

Antimalarial Dyes Revisited: Xanthenes, Azines, Oxazines, and Thiazines

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Received 23 June 1995/Returned for modification 11 September 1995/Accepted 25 September 1995

In 1891 Guttman and Ehrlich (P. Guttman and P. Ehrlich, Berlin Klin. Wochenschr. 28:953-956, 1891) were the first to report the antimalarial properties of a synthetic, rather than a natural, material when they described the clinical cure of two patients after oral administration of a thiazine dye, methylene blue. Since that time, sporadic reports of the antimalarial properties of several xanthene and azine dyes related to methylene blue have been noted. We report here the results from a reexamination of the antimalarial properties of methylene blue, Janus green B, and three rhodamine dyes and disclose new antimalarial data for 16 commercially available structural analogs of these dyes. The 50% inhibitory concentrations for the chloroquine-susceptible D6 clone and SN isolate and the chloroquine-resistant W2 clone of *Plasmodium falciparum* were determined by the recently described parasite lactate dehydrogenase enzyme assay. No cross-resistance to chloroquine was observed for any of the dyes. For the 21 dyes tested, no correlation was observed between antimalarial activity and cytotoxicity against KB cells. No correlation between log P (where P is the octanol/water partition coefficient) or relative catalyst efficiency for glucose oxidation and antimalarial activity or cytotoxicity was observed for the dyes as a whole or for the thiazine dyes. The thiazine dyes were the most uniformly potent structural class tested, and among the dyes in this class, methylene blue was notable for both its high antimalarial potency and selectivity.

In 1891 Guttman and Ehrlich (25) were the first to report the antimalarial properties of a synthetic, rather than a natural, material when they described the clinical cure of two patients after oral administration of a thiazine dye, methylene blue. The only side effect that they noted was a "spastic irritation of the bladder," which was partially alleviated by the administration of powdered nutmeg. They selected methylene blue because it had been previously demonstrated by Celli and Guarnieri to specifically stain plasmodia (25) and also because Ehrlich and Leppmann had prior experience in the therapeutic use of this dye in the successful clinical treatment of neuralgias (32). Guttman and Ehrlich (25) apparently made no further attempt to pursue this interesting observation, although as late as 1956, Dale (14), in his introduction to *The Collected Papers of Paul Ehrlich*, noted that "methylene blue itself has there [in the Balkan countries] retained a fairly extensive use, for the treatment of malaria in cases which have been found refractory to quinine." Moreover, methylene blue, along with quinine, served as structural prototypes (48) which led to the subsequent development of the 8-aminoquinoline pamaquine and the 9-aminoacridine mepacrine (quinacrine) in 1925 and 1930, respectively (Fig. 1).

In the past decade, sporadic reports (7, 18, 19, 21, 27) of the antimalarial properties of several xanthene (rhodamines 123, 6G, and B) and azine (Janus green B) dyes related to methylene blue have been noted. With this background, we decided to reinvestigate the antimalarial properties of methylene blue, Janus green B, and rhodamine 123 and to screen several structural analogs of these dyes (Fig. 2 to 5) against *Plasmodium falciparum* in vitro. We were especially intrigued by the re-

ported lack of cross-resistance with chloroquine for several of these dyes.

(This study was presented in part at the 43rd Annual Meeting of the American Society of Tropical Medicine and Hygiene, Cincinnati, Ohio, 1994.)

MATERIALS AND METHODS

Dyes. All dyes were purchased from Aldrich Chemical Company and were used as received. Dyes were tested for purity by thin-layer chromatography with silica gel plates with an *n*-butanol-acetic acid-water (4:1.6:5) solvent system. This mobile phase solvent system was based on a report by Allison and Garratt (1) in which they had used the same solvents but in a 4:1:5 proportion. We observed that this proportion of solvents afforded a two-phase mixture; the addition of additional acetic acid to the indicated proportion was required to produce a miscible solution. All dyes except methylene violet, toluidine blue O, indoline blue, and methylene green were either homogeneous or were $\geq 90\%$ pure.

Antimalarial assay. A recently described (40) chloroquine-susceptible, mefloquine-resistant *P. falciparum* isolate (SN) and two *P. falciparum* clones (45), designated Sierra Leone (D6) and Indochina (W2), were used in the susceptibility tests. Clone D6 is resistant to mefloquine, and clone W2 is resistant to chloroquine, pyrimethamine, sulfadoxine, and quinine. The initial levels of parasitemias were 2% for all experiments. Stock solutions (100 $\mu\text{g/ml}$) of each dye in absolute ethanol were used for the drug inhibition experiments. Parasite viability was measured by determining the activity of the parasite lactate dehydrogenase enzyme by using the assay of Makler and colleagues (39, 41). Log-logit dose-response curves were calculated from the raw data by using Softmax software (Molecular Devices).

