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Mode of Antimalarial Effect of Methylene Blue and Some of its Analogues on *Plasmodium falciparum* in Culture and Their Inhibition of *P. vinckei petteri* and *P. yoelii nigeriensis in Vivo*

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ABSTRACT. The antimalarial action of methylene blue (MB) was first noted by Paul Ehrlich in the late 19th century. Although it has only sporadically been adopted as a serviceable drug, the resolution of its antimalarial action seems warranted, as it is currently used for the treatment of various methemoglobinemias. In this work we have used MB, and its analogues Azures A (AZA), B (AZB), C (AZC), and thionin (TH), as well as the oxazine Celestine blue (CB) and the azine Phenosaphranin (PS). All MB analogues inhibit the growth of various strains of *Plasmodium falciparum* in culture with IC_{50}s in the $2 \cdot 10^{-9}$–$1 \cdot 10^{-7}$ M range, with the rank order MB ≈ AZA > AZB > AZC > TH > PS > CB. The IC_{50}s for a mammalian cell line were in the $3 \cdot 10^{-6}$–$4 \cdot 10^{-5}$ M range, and the rank order was TH ≈ AZB > AZA ≈ PS > AZC ≈ CB > MB. As MB could affect cell growth through the oxidation of NADPH, we tested the action of the various compounds on the hexose-monophosphate shunt activity. Appreciable activation of the shunt was observed at $1 \cdot 10^{-5}$ M in both cell types, thus accounting for inhibition of growth of mammalian cells but not of parasites. All compounds were found to complex with heme in a rank order similar to their antimalarial effect. It is therefore suggested that MB and its congeners act by preventing the polymerization of heme, which is produced during the digestion of host cell cytosol in the parasite food vacuole, into hemozoin. In this respect, these compounds seem to act similarly to the 4-aminoquinoline antimalarials. All compounds effectively suppressed the growth of *P. vinckei petteri in vivo* with IC_{50} in the 1.2–5.2 mg/kg range, and MB and AZB suppressed *P. yoelii nigeriensis* in the 9–11 mg/kg range (i.e. at doses similar to those of chloroquine). The potential toxicity of these compounds may restrict their clinical use, but their impressive antimalarial activities suggest that the phenothiazine structure could serve as a lead compound for further drug development. BIOCHEM PHARMACOL 51;5:693–700, 1996.

KEY WORDS. phenothiazines; methylene blue; antimalarial action; *Plasmodium falciparum*; murine malarias; hexose-monophosphate shunt

The first test of MB§ for the cure of malaria was reported by Guttman and Ehrlich [1]. Its successful use was reported in Brazil, but it was subsequently abandoned due to toxic episodes (reviewed in Ref. [2]). The consideration of MB, the azure dyes, and thionin as potential antimalarial drugs has been abandoned by the U.S. Army due to a therapeutic index of 1, as determined by the Rane's test (communicated by E.O. Nuzum and D.E. Kyle, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington D.C., U.S.A.) and the demonstration of MB's potential for inducing drug resistance in mice [3]. Interestingly, the use of MB as an antimalarial drug still appeared in a French pharmacopea in 1984 [4], but not later. Subsequent chemical modifications by the addition of dialkyl and/or aminoalkyl groups has increased the antimalarial activity. Further modifications and testing led to the development of the 8-aminoquinolines [5], of which only primaquine is currently used as a tissue schizontocide in the cure of vivax and ovale malarias (reviewed in Ref. [6]).

The precise antimalarial mode of action of MB and its congeners is not known. Studies on the ability of MB to reduce methemoglobin to oxyHb revealed that MB penetrates into erythrocytes, where it is enzymatically reduced by NADPH

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§ *Abbreviations:* AZA, azure A; AZB, azure B; AZC, azure C; CB, celestine blue; HMS, hexose-monophosphate shunt; MB, methylene blue; PS, phenosaphranin; RBC, red blood cells; and TH, thionine.

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reductase to leucoblue at the expense of NADPH, and the reduced MB acts to reduce metHb [7]. MB also activates the hexose-monophosphate shunt of mammalian cells due to the oxidation of NADPH to NADP, and the consequent activation of glucose-6-phosphate dehydrogenase, which is the rate-limiting enzyme of the shunt [8]. We recently demonstrated that MB also activates the shunt of *Plasmodium falciparum* [9]. It is possible to infer from these studies that the antimalarial effect of MB could involve the oxidation of the parasite's NADPH, a co-factor needed for various essential metabolic activities, such as reduction of oxidized glutathione (through glutathione reductase), the activation of diribonucleotide reductase that is the rate-limiting enzyme of DNA synthesis, for the proper function of catalase and for the synthesis of pyrimidine bases [10].

