose/response curves (Fig. A-F) showed that for CQ and QN, the percentage parasitaemia was fairly constant and only started falling after a high concentration was reached. Whereas with MB, the curve was steep as parasitaemia fell steadily with increasing concentration. This indicated a relatively high antimalarial activity MB against *P. falciparum* isolates.

The patient from whom the isolate having cross-resistance to CQ and QN and an elevated IC_{50} for MB was obtained, also failed treatment with CQ *in vivo (*data not shown). However, in some cases the *in vivo* outcome did not correspond with the *in vitro* results (data not shown). This is because parasite clearance in the host depends on various pharmacokinetic and pharmacodynamic parameters, the level of acquired immunity, which interact with and enhances drug efficacy. A patient harbouring CQ-resistant population of *P. falciparum* may thus eliminate all parasites after adequate treatment with CQ due to 'booster effect' of the immune system. Also, a CQ-sensitive patient may fail to clear *P. falciparum* within 14 days because of an inadequate plasma CQ-concentration level or re-infection[22].

The response of the CQ-sensitive isolates to MB was similar to that of the CQ-resistant isolates. It confirms the high antimalarial activity of MB as a potent schizonticidal drug. However, accurate determination of the IC_{50} cut-off value for resistance will require correlation between responses observed *in vitro* and results obtained after chemotherapy *in vivo*. The culture of fresh isolates of *P. falciparum* showed that only 14 of 20 isolates grew to maturity within 48 h. The retarded or lack of growth in the remaining six might be due to presence of drug in the blood of the patients or certain serum factors that may inhibit parasite growth *in vitro*. A limitation to this study was that laboratory adapted clones were not used. However, results obtained using wild parasites have been demonstrated to be reliable[23,24].

This preliminary study showed that MB has a potential to be used as a potent schizonticidal antimalarial. However, its potential toxic effect is a concern. This needs further elucidation. MB is readily available and affordable. In view of its antimalarial activity and its use in the treatment of methemoglobinaemia at concentrations within the antimalarial activity range, it will be appropriate to carry out further studies on its potential antimalarial activity and the degree of toxicity.

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