Cytotoxicity assay. The cytotoxicity of each of the dyes was measured by the sulforhodamine B assay described by Skehan et al. (50) KB cells (ATTC CCL 17, a human epidermoid carcinoma of the mouth) were grown to confluency under standard conditions and were harvested by trypsinization as described by Likhitwitayawuid et al. (37). After appropriate dilution of the harvested KB cells, the cells were added to 96-well microtiter plates containing the dyes dissolved in dimethyl sulfoxide (DMSO); negative control wells contained DMSO. The plates were incubated for 3 days at 37°C in 5% CO₂. On the third day, the cells were fixed to the plates by the addition of cold 50% trichloroacetic acid and incubation for 1 h. The plates were washed with tap water and were dried overnight. The fixed cells were dyed with a dilute acetic acid solution of sulforhodamine B, an anionic protein stain, and were incubated for 30 min. The plates were washed with dilute acetic acid and were allowed to dry. The dye was solubilized with

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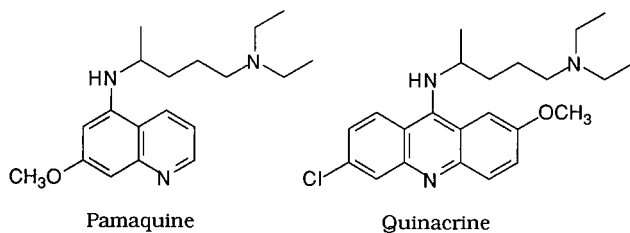


FIG. 1. Structures of pamaquine and quinacrine.

dilute Tris base, and the plates were read at 515 nm with an enzyme-linked immunosorbent assay reader. The absorbance due to the initial concentration of cells added to the plates was determined by fixing a separate plate on the day that the cells were seeded; this plate was dyed and the results were read along with those for the test plate(s). The percent cell survival was determined by subtracting the absorbance due to the initial concentration from the absorbance of the test plate and dividing this quotient by the absorbance of the plate containing DMSO minus the initial absorbance. The 50% effective doses were calculated by using semilogarithmic plots.

Calculation of selectivity index. An in vitro selectivity index was expressed for each dye as the 50% effective dose (in KB cells)/50% inhibitory concentration (IC_{50} ; in *P. falciparum*) (2) in an attempt to discriminate between those compounds which might selectively inhibit the malaria parasite versus those that exhibit nonspecific cellular toxicity.

Calculation of log P values: Log P values (where P is the octanol/water partition coefficient) for the dyes were calculated by using the log-linear solubilization model of Li and Yalkowsky (36), which relates the solubilization of solutes of known hydrophobicity in ethanol-water cosolvent systems by the following equation: $\sigma = 0.402 + 0.903 \log P$, where σ is $\log (S_c/S_w)$ (S_c is the solubility of the solute in ethanol, and S_w is the solubility of the solute in water). Data on the solubilities of thiazine dyes in water and ethanol were available from Green (24). A potential source of error in these data is the tendency of thiazine dyes to form dimers via hydrophobic bonding in water (8, 26, 53) but not in ethanol (20). For example, methylene blue forms face-to-face dimers with a dissociation constant of 1.7×10^{-4} (8). Indeed, substantial variation in the data for the solubilities of thiazine dyes in water and ethanol exists (38).

Statistical analysis of data. The program InStat 2.01 for the Macintosh was used to calculate the means \pm standard deviations and two-tailed *P* values by an unpaired Student's *t* test. Spearman's rank order correlation method (51) was used to test the hypothesis of independence (22).

RESULTS

For 18 of the 21 dyes tested, there was no statistical difference ($P \leq 0.05$) in the IC_{50} s (Table 1) for the D6 and W2 clones of *P. falciparum*. The exceptions were rhodamine B (P

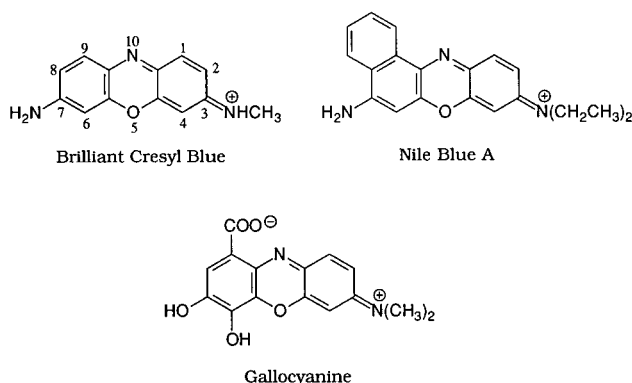


FIG. 3. Structures of oxazine dyes.

