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In Vitro Activity of Proveblue (Methylene Blue) on *Plasmodium falciparum* Strains Resistant to Standard Antimalarial Drugs▽

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The geometric mean 50% inhibitory concentration (IC_{50}) for Proveblue, a methylene blue complying with the European Pharmacopoeia, was more active on 23 *P. falciparum* strains than chloroquine, quinine, mefloquine, monodesethylamodiaquine, and lumefantrine. We did not find significant associations between the Proveblue IC_{50} and polymorphisms in the *pfcrt*, *pfmdr1*, *pfmdr2*, *pfmrp*, and *pfnhe-1* genes or the copy numbers of the *pfmdr1* and *pfmdr2* genes, all of which are involved in antimalarial resistance.

In 1891, Guttmann and Ehrlich were the first to report the antimalarial properties of a synthetic thiazine dye, methylene blue (MB), when they described the clinical cure of two patients after oral administration of MB (11). Cardamatis wrote in *Progrès Médical* that he had found MB to be very effective in the early stages of severe malaria cachexia in cases resistant to quinine (4). MB has shown *in vitro* activity against *Plasmodium falciparum* strains (2, 10) and isolates (1) and *in vivo* activity against *P. vinckei* and *P. yoelii* parasites (5).

Currently, there is no available MB across the world that complies with the European Pharmacopoeia. Indeed, up to now, the pharmaceutical use of MB has been stymied by contamination with organic impurities as well as heavy metals of recognized toxicity. Provence Technologies and its subsidiary, Provepharm, have conducted 4 years of research that resulted in the first European Pharmacopoeia-grade MB, Proveblue, obtained using a new innovative synthetic pathway with a heavy-metal-free process involving pharmaceutical-grade reagents (patent application no. FR06/06330 [July 2006, France], which has been extended to the international PCT reference PCT/FR/2007/001193). The sum of metals is <20 ppm, the quantity of azure B is <2% (the most important impurity in MB), and the quantity of other impurities is <0.5%. An analysis performed on commercial MB by an independent laboratory showed that officinal MB contained a quantity of cadmium higher than the level authorized by the European Pharmacopoeia. The industrial MB contained 94.45% of MB, while the European Pharmacopoeia requires a greater than 95% quantity of MB. Moreover, it contained 5.55% of azure B impurities, while the required quantity is below 5%. Those results showed that the currently marketed officinal and industrial MB do not comply with European Pharmacopoeia standards.

The aim of the present work was to assess the following: (i) the *in vitro* activity of Proveblue in comparison to that of commercial MB, to ensure that the previously described antimalarial activity was due to MB and not to contaminants; (ii) the *in vitro* activity of Proveblue in comparison to those of standard antimalarial drugs such as chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ) (the active metabolite of amodiaquine), mefloquine (MQ), lumefantrine (LMF), and dihydroartemisinin (DHA) (the active metabolite of artemisinin derivatives); (iii) the *in vitro* cross-resistance between Proveblue and standard antimalarial drugs; and (iv) the potential association of *in vitro* Proveblue responses with genetic polymorphisms in genes that are known or supposed to be associated with reduced quinoline susceptibility, such as the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*), the *P. falciparum* multidrug resistance-associated protein gene (*pfmrp*), the *P. falciparum* multidrug resistance protein 1 gene (*pfmdr1*), the *P. falciparum* Na^+/H^+ exchanger gene (*pfnhe1*), and the *P. falciparum* multidrug resistance protein 2 gene (*pfmdr2*) (3, 13, 14, 16).

Materials and methods. A total of 23 preidentified parasite strains from a wide panel of countries (Brazil, Cambodia, Cameroon, Comoros, Djibouti, the Gambia, French Guyana, Honduras, Indochina, Niger, Republic of Congo, Senegal, Sierra Leone, Sudan, Thailand, and Uganda) (15, 21) were maintained in culture as previously described (27) and verified using PCR genotyping of polymorphic genetic markers, including *msp1*, *msp2*, and microsatellite loci (6, 12). Each strain was tested for antimalarial activity in 6 to 21 experiments.

Proveblue was obtained from Provepharm SAS (Marseille, France). The two standard MB varieties were purchased from Cooper (France), one of "officinal" quality from a purified source (MBO) and the other of "industrial" quality (MBI).

The three methylene blue dyes were assessed in blind tests. The *in vitro* isotopic microtest used was previously described (21).