Our findings that MB was extremely effective in mouse malaria using the 4-days suppressive test, and the possibility to grow parasite in culture, and hence, their amenability to biochemical, physiological, and pharmacological studies, has prompted us to test the antimalarial mode of action of MB.

MATERIALS AND METHODS

Materials

MB, AZA, AZB, AZC, TH, PS, and CB were purchased from Sigma. See Fig. 1 for structural formulae. RPMI-1640 and fetal calf serum were from Biological Industries, Beit-Haemek, Israel. Heme (ferriprotoporphyrin IX) was from Porphyrin Products (Logan, UT). Fresh blood and plasma were kindly donated by the Shaarei Zedek and the Hadassah Hospitals, Jerusalem. All other chemicals used were of the best available grade.

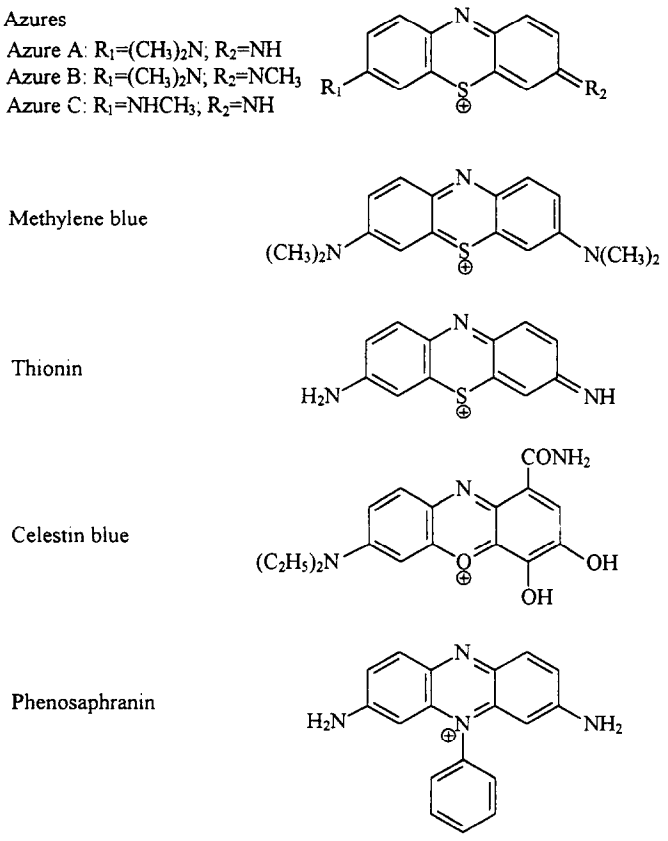

FIG. 1. Structural formulas of compounds used.

Effect of drugs on P. falciparum *in vitro*

Various P. falciparum strains (FCR-3, D6, and W2) were cultivated using human erythrocytes and heat-inactivated compatible human plasma, as previously described [11]. Drug action was tested on cultures of 1% hematocrit and 1% parasitemia, and the IC_{50} was determined as before [12].

Effect of Drugs on Human Cells

The human monocytic leukemia-derived cell line J-111 [13] was used as a model mammalian cell. One mL of cell suspension containing $2.5 \cdot 10^5$ cells/mL of RPMI-1640, supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 20 mM $NaHCO_3$, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), was aliquoted into each well of a Nunc 24-well plastic tissue culture plate. Cells were allowed to adhere to the plate at 37°C in a humidified 5% CO_2 and 95% air atmosphere. Thereafter, drugs were added at 10^{-8} to 10^{-4} M, and after an additional 1-hour incubation in culture, [^{3}H]thymidine was added (0.5 μCi/mL), and monolayers were cultivated overnight. The wells were washed twice with 1 mL lukewarm PBS supplemented with 5 mM glucose per well to remove non-incorporated radioactivity. The monolayers were resuspended by incubation in 0.2 mL of phosphate-buffered saline containing 0.25% (w/v) trypsin + 0.05% (w/v) EDTA at 37°C for 15 min, and were mixed with 3 mL Quicksafe A, and the radioactivity was counted. The IC_{50} values of growth inhibition were calculated on the Enzfitter program.