= 0.04), rhodamine 6G ($P = 0.04$), and Janus green B ($P = 0.0003$), each of which was more potent against the W2 clone, suggesting that these three dyes might be cross-resistant with mefloquine. For the 21 dyes tested, no correlation (Fig. 6) between antimalarial activity and cytotoxicity was observed, as measured by the Spearman rank order correlation coefficient ($r_s = 0.20$ and 0.29 for the D6 and W2 clones of *P. falciparum* versus KB cells, respectively).

In the xanthene structural class (Fig. 2), the three rhodamine dyes, with either free acid or esterified carboxyphenyl substituents at the 9 position, were two- to threefold more potent than the unsubstituted pyronine Y. There was no significant difference in antimalarial potency between the zwitterionic rhodamine B and the cationic rhodamines 123 and 6G; only rhodamine B was not cytotoxic. Of the dyes in the oxazine structural class (Fig. 3), gallocyanine, a zwitterionic carboxycatechol which lacks a C-7 amino functional group, was significantly less potent than either brilliant cresyl blue or niles blue A. For these three oxazines, antimalarial activity and cytotoxicity correlated well; however, this is not especially meaningful with these limited data. There was no statistical difference in potency for three azine dyes (phenosafranin, safranin O, methylene violet 3RAX) without an azo functional group. One of the azine azo dyes, indoine blue, was four- to sixfold less potent

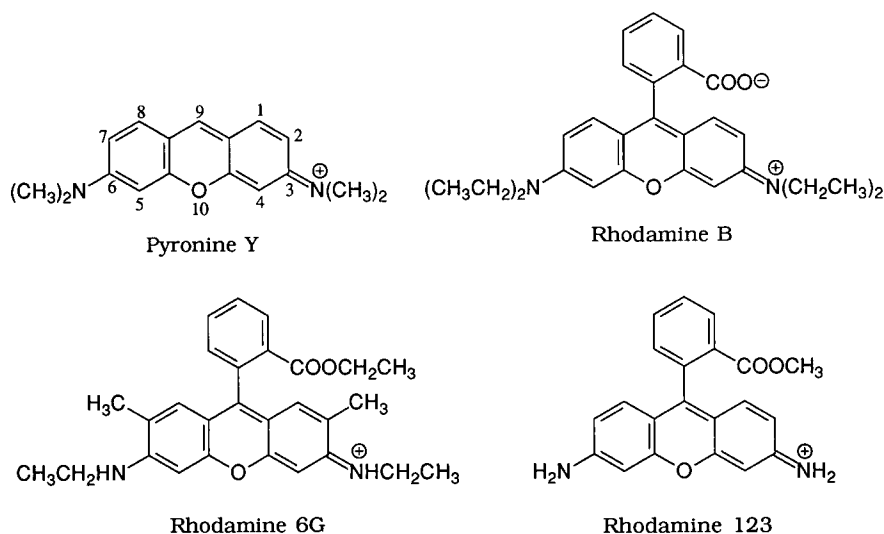


FIG. 2. Structures of xanthene dyes.

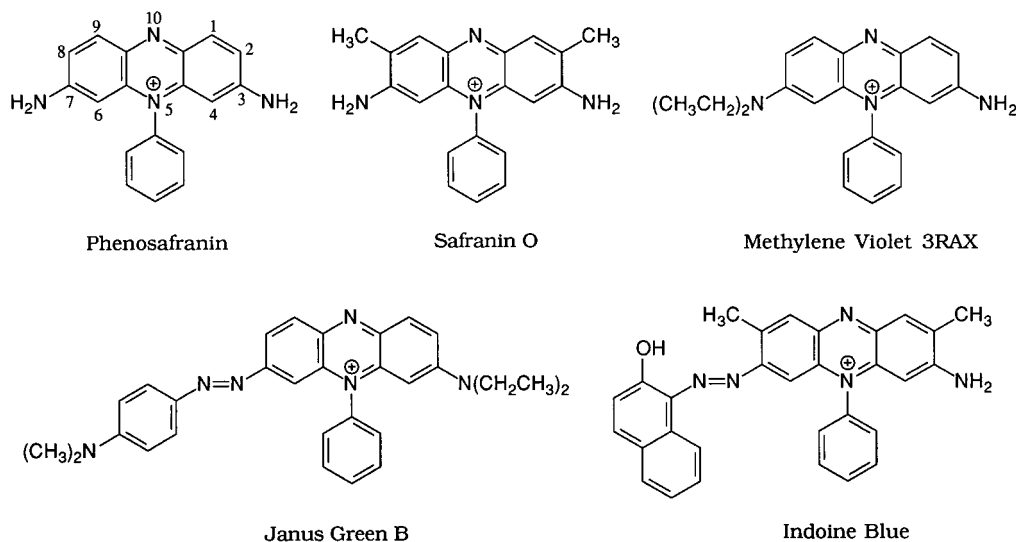


FIG. 4. Structures of azine dyes.