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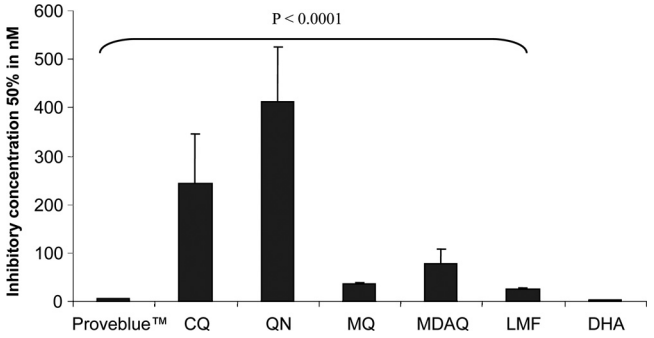

FIG. 1. *In vitro* activity of Proveblue (geometric mean) against 23 *P. falciparum* strains in comparison to chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ), mefloquine (MQ), lumefantrine (LMF), and dihydroartemisinin (DHA). Bars represent the 95% confidence interval.

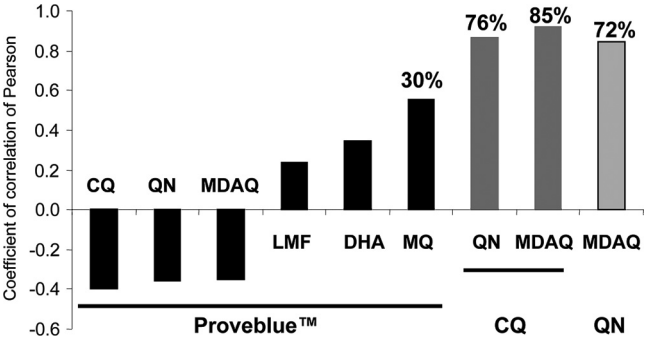

FIG. 2. *In vitro* Pearson's coefficients of correlation between the Proveblue responses (IC_{50}) and those to chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ), mefloquine (MQ), lumefantrine (LMF), and dihydroartemisinin (DHA), between the CQ responses and those to MDAQ and QN and between the QN and MDAQ responses. Coefficients of determination are indicated in boldface.

The methods for single nucleotide polymorphism (SNP) identification of *pfcrt*, *pfmdr1*, *pfmdr2*, and *pfmrp* (27), for the identification of *pfnhe-1* microsatellite profiles (9, 16), and for the estimation of the copy numbers of *pfmdr1* and *pfmdr2* (27) were previously described.

An assessment of the cross-resistance of standard antimalarial drugs with Proveblue was estimated by Pearson's coefficient of correlation (r) and the coefficient of determination (r^2). The Kruskal-Wallis test or the Mann-Whitney U test was used, when appropriate, to test for associations between the 50% inhibitory concentration (IC_{50}) and mutations.

Results and discussion. Twenty-three *P. falciparum* strains were tested for their *in vitro* susceptibilities to Proveblue, MBI, MBO, CQ, QN, MQ, MDAQ, LMF, and DHA. The geometric mean IC_{50} was 3.62 nM (95% confidence interval [CI]= 2.82 to 4.66) for Proveblue, 3.87 nM (CI = 3.08 to 4.87) for MBI, and 3.97 nM (CI = 2.83 to 5.57) for MBO. The same antimalarial activities were observed with the three qualities of MB. Proveblue was significantly more active than the officinal MB (P = 0.0020). Proveblue demonstrated a high antimalarial potency. The mean IC_{50} for each strain ranged from 0.6 nM to 9.4 nM. These data are consistent with previous findings for MB with organic impurities as well as inorganic impurities (heavy metals), with mean IC_{50}s ranging from 3 to 11 nM on *P. falciparum* strains (2, 10, 26) and of about 10 nM on Nigerian isolates (1). We proved that the antimalarial activity is not linked to the metal impurities but to the phenothiazine.

Moreover, this activity appeared to be 20 to 50 times higher than those of standard antimalarial drugs such as CQ, QN, MDAQ, MQ, and LMF ($P < 0.0001$) (Fig. 1). Proveblue is highly effective on *P. falciparum* strains with reduced susceptibilities to CQ, QN, MDAQ, or MQ, but it is significantly less active than DHA (P = 0.0004; geometric mean IC_{50} = 1.77 nM; CI = 1.39 to 2.26).