Effect of Drugs on Hexose-Monophosphate Shunt Activity

The effect of MB and its analogues was also tested on the HMS activity in *P. falciparum*-infected cells and in J-111 cells. Infected cells (IRBC) were separated from cultures at the trophozoite stage by gelatin flotation [14]. IRBC were suspended at $1 \cdot 10^8$ cells/mL in 400 μL of glucose-free RPMI-1640 supplemented with 5 mM glucose, 10 mM $NaHCO_3$, 100 μM hypoxanthine, and 0.24 μCi of D-[1-^{14}C]glucose and various concentrations of MB or its analogues, and HMS activity was monitored measuring the evolution of $^{14}CO_2$ as previously described [9]. Similarly, J-111 cells were detached from culture bottle walls by incubation in PBS/trypsin/EDTA buffer, and suspended at $1 \cdot 10^6$ cells/mL in the RPMI-1640/5 mM glucose medium, and the evolution of $^{14}CO_2$ from D-[1-^{14}C]glucose and from D-[6-^{14}C]glucose (reflecting oxidation of glucose by mitochondria) was measured in parallel samples. The latter value was subtracted from the first to evaluate the HMS activity in these cells.

Measurement of the Reduction Potential of Drugs

Solutions of 0.1 mM of drugs in 0.1 M potassium phosphate, pH 7.4, were bubbled with argon, and the reduction potential

was measured by cyclic voltametry using a gold electrode and a sweep rate of 100 mV/sec between −0.4 and +0.25 V. The reduction potential was recorded at the maximal positive current.

In vivo *experiments*

The *in vivo* antimalarial activity of MB and its analogues was determined by the classical 4-days suppressive test [15] against *Plasmodium vinckei petteri*, Carter and Walliker 1975 (279 BY), and *P. yoelii nigeriensis*, Killick-Kendrick 1975 (NIG). Swiss male mice (Charles River, France), mean body weight 20 ± 2 g, were infected with 10^7 parasitized cells in 0.9% saline, on day 0. Groups of 3 mice were treated intraperitoneally from day 0 to day 3 with increasing doses of the various compounds dissolved in 0.9% saline. The suppressive effect was estimated on day 4 by examining Giemsa-stained thin blood smears made from the tails of control mice injected with saline and drug-treated mice. The stained thin blood smears were examined under ×1,000 magnification, counting at least 9,000 cells, and the percentage of parasitized red blood cells was calculated for each concentration and compared to that observed in untreated animals.

Formation of Complexes Between Heme and Drugs

The complexation of heme with MB and its congeners was tested spectrophotometrically [16]. A stock solution of 1 mM heme was prepared fresh in 50 mM NaOH, and the final concentration was determined by measuring the absorption at 605 nm, using a molar absorption coefficient of $4.6 \cdot 10^3$. Heme was diluted to 5 μM in PBS, and its absorption spectrum was recorded in a Milton Roy Spectronic 3000 Array spectrophotometer against a blank of PBS. Then the test compound was added to 5 μM, and the spectrum was recorded again against a blank of the compound in PBS.

RESULTS

Effect of Drugs on P. falciparum *in Culture*

Three strains that display differential sensitivity to chloroquine were used (IC_{50} to chloroquine in nM: D6, 25.6 ± 3.7; FCR3, 155.6 ± 26.4; W2, 582.1 ± 14.9). All drugs tested inhibited the growth of *P. falciparum* in culture (Fig. 2). Of the thiazine drugs, AZA and MB were found to be the most potent drugs. There was a graded decline in the potency of the Azures in going from AZA to AZC: AZB was significantly ($P < 0.05$) less toxic than AZA for the D6 and the FCR3 strains; AZC is significantly less toxic than AZB to D6 and W2. TH was as active as AZC. The azine dye PS was less effective than AZA, AZB, AZC, and MB for all strains. The least effective drug was the oxazine derivative CB. For some of the compounds, similar results were recently reported [17]. Some cross-resistance between AZA or MB and chloroquine could be inferred, as the D6 clone is sensitive to chloroquine and the W2 clone is resistant to it. CB is apparently cross-resistant with meflo-