than the three azine dyes. The other azine azo dye, Janus green B, was unique for its conspicuously higher potency against the W2 clone than against the D6 clone of *P. falciparum*. The SN isolate, like the W2 clone, was strongly inhibited by Janus green B (data not shown), which suggests that Janus green B is not cross-resistant with either mefloquine or chloroquine. The entire group of azine dyes (Fig. 4) had very low levels of antimalarial selectivity.

As a structural class, the thiazine dyes (Fig. 5) had the highest level of antimalarial activity against both the D6 and W2 clones (Table 1) and a recently described (40) chloroquine-susceptible, mefloquine-resistant isolate (SN) of *P. falciparum* (data not shown). The thiazine dyes with the exception of new methylene blue were equally effective against both clones and the SN isolate; new methylene blue was an order of magnitude more potent than the other thiazine dyes against the SN isolate (data not shown). Only three thiazine dyes, methylene violet, new methylene blue, and 1,9-dimethyl methylene blue, had

IC₅₀s of ≥ 50 nM. Methylene blue, azure A, azure B, toluidine blue O, and brilliant cresyl blue had IC₅₀s of ≤ 20 nM. Only one other dye, the oxazine brilliant cresyl blue, was as potent as these thiazine dyes. The antimalarial potencies of the thiazine dyes varied little at incubation times of 72 and 48 h (data not shown). The order of antimalarial selectivity for this group of dyes was methylene blue > thionin \approx azure A > azure B \approx methylene green > toluidine blue O \approx brilliant cresyl blue. No rank order correlation between antimalarial activity and cytotoxicity ($r_s = -0.25$ and -0.28 for the D6 and W2 clones of *P. falciparum* versus KB cells, respectively) was evident for the thiazine dyes (data not shown).

With the exception of methylene violet, all of the thiazine dyes (Fig. 5) had amino or alkylamino functional groups at both the 3 and 7 positions; this lack of an amino functional group at the 3 position, as in methylene violet versus methylene blue, lowered the potency by an order of magnitude. There was no clear relationship between the type of alkylamino functional

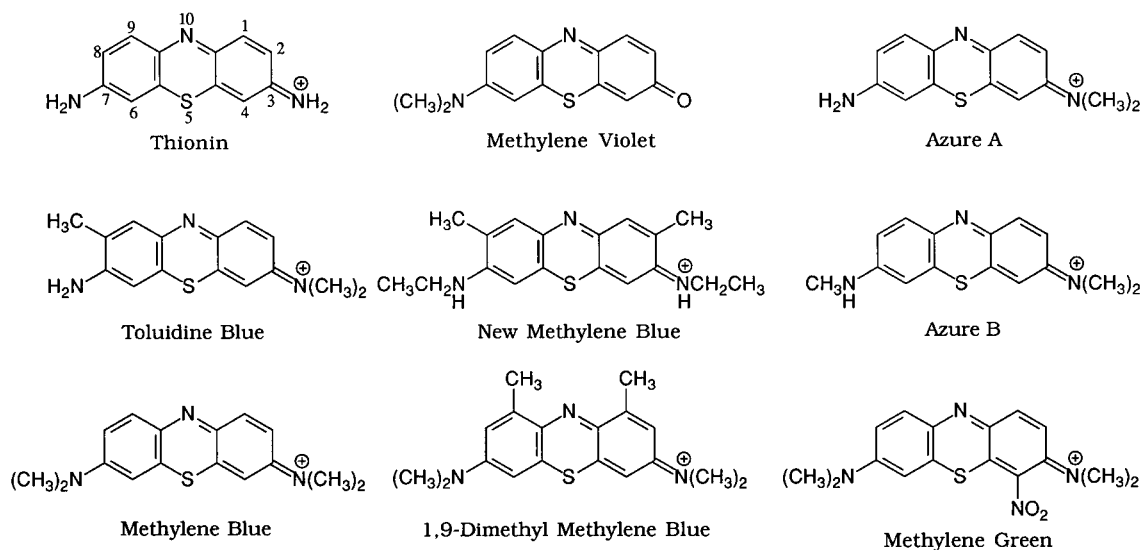


FIG. 5. Structures of thiazine dyes.