Encouragingly, no significant correlations were found between the Proveblue responses and CQ, QN, MDAQ, LMF, or DHA (Fig. 2), suggesting that no cross-resistance exists between Proveblue and the standard antimalarial drugs. This absence of cross-resistance suggests that Proveblue and CQ, QN, MDAQ, LMF, and DHA have different modes of action or that different mechanisms of resistance are involved. A significant positive correlation was shown between responses to Proveblue and MQ (r = 0.5519; P = 0.0063). Thirty percent of the variation in the response to Proveblue can be explained by variations in the responses to MQ.

The differences in the drug uptakes and/or modes of action of Proveblue and the other commonly used antimalarial drugs are reinforced by the lack of a significant association between the Proveblue IC_{50} ($0.0556 < P < 0.8248$) and the polymorphisms or copy numbers of genes involved in quinoline resistance, such as *pfcrt* for CQ and MDAQ (24), *pfmdr1* for MQ (23), and LMF (8), *pfnhe-1* for QN (16, 22), and *pfmrp* for CQ and QN (25) (Table 1), while significant associations were

TABLE 1. Associations of the *in vitro* responses (IC_{50}) of Proveblue and the polymorphisms in the *pfcrt*, *pfmdr1*, *pfmrp*, *pfnhe1*, and *pfmrp2* genes and copy numbers of the *pfmdr1* and *pfmdr2* genes of 23 strains of *Plasmodium falciparum*[a]

Genotype	*P* value	Significance
pfnhe-1 ms4760 profiles	0.2975	NS
pfnhe-1, no. of DNNND repeats	0.1768	NS
pfnhe-1, no. of NHNDNHNNDDD repeats	0.1026	NS
Mutation in codon 72 of the *pfcrt* gene	0.7628	NS
Mutation in codon 74 of the *pfcrt* gene	0.1011	NS
Mutation in codon 75 of the *pfcrt* gene	0.1011	NS
Mutation in codon 76 of the *pfcrt* gene	0.1654	NS
Mutation in codon 220 of the *pfcrt* gene	0.1210	NS
Mutation in codon 271 of the *pfcrt* gene	0.2370	NS
Mutation in codon 326 of the *pfcrt* gene	0.2911	NS
Mutation in codon 356 of the *pfcrt* gene	0.9394	NS
Mutation in codon 371 of the *pfcrt* gene	0.1011	NS
Mutation in codon 86 of the *pfmdr1* gene	0.5347	NS
Mutation in codon 184 of the *pfmdr1* gene	0.0926	NS
Mutation in codon 1034 of the *pfmdr1* gene	0.1939	NS
Mutation in codon 1042 of the *pfmdr1* gene	0.2962	NS
Mutation in codon 1246 of the *pfmdr1* gene	0.1554	NS
Copy no. of the *pfmdr1* gene (1 and >1)	0.5977	NS
Mutation in codon 208 of the *pfmdr2* gene	0.0676	NS
Mutation in codon 423 of the *pfmdr2* gene	0.4940	NS
Copy no. of the *pfmdr2* gene (only 1)	ND	
Mutation in codon 191 of the *pfmrp* gene	0.5793	NS
Mutation in codon 437 of the *pfmrp* gene	0.5793	NS

[a] Values are based on the Mann-Whitney U test or the Kruskal-Wallis test. NS, not significant; ND, not determined.

shown between the CQ, MDAQ, QN, and MQ responses and polymorphisms in the *pfcrt* gene and between the MDAQ and QN responses and polymorphisms in the *pfmrp* gene (not shown).

Twenty-three strains may not be a sufficient number on which to base definitive conclusions. The validity of the conclusions should be further investigated by analyzing more strains or isolates. Nevertheless, the high level of activity of Proveblue against all of the *P. falciparum* strains with reduced susceptibility to CQ, QN, MDAQ, or MQ, the absence of cross-resistance, and the lack of association with the proteins involved in quinoline resistance suggest that Proveblue could be a good partner in combination with current antimalarial drugs. Recent trials with different partner drugs, such as amodiaquine and artesunate (7, 28), conducted with children and adults in Burkina Faso provided evidence that MB (despite not complying with the European Pharmacopoeia) is safe and relatively effective in uncomplicated falciparum malaria. Nevertheless, the combination of MB and CQ is safe but not effective in malaria (17–20). In addition, MB showed a gametocytocidal effect that appeared to act on both existing and developing *P. falciparum* gametocytes (7).

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