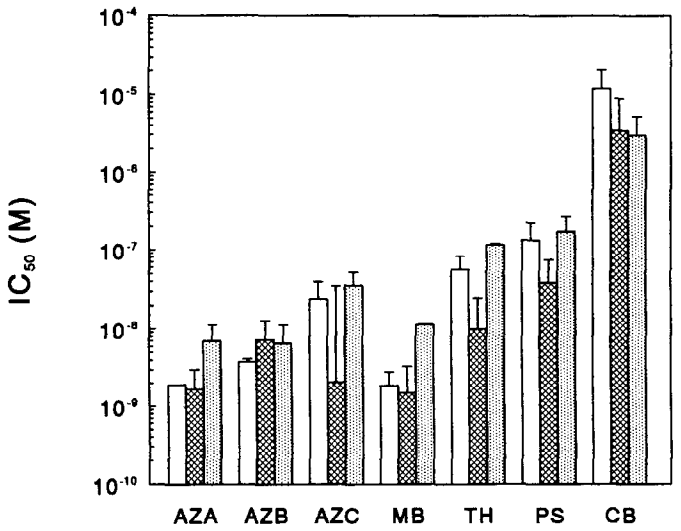

FIG. 2. Effect of drugs on the growth of *P. falciparum* strains in culture. Drug test and the calculation of IC_{50} were performed as described in Materials and Methods. Each individual experiment was performed in triplicate, and results show means ± SD of IC_{50} of several (2–4) experiments. D6, open bars; FCR-3, cross-hatched bars; W2, dotted bars.

quine, which shows the inverse pattern of drug sensitivity to that of chloroquine with the tested strains [12].

Effect of Drugs on a Mammalian Cell Line

The use of compounds that show antimalarial activity in culture dictate that the same compounds should have low toxicity against the host. The human monocytic leukemia-derived cell line J-111 was used in the present work as a model host cell. All drugs tested inhibited cell growth as tested by [^{3}H]thymidine incorporation. All drugs were much less potent against the mammalian cell, the IC_{50} values being 1–4 orders of magnitude higher than their effect on parasites (Table 1). The rank order of potency was TH ≈ AZB > AZA ≈ PS > AZC ≈ CB > MB (where ≈ signifies $P > 0.05$ and > stands for $P <$

TABLE 1. Effect of drugs on the viability of J-111 monocytes. Cells were grown and treated with drugs as described in Materials and Methods. The incorporation of [^{3}H]thymidine into nucleic acids was measured as a function of drug concentration in 3 separate experiments done in triplicates, and the IC_{50} for each drug in (μM ± SE) is shown. The selectivity indices (IC_{50}(J-111)/IC_{50} (parasite)) were calculated for each parasite strain from data shown in Fig. 2).

		Selectivity index		
Compound	**IC_{50} (μM)**	**D6**	**FCR3**	**W2**
Azure A	6.56 ± 0.83	3434	3928	935
Azure B	3.72 ± 0.72	979	511	571
Azure C	17.82 ± 8.12	742	8526	501
Methylene blue	26.70 ± 10.72	14278	17682	2342
Thionine	3.09 ± 0.95	54.5	310	26.6
Phenosaphranin	7.84 ± 0.73	58.5	204	45.6
Celestine blue	20.39 ± 3.68	1.7	6	7

0.05), clearly different from the rank order of their antimalarial activity. This discrepancy implies a different mode of action on the two cell types.

The selectivity indices (IC_{50}(J-111)/IC_{50}(parasite)) for the 3 parasite strains are also shown in Table 1. MB stands out as the most selective drug for *P. falciparum*, while CB is the least selective. Whereas the absolute indices vary from one strain to the other, their rank order remains virtually the same. Similar experiments using KB (ATTC CCL 17, human epidermoid carcinoma of the mouth) were reported recently [17]. These cells were apparently more sensitive to MB, AZA, AZB, and THI than the J-111 cells, yielding much smaller selectivity indices. Although the reason for this discrepancy is not known, it underscores the potential pitfalls that may arise from the choice of the particular mammalian cell for such comparisons, and the need for *in vivo* testing as has been done in this work.