TABLE 1. Antimalarial activities and cytotoxicities of the dyes tested

Dye	IC ₅₀ (nM)		KB cells ^c	Selectivity index ^e
	<i>P. falciparum</i> ^b			
	D6	W2		
Pyronine Y	522 ± 117	449 ± 201	6,600	12.6, 14.7
Rhodamine 123	111 ± 49.1	153 ± 37.5	788	7.09, 5.15
Rhodamine B	146 ± 33.8	80.7 ± 30.2	29,400	201, 364
Rhodamine 6G	143 ± 52.2	66.8 ± 22.9	209	1.46, 3.13
Brilliant cresyl blue	9.66 ± 4.17	5.52 ± 3.06	259	26.8, 46.9
Niles blue A	51.0 ± 12.9	42.2 ± 24.9	1,640	32.2, 38.9
Gallocyanine	588 ± 34.2	674 ± 159	43,400	73.8, 64.4
Phenosafranin	88.2 ± 31.9	65.1 ± 18.1	310	3.51, 4.76
Safranin O	43.3 ± 29.3	34.8 ± 17.2	570	13.2, 16.4
Methylene violet 3RAX	73.9 ± 24.6	52.8 ± 16.5	528	7.14, 10.0
Janus green B	44.0 ± 9.86	4.85 ± 2.54	196	4.45, 40.4
Indoine blue	192 ± 63.4	239 ± 69.2	1,580	8.23, 6.61
Thionin	25.2 ± 6.51	34.6 ± 10.0	4,180	166, 121
Methylene violet	106 ± 30.2	122 ± 75.7	1,130	10.7, 9.26
Azure A	10.2 ± 4.8	10.5 ± 4.80	1,710	168, 163
Toluidine blue O	18.9 ± 9.22	10.2 ± 8.08	654	34.6, 64.1
New methylene blue	42.1 ± 15.5	57.7 ± 8.53	481	11.4, 8.34
Azure B	8.31 ± 4.32	12.7 ± 7.36	654	78.7, 51.5
Methylene blue	3.58 ± 2.22	3.99 ± 2.31	1,610	450, 404
1,9-Dimethyl methylene blue	60.6 ± 1.49	82.5 ± 38.0	287	4.74, 3.50
Methylene green	22.8 ± 8.18	25.9 ± 2.09	1,850	81.1, 71.4
Chloroquine	24.0 ± 4.92	122 ± 65.5	33,800 ^d	1,410, 277

^a Values are for clone D6, clone W2.

^b Values are means ± standard deviations ($n = 4$ to 6).

^c Values are averages for $n = 2$.

^d Data are from reference 37.

group and activity, although thiazines with alkylamino groups at these positions were more active than those with unsubstituted amino groups, as in azure A, azure B, and methylene blue versus thionin. The addition of two methyl groups on the thiazine ring, as in new methylene blue and 1,9-dimethyl methylene blue versus methylene blue, lowered the potency by an order of magnitude. The addition of a single methyl group, as in toluidine blue versus azure A, however, did not decrease the potency. A 4-nitro functional group lowered the potency, as in methylene green versus methylene blue.

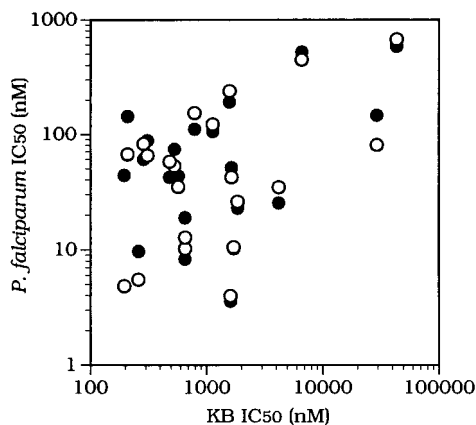


FIG. 6. Antimalarial activity against the D6 (●) and W2 (○) clones of *P. falciparum* versus cytotoxicity against KB cells for all dyes tested.

Correlations between antimalarial activity and three physicochemical properties of the thiazine dyes were examined. First, no correlation between thiazine dye log P and antimalarial activity was observed (data not shown). There was also no correlation between log P and either antimalarial activity or cytotoxicity for the dyes as a whole (data not shown). A log-linear solubilization model (36) was used to calculate log P values from the known, experimentally determined solubilities (24) of all dyes except rhodamine 123, brilliant cresyl blue, phenosafranin, and 1,9-dimethyl methylene blue in ethanol and water. Solubility data were unavailable for these dyes. All of the thiazine dyes had been previously tested (13) for their relative abilities to serve as catalysts for glucose oxidation at basic pH, data which might be expected to correlate with the relative efficiency of thiazine dye-catalyzed stimulation of the hexose monophosphate shunt (HMS) (3). There was no correlation, however, between the relative efficiency of dye-catalyzed glucose oxidation and antimalarial activity (data not shown).