Effect of Drugs on Murine Parasites in vivo

Using the suppressive 4-day test, drugs were found to be effective against *P. vinckei petteri* and *P. yoelii nigeriensis*. MB, AZB, and TH were equally effective, and AZA and PS were less efficient (Table 2). The doses that inhibited these murine parasite species are well within the suppressive concentration range of chloroquine [18]. The IC_{50} values for CB and for AZC could not be calculated accurately, but they were definitely lower than 5 mg/kg. MB and AZB were found to be less effective against *P. yoelii nigeriensis*. This result is comparable to the relative sensitivity of these parasite species to chloroquine, which is correlated to the degree of synchronicity of infection. For chloroquine, it has been shown to act almost exclusively on the mid-term trophozoite stage [19]. Hence, given the rapid pharmacokinetics of chloroquine in the mouse [20], in the synchronous *P. vinckei petteri*, all parasites will be eradicated if the drug is administered when mid-term trophozoites are prevalent in the blood, whereas in the asynchronous *P. yoelii nigeriensis*, the drug-refractive stages present during drug treatment will be spared, resulting in an apparent drug resistance [21].

Effect of Drugs on the Hexose-Monophosphate Shunt (HMS) Activity of Malaria-Infected Erythrocytes

The oxidation of NADPH to NADP by MB is considered to underlie the potentiation of HMS activity, since the activity of the rate-limiting enzyme of this pathway, glucose-6-phosphate dehydrogenase (G6PD), is determined by the ratio of NADP/NADPH concentrations. If the tested drugs inhibit parasite growth by the reduction of NADPH levels, then one should expect to find an activation of the HMS at those concentrations that inhibit parasite growth. The results depicted in Fig. 3 clearly indicate that a substantial (15–30%) increase in HMS activity occurred only at 1 μM for all drugs except PS, which inhibited HMS. This concentration is at least one order of magnitude above the IC_{50} for inhibition of parasite growth, except for CB. This discrepancy in effective concentrations in conjunction with the fact that PS inhibits parasite growth and also curtails its HMS activity, solidly hints that the antimalarial mode of action (except for CB) has probably nothing to do with the pro-oxidant activity of the tested drugs.

TABLE 2. **Effect of drugs on the murine malarial parasites *P. vinckel petteri* and *P. yoelii nigeriensis***

Drug	*P. vinckei petteri*	*P. yoelii nigeriensis*
Methylene blue	1.21 ± 0.17	11.03 ± 2.88
Azure A	5.23 ± 0.24	–
Azure B	1.19 ± 0.17	9.08 ± 3.42
Thionine	1.74 ± 0.25	–
Phenosaphranin	4.45 ± 1.06	–

Effect of Drugs on the HMS Activity of J-111 Monocytes

The dose-dependent activation of the HMS by the various drugs is depicted in Fig. 4. The rank order of activation potency is somewhat different than that in malaria-infected RBC, in that AZA and AZB are the most potent, next come TH and MB, and last are AZC and CB, the latter being also the least effective in infected RBC. Here also, PS does not cause any activation of HMS.

The Redox Potential of the Drugs

In an attempt to understand the differential activation of HMS by the drugs, their reduction potential has been mea-

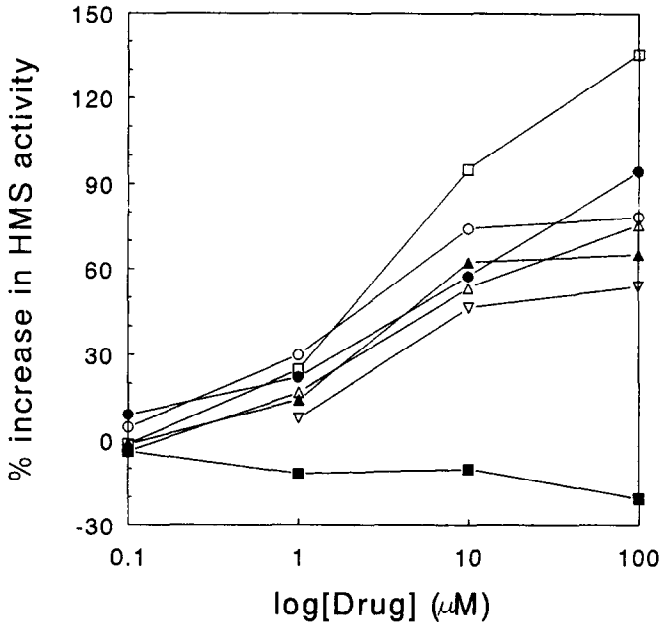

FIG. 3. **Effect of drugs on the HMS activity of *P. falciparum*-infected RBC. Synchronized cultures of the FCR-3 strain were cultured to the trophozoite stage, infected cells were separated from uninfected RBC, and their HMS activity in presence of increasing drug concentrations measured as described in Materials and Methods. The % increase in HMS activity due to drug is plotted against the logarithm of drug concentration. Azure A, ○; azure B, ●; azure C, △; methylene blue, ▲; thionine, □; phenosaphranin, ■; celestine blue, ▽.**