DISCUSSION

As a structural class, the xanthenes were the least effective dyes, and the thiazine dyes were the most uniformly active. Only one of the three oxazine dyes tested, brilliant cresyl blue, was as effective as the more potent thiazine dyes. For all of the dyes tested, there was no observed cross-resistance with chloroquine, although the mitochondrial inhibitors rhodamines B and 6G and Janus green B were cross-resistant with mefloquine. Extensive screening against a number of drug-resistant

clones and isolates of *P. falciparum* will be required to confirm this observed lack of cross-resistance with chloroquine. Despite a lack of correlation between antimalarial activity against *P. falciparum* and cytotoxicity against the KB cell line for the group of dyes as a whole, the majority of the dyes were rather unselective antimalarial agents. Only rhodamine B, gallocyanine, thionin, azure A, azure B, methylene blue, and methylene green had selectivity indices of ≥ 50 . Of these dyes, two thiazines, methylene blue and azure A, had the best combination of intrinsic antimalarial activity and good selectivity. It is likely that other human cell lines may be more or less susceptible to this same group of dyes. For example, four of the same thiazines (thionin, azures A and B, and methylene blue) and one azine, phenosafranin, were not only much less potent against a human monocytic leukemia J111 cell line (IC_{50} range, 3,000 to 27,000 nM) (3), compared with our data for the KB human epidermoid carcinoma cell line (IC_{50} range, 300 to 4,000 nM), but they also had a quite different rank order of cytotoxic potency.

Our results with the rhodamines contrast somewhat with existing antimalarial data for these dyes (7, 27). Basco and Le Bras (7) report that rhodamines 123 and 6G are 3- to 10-fold less potent against the chloroquine-susceptible L-3 and chloroquine-resistant FCM29 clones of *P. falciparum* in comparison with the potency against the D6 and W2 clones indicated by our inhibition data. These two rhodamines were also significantly less potent against the FCM29 clone, suggestive of cross-resistance with chloroquine. Izumo and Tanabe (27) demonstrated that rhodamine 123 has an IC_{50} of 600 nM for the GGG strain of *P. falciparum*, and that 10 μ M rhodamine B and 10 μ M rhodamine 6G showed a 3-fold decrease and a 2.5-fold increase, respectively, in potency relative to the potency of rhodamine 123 at that same concentration. Rings and trophozoites were more susceptible than schizonts to rhodamine 123. In comparison, we found that all three rhodamines had similar antimalarial potencies. Our inhibition data for rhodamine 123 are, however, similar to those reported by Divo et al. (19). As estimated from their dose-response curves, rhodamine 123 had an IC_{50} of 150 nM for the chloroquine-resistant FCR3TC isolate of *P. falciparum* at 48 h of incubation. Those investigators also reported that the potency of rhodamine 123 increased by 2 orders of magnitude with an extended incubation time (96 h) but by only 1 order of magnitude with both an extended incubation time and high oxygen (17 versus 1%) tension.

Mitochondrial staining (28) in living cells is observed for positively charged rhodamines such as rhodamines 123 and 6G but not for zwitterionic rhodamines such as rhodamine B. Similarly, Divo et al. (19) and Kato et al. (30), using epifluorescence microscopy, revealed that rhodamine 123 selectively stains cellular structures resembling mitochondria in intraerythrocytic stages of *P. falciparum*. Fluorescence microscopy reveals that the GGG and FCR3 strains of *P. falciparum* accumulate rhodamine 123 and 6G but not rhodamine B (27, 29). Nevertheless, we observed a comparable inhibition of *P. falciparum* by each of these three rhodamine dyes, although only the zwitterionic rhodamine B had good antimalarial selectivity. Our results suggest that either an analogous drug accumulation differential does not exist for the rhodamines in the D6 and W2 clones of *P. falciparum* or, if it does, the parasite is more susceptible to rhodamine B than to rhodamines 123 and 6G. We can also infer that drug resistance in *P. falciparum* does not depend on the net charge of the rhodamine dye, as it does in mammalian cells (34), because both rhodamine B and 6G were nearly equipotent against both the D6 and the W2 clones.

The lack of antimalarial selectivity for the oxazines brilliant cresyl blue and Nile blue A is consistent with their known antitumor activities (43). Moreover, antimalarial activity and cytotoxicity correlated well for the three oxazine dyes tested (Fig. 3). Of possible interest is a discovery by Wagner and Maier (55) that Nile blue A is a selective stain for exoerythrocytic forms of *Plasmodium yoelli*, which hints that this or other oxazine dyes might inhibit tissue forms of plasmodia. Brilliant cresyl blue was the only nonthiazine dye with IC_{50} s of ≤ 10 nM for *P. falciparum* and is an isostere of azure C, a dye that we did not test. However, new data disclosed by Atamna et al. (3) indicate that azure C is as active as is thionin. These data combined suggest that oxazine isosteres of the azures and methylene blue might possess interesting antimalarial properties.

A similar lack of antimalarial selectivity was observed for each of the azine dyes (Fig. 4). The data suggested ($P = 0.06$) that safranin O is the most potent azine dye against both the D6 and the W2 clones of *P. falciparum*; safranin O also had the highest antimalarial selectivity. The azo azine dye Janus green B, a known mitochondrial poison (11, 35), was as active as safranin O against the D6 clone, but it was an order of magnitude more potent against both the W2 clone and the SN isolate, which suggests that the mechanisms of drug resistance to Janus green B, chloroquine, and mefloquine are dissimilar. Previous work (7, 18, 21) demonstrated that Janus green B is not cross-resistant with chloroquine. Basco and Le Bras (7) report that Janus green B is equipotent against the chloroquine-susceptible L-3 and chloroquine-resistant FCM29 clones of *P. falciparum* with IC_{50} s of 23 and 25 nM, respectively. As estimated from dose-response curves reported by Divo et al. (18), Janus green B had IC_{50} s of 5 nM for the chloroquine-resistant FCR3TC isolate of *P. falciparum* at 48 h of incubation. For this dye, an IC_{50} of approximately 1 pM was attained (18) with both an extended incubation time (96 versus 48 h) and a high oxygen (17 versus 1%) tension. In contrast, we noted a modest but significant ($P \leq 0.05$) decrease in the potency of Janus green B at incubation times of 72 and 48 h (data not shown). This decreased potency may have resulted from the inoculum effect (23), because we started with a higher initial percent parasitemia (2% versus 0.2 to 0.3%) (18). Ginsburg et al. (21) later reported IC_{50} s of Janus green B of between 0.05 to 0.16 nM for three chloroquine-resistant strains (FCR1, FCC1, FCR8) of *P. falciparum* cultivated in candle jars (17% oxygen). These IC_{50} s did not correlate with the known susceptibilities of the same strains to chloroquine (21), consistent with our observation that Janus green B was not cross-resistant with chloroquine.

As a structural class, the thiazine dyes (Fig. 5) were the most uniformly active antimalarial agents. Of these, methylene blue, azure A, azure B, and toluidine blue O were the most effective. The evident lack of correlation between antimalarial activity and cytotoxicity for the thiazine dyes implies that the cytotoxicity and antimalarial mechanism(s) of action may be independent. Methylene blue was notable for both its high level of antimalarial potency and its selectivity.

No significant difference in potency against the D6 and W2 clones and the SN isolate of *P. falciparum* was observed for any of the thiazines tested with the exception of new methylene blue, which was substantially more potent against the SN isolate than against either the D6 or the W2 clone. Atamna et al. (3) noted a similar lack of cross-resistance for three of these same thiazine dyes and azure C against these two clones, in addition to the chloroquine-resistant FCR3 clone. Although both the D6 clone and SN isolate are susceptible to chloroquine, this result with new methylene blue suggests a dissimi-

larity between the drug resistance mechanisms for the thiazine dyes, chloroquine, and mefloquine. This hypothesis is borne out by the results reported by Thurston (52), who succeeded in inducing only a modest drug resistance to methylene blue in *Plasmodium berghei* in vivo; after 4 months of treating acute infections with low doses of methylene blue, the minimum effective dose of methylene blue increased from 15 to 35 mg/kg of body weight. This *P. berghei* strain retained its susceptibility to pamaquine, quinacrine (a chloroquine structural analog), sulfadiazine, proguanil, and pyrimethamine.