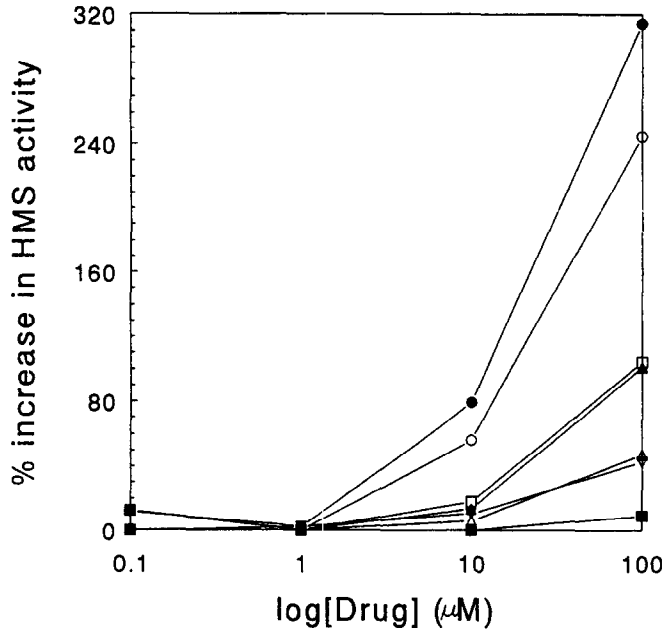

FIG. 4. Effect of drugs on the HMS activity of J-111 monocytes. Logarithmically growing J-111 monocytes were suspended in RPMI-1640 medium containing 5 mM glucose, and their HMS activity in presence of increasing drug concentrations measured as described in Materials and Methods. The % increase in HMS activity due to drug is plotted against the logarithm of drug concentration. Azure A, ○; azure B, ●; azure C, △; methylene blue, ▲; thionine, □; phenosaphranin, ■; celestine blue, ▽.

sured by cyclic voltametry. As shown in Table 3, all compounds had very similar reduction potentials in the range of −0.205 to −0.245 volt. The reduction potential of MB was previously reported to be −0.25 volt [22].

Complex Formation Between Drugs and Heme

Considering the structure of MB and its congeners, it was expected that they should complex with heme. Indeed, the mixing of heme with the various drugs at equi-molar ratios produced distinct alterations in the absorption spectrum, indicating complex formation (Fig. 5): red-shifts in the absorption maxima and reduction of the latter, as well as increased absorption above 400 nm.

DISCUSSION

MB and its analogues were found to have 4 orders of magnitude difference in their inhibitory effect of *P. falciparum* growth in culture, MB being the most active and CB the least. No consistent differences were found in the sensitivity of the 3 strains that were tested, indicating that there is no cross-resistance between these compounds and chloroquine or mefloquine. All compounds were less toxic to the monocyte cell line J-111, with selectivity indices as high as $1.7 \cdot 10^4$ for MB and as low as 1.7 for CB. The high selectivity indices of some of the compounds recommend their further testing, and we shall now discuss plausible mechanisms that could rationalize them.

TABLE 3. Reduction potentials of drugs

Drug	Reduction potential (volts)
Azure A	−0.23
Azure B	−0.225
Azure C	−0.24
Methylene blue	−0.21
Thionin	−0.205
Phenosaphranin	−0.215
Celestine blue	−0.245

Since MB has been shown before to activate the HMS of both host cell and parasite [9], it was plausible to assume that the tested compounds act by depleting cellular NADPH with consequent inhibition of processes that depend on adequate levels of this cofactor. However, direct measurements of HMS in presence of increasing concentrations of the various drugs indicated that there was no correlation between the HMS potentiating activity and the antimalarial effect. Thus, TH was the best HMS potentiator, whereas it was ca. 100-fold less inhibitory than MB to parasite growth. On the other hand, PS that inhibited HMS activity still had an appreciable effect on parasite growth. Most importantly, the minimal drug concentration affecting HMS activity (1 μM) was substantially higher than the IC_{50} of all compounds except CB.

Contrary to this observation, all compounds (except PS) inhibited the growth of the mammalian J-111 cells at concentrations that appreciably activated HMS. In these cells the rank order of inhibition generally followed the HMS-potentiating activity, except for MB and PS, the first being less inhibiting than expected according to this paradigm, whereas the second inhibited cell growth despite the fact that it did not activate HMS. Since all drugs have a very similar reduction potential, it seems that the differential HMS potentiation should be related to the rate of drug uptake and/or to the retention of the reduced drug (see below). Whatever may be the case, it seems that in J-111 monocytes, the depletion of NADPH may be a major factor that underlies their inhibitory effect on cell growth, although toxicity due to binding to and intercalation into DNA [23] cannot be excluded. Given this scrutiny, alternative mode(s) of drug action that may explain the specific antimalarial effect of the drugs were investigated.

Consideration of the acid/base properties of the compounds tested in this work seems essential. MB is an exceptionally weak base (pKa 0–1 [24, 25]), but inside cells it can undergo one electron reduction, and then its pKa would be around 9 [26]. The fact that MB accumulates inside RBC [27, 28], and much more so in the malaria-infected RBC in which it is reduced [9], suggests that it accumulates after having been reduced and subsequently protonated (assuming that the protonated form cannot translocate across membranes). Hence, although it has not been verified where in the infected cell MB accumulates, most likely much of it could amass in the food vacuole. Such may also be the case of thionine, whose pKa at the reduced stage is 8.1 [26]. The high pKa values of the azures (10–12 [29, 30]) indicates that they also could accumulate inside the acid food vacuole, but they would do so to the same extent, not explaining their differential antimalarial effect.

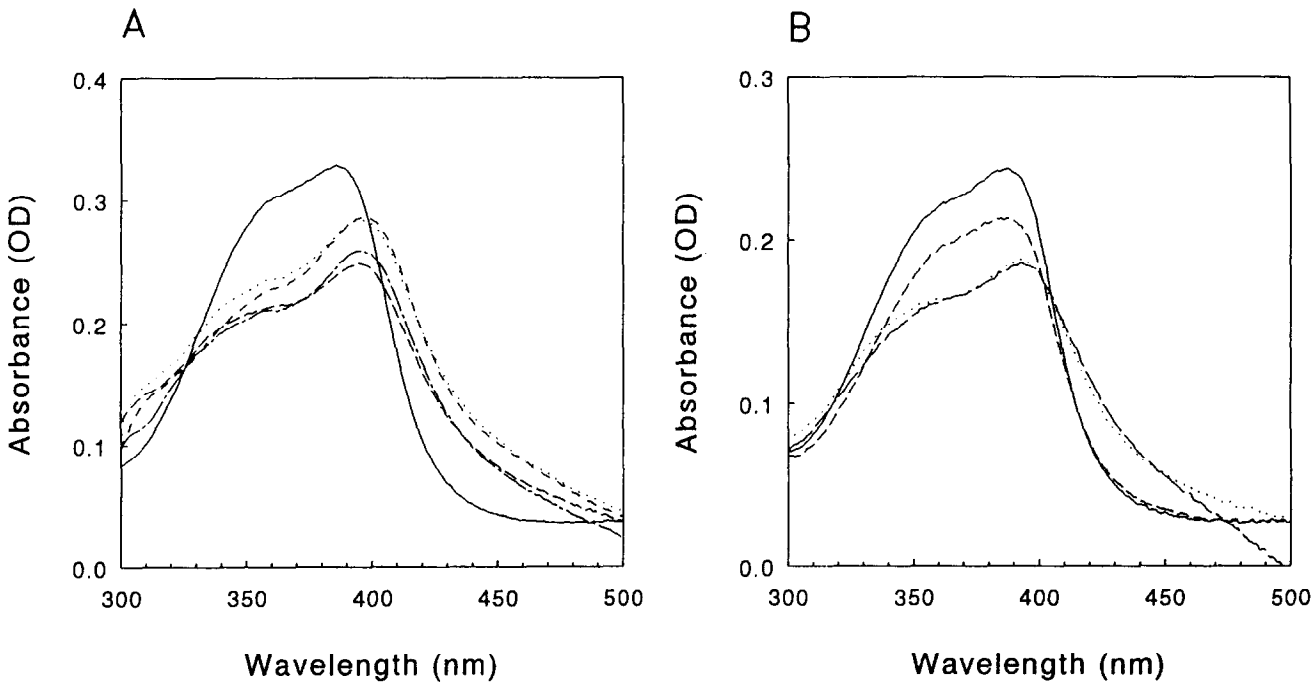

FIG. 5. Complex formation between heme and drugs. Heme and drugs were diluted in PBS to 5 μM at equimolar concentration, and the absorption spectrum between 300 and 500 nm was recorded against a blank containing drug in PBS alone. (A) heme ——; methylene blue – – –; azure A —; azure B · · · ·; thionine — – —; (B) heme ——; azure C · · · ·; celestine blue – – –; phenosaphranin —.