Thiazine dyes (Fig. 5) with alkylamino functional groups at either or both the 3 and 7 positions had the highest intrinsic antimalarial activities. For example, two desmethyl methylene blue analogs, azures A and B, were only slightly less potent than methylene blue. Interestingly, among these dyes, only methylene blue is not a potential hydrogen bond donor. 1,9-Dimethyl methylene blue, with its two additional methyl groups on the thiazine ring, and methylene green, with its additional 4-nitro functional group, were each significantly less potent than methylene blue. On the basis of a reported (3) correlation between antimalarial potency and hematin (ferri-protoporphyrin-IX) binding for several of the same thiazine dyes, we hypothesize that the decreased potencies observed for 1,9-dimethyl methylene blue, methylene green, and new methylene blue results from steric inhibition to thiazine dye-hematin binding. It should be noted, however, that enhanced antimalarial selectivity has been reported for several methylene blue analogs with considerably greater steric bulk. These methylene blue analogs included those with higher alkyl and aminoalkyl groups substituted for the dimethylamino methyl groups in methylene blue (56). In any event, additional data on a more complete set of methylene blue analogs will be required before definitive statements can be made on antimalarial thiazine dye structure-activity relationships.

The relative lipophilicity of the thiazine dyes may be rather unimportant, because no correlation between log P and antimalarial activity was observed. In this respect, pharmacokinetic data for methylene blue reveal that, despite its cationic nature, the dye is rapidly and widely distributed throughout the body (16, 17). It is interesting that methylene blue can be converted to the azures thionin and methylene violet via a progressive oxidative demethylation process (38). However, the only urinary metabolite of methylene blue isolated in humans, in addition to unchanged methylene blue, is its reduction product, leukomethylene blue (16), although a portion of a given methylene blue does may undergo oxidative demethylation.

Although vital dyes (38) such as methylene blue are classified as "basic" dyes, this classification refers only to their cationic nature (5) and not to their acid-base properties. Indeed, methylene blue is an extremely weak base (pK_a , 0 to 1) (31, 46, 47). In contrast, desmethyl methylene blue analogs such as the azures, thionin, and toluidine blue O can undergo proton transfer to form a neutral species, a process with no counterpart in methylene blue (54), and are weak bases with pK_a s of between 10 and 12 (8, 10). Thus, it is unlikely that the antimalarial activities of thiazine dyes are a function of their acid-base properties; however, the weak base properties of the reduced forms of these dyes may be relevant. For example, pK_a s of between 8 and 9 for the one-electron reduction products, the radical cations (9, 31, 54), and two pK_a s of approximately 5 and 6 for the dibasic two-electron, fully reduced forms (leuko dyes) have been noted (12, 44). In contrast to the cationic thiazine dyes, the leuko forms of these dyes exist largely as the neutral species at physiological pH and as a mixture of cationic and dicationic forms in acidic cellular compartments such as the parasite food vacuole (pH 5.0 to 5.4) (33, 57). Given this pH

differential, the thiazine dyes would tend to concentrate inside infected rather than inside noninfected erythrocytes on the assumption that ionized forms of the leuko dyes are relatively membrane impermeant.

It is well known that methylene blue stimulates HMS (42) in erythrocytes. Methylene blue also stimulates HMS in erythrocytes infected with *Plasmodium knowlesi* (6, 49), *P. berghei* (15), and *P. falciparum* (4). At high levels of parasitemia (>90%) the HMS activity of *P. falciparum*-infected erythrocytes, although not fully activated, is 78-fold greater than that of normal erythrocytes; the HMS activity of the parasite itself contributes 82% of the total HMS activity of infected erythrocytes; the erythrocyte host cell HMS activity is increased 24-fold (4). Yet, it is unlikely that methylene blue or other thiazine dyes exert their antimalarial action via stimulation of the HMS, as shown by Atamna et al. (3), who found no correlation between HMS-potentiating activity and antimalarial activity for five thiazine dyes. Moreover, the minimum concentration of these same dyes which stimulate the HMS (1 μ M) was much higher than their antimalarial IC_{50} s (3). We also found a lack of correlation between the relative ability of the thiazine dyes to serve as catalysts (13) for glucose oxidation (data which might be expected to correlate with the relative ability to stimulate the HMS) and antimalarial activity.

The concluding sentence in the English translation of the famous paper by Guttman and Ehrlich (25) on the treatment of malaria with methylene blue was remarkably predictive of the present challenge of multiple drug resistance in malaria: "Likewise, the future will have to answer the very important question whether, with tropical forms of malaria which are so often resistant to quinine, a combination of quinine treatment with methylene blue treatment will bring about a cure." We are now investigating this and other methylene blue-drug combinations and will report our results shortly.

ACKNOWLEDGMENTS

This work was supported in part by DOD contract DAMD 1719C1015 (to Flow, Inc.), by NIAID grant 1 R29 AI34408-01A1 (to Cindy K. Angerhofer), and by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases grant M20/181/116 (to Jonathan L. Vennerstrom).

J.L.V. acknowledges helpful discussions with Hagai Ginsburg, particularly on the acid-base properties of the thiazine dyes.

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