The accumulation of a weak base can be assessed by calculating the accumulation using the equation

$$C_i/C_o = (1 + 10^{pKa-pHi})/(1 + 10^{pKa-pHo}),$$

where C is drug concentration, *i* and *o* symbolize any intracellular compartment and the extracellular medium, respectively [31]. As the exponential term in the denominator becomes much larger than 1 (e.g. pKa > pHo + 2), C_i/C_o remains essentially constant. The pKa values of PS and CB are not available, but the fact that at the IC_{50} of CB, a 50% enhancement of HMS activity was observed, suggests that it accumulates minimally and exerts its antimalarial action through the depletion of NADPH.

Once inside the vacuole, compounds could interact with heme produced during the digestion of host cell hemoglobin. All drugs were found to complex with heme. Based on the red shift and the decrease in absorbance observed with the various complexes of heme, the rank order of complexation is: MB > AZB > TH > AZA > AZC > PS > CB. This is rather close to the order of antimalarial potency, although no straight correlation should be expected because of differential accumulation inside the food vacuole. The complexation of heme with drugs is reminiscent with that observed with the 4-aminoquinolines [16, 32–38], suggesting a similar mode of action.

The current view on the antimalarial mode of action of 4-aminoquinolines (e.g. chloroquine, quinine, or mefloquine) is that they accumulate inside the acidic food vacuole of the parasite, as alluded for the presently investigated compounds, where they complex with heme (as is true for MB and its analogues) and prevent its sequestration into hemozoin [39, 40], which is essentially β-hematin [41]. Irrespective of whether heme polymerization occurs spontaneously in the acidic environment of the food vacuole [42, 43] or is mediated by heme polymerase [41], it is invariably inhibited by quinoline-containing antimalarials, which are able to complex with heme. Unsequestered heme is believed to be cytotoxic to the parasite. This may also be the case of the present compounds. Under certain conditions [41], and with a two-fold excess of drug over heme, the production of β-hematin by MB was reduced by a factor of about 6, whereas in presence of CB its formation decreased by only a factor of 1.7 (G. Blauer and M. Akkawai, personal communication*). These results are in line with the high inhibitory effect of MB on parasite growth compared to weaker effect of CB. We suggest, therefore, that the antimalarial mode of action of MB and its analogues is similar, if not identical, to that of the 4-aminoquinolines. Further support for this proposition is the reported resemblance of ultrastructural alterations caused by MB, chloroquine, and quinine in the murine parasite *P. berghei* [44].

As previously shown with *P. berghei* [3], MB also inhibited the development of *P. vinckei petteri* and *P. yoelii nigeriensis*, as did some of the analogues. Structure-activity relationships suggest that 2 dimethyl amines (as in MB) or 1 methylamine and 1 dimethylamine (AZB) are required for maximal antimalarial activity. Substitution of either of these groups with NH_2 reduced the antimalarial effect. TH, however, is an exception. The concentrations used were well within the range of those of 4-aminoquinolines. The structure-activity relationships of

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MB and its congeners *in vivo* may be further affected by the pharmacokinetics of the individual compounds.

The present *in vivo* results stand in sharp contrast to those obtained using the Rane's test, where substantially higher concentrations had to be used, reaching in many cases toxic levels. A comparison of the ED_{90} obtained by the suppressive test with the minimal effective dose obtained by the Rane's test shows that for many types of drugs, the latter parameter is considerably higher (7–800 fold) than the former, and much closer to the CD_{50} [15]. This is probably the reason why a therapeutic index of ≈1 found using the Rane's test (although its validity has been questioned [45]) has excluded the further probing of MB and its congeners as potential antimalarials.

The barring of these compounds as antimalarials may have been too hasty and not adequately founded, and only a limited number of MB derivatives have ever been tested. In view of the present results and the use of MB for the treatment of various methemoglobinemias in humans at concentrations within the antimalarial efficacy in the mouse model [7, 46], we suggest that MB and its analogues could be reconsidered as potential antimalarial drugs, or at least as a lead compound for the production of more promising derivatives.

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