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Proveblue (Methylene Blue) as an Antimalarial Agent: *In Vitro* Synergy with Dihydroartemisinin and Atorvastatin

Proveblue (international patent PCT/FR/2007/001193), which is a methylene blue preparation that complies with the European Pharmacopoeia and contains limited organic impurities and heavy metals of recognized toxicity, has previously been demonstrated to possess *in vitro* antimalarial activity (at a geometric mean 50% inhibitory concentration [IC_{50}] of 3.62 nM) against 23 *Plasmodium falciparum* strains that are resistant to various other antimalarials (11). No significant association was found between Proveblue IC_{50}s and polymorphisms in the genes that are involved in quinoline resistance, such as *pfcrt*, *pfmdr1*, *pfmdr2*, *pfmrp*, and *pfnhe-1*; furthermore, there was no significant association between Proveblue IC_{50}s and the copy numbers of *pfmdr1* and *pfmdr2* (11).

In the present study, we tested the effects of Proveblue in combination with the standard antimalarial drugs chloroquine (CQ), monodesethylamodiaquine (MDAQ; the active metabolite of amodiaquine), quinine (QN), mefloquine (MQ), and dihydroartemisinin (DHA) and with atorvastatin (AVA), a potential antimalarial drug (9, 12).

The methodology of the *in vitro* potentiating test was previously described (7). We used nine well-established *Plasmodium falciparum* strains that had different phenotypic profiles: 3D7, W2, Palo Alto, FCR3, FCM29, ImtVol, ImtK2, ImtL1, and Imt10500 (3). Each strain was assessed once in triplicate for eight concentrations of standard drugs in combination with 10 concentrations of Proveblue ranging from 0.004 to 10 nM.

While Proveblue was shown to have antagonistic effects in combination with CQ and additive effects in combination with MDAQ against the nine *P. falciparum* strains (Fig. 1), Proveblue exhibited noticeable synergistic effects in combination with MQ and QN but high synergistic effects in combination with DHA and AVA. CQ IC_{50}s were not significantly reduced in combination with Proveblue (Table 1). MQ and DHA IC_{50}s were significantly reduced from 12.6% to 31.54% and from 18.9% to 48%, respectively, when Proveblue was added at concentrations ranging from 0.04 to 0.63 nM (9- to 140-fold less than the mean Proveblue IC_{50} for the nine strains and 0 to 2% of the growth inhibition obtained when used alone).

These results were in agreement with the previous data on methylene blue noncompliant with the European Pharmacopoeia (Neph MB) that presented an antagonistic effect of Neph MB in combination with CQ against a CQ-resistant K1 strain but additive effects in combination with MQ and QN (2). More interestingly, the combination of Neph MB with artemisinin, artesunate, or artemether was found to act synergistically on the K1 strain (2). Garavito et al. found antagonism of Neph MB in combination with amodiaquine; additive effects in combination with CQ, MQ, and artemether; and synergy in combination with QN (5). Artemisinin induces a synergistic interaction with methylene blue; i.e., artemisinin reoxidizes leucomethylene blue, which is produced by reduction of methylene blue in parasites by the NADPH-flavin reductase system, in methylene blue, which both together oxidize $FADH_2$ (6). This oxidation is inhibited by CQ, which interferes with redox processes.

In a previous study, we demonstrated that there was no significant correlation between DHA and Proveblue IC_{50}s ($r^2 = 0.056$; $P = 0.275$) (11). All of these data suggest that Proveblue could be effective as a good partner with artemisinin derivatives. Recent trials using artesunate provided evidence that Neph MB (despite not complying with the European Pharmacopoeia) is safe and relatively effective in uncomplicated falciparum malaria (4, 15). In addition, Neph MB has a gametocytocidal effect both *in vitro* and *in vivo* (1, 4). As suggested by *in vitro* combination data, the combination of Neph MB and CQ is not sufficiently effective against malaria *in vivo* (8).

Proveblue demonstrated synergistic effects in combination with AVA, a synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A. AVA IC_{50}s were significantly reduced from 24.6% to 63.1% when Proveblue was added at concentrations ranging from

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TABLE 1 Reduction of the *in vitro* IC_{50}s of CQ, MDAQ, QN, MQ, DHA, and AVA in combination with Proveblue[a]

Antimalarial	Avg % IC_{50} reduction [95% CI] (*P* value) with Proveblue at:				
	0.04 nM[b]	0.08 nM[c]	0.16 nM[d]	0.31 nM[e]	0.63 nM[f]
CQ	4.3 [0.9–7.7] (0.250)	4.1 [0.6–7.6] (0.441)	8.8 [2.9–14.7] (0.130)	9.2 [1.1–17.4] (0.054)	11.8 [2.2–21.3] (0.054)
MDAQ	6.2 [0.–12.6] (0.859)	15.1 [6.1–24.0] (0.075)	15.4 [7.4–23.2] (**0.044**)	19.3 [8.3–30.3] (**0.039**)	17.4 [4.3–30.6] (**0.008**)
QN	3.0 [0.–6.3] (0.820)	7.5 [0.–17.4] (0.383)	8.3 [1.8.–15.7] (0.074)	15.3 [5.6–24.9] (**0.004**)	20.6 [12.1–29.0] (**0.009**)
MQ	12.6 [5.0–20.1] (**0.027**)	15.1 [5.3–25.0] (**0.020**)	20.9 [8.4–33.5] (**0.004**)	25.6 [14.0–37.3] (**0.004**)	31.5 [22.7–40.3] (**0.004**)
DHA	18.9 [8.3–29.4] (**0.012**)	23.7 [11.8–35.5] (**0.008**)	33.0 [19.3–46.7] (**0.008**)	41.2 [27.9–54.5] (**0.004**)	48.0 [32.6–63.3] (**0.009**)
AVA	24.6 [13.8–35.4] (**0.020**)	37.0 [18.1–56.0] (**0.020**)	43.6 [28.9–58.2] (**0.020**)	56.3 [40.8–71.8] (**0.020**)	63.1 [51.7–74.4] (**0.020**)

[a] CI, confidence interval. *P* values (for antimalarial plus Proveblue versus antimalarial alone) were determined by the Wilcoxon signed rank test. Significant *P* values (<0.05) are in bold.
[b] Mean IC_{50}/140.
[c] Mean IC_{50}/70.
[d] Mean IC_{50}/35.
[e] Mean IC_{50}/18.
[f] Mean IC_{50}/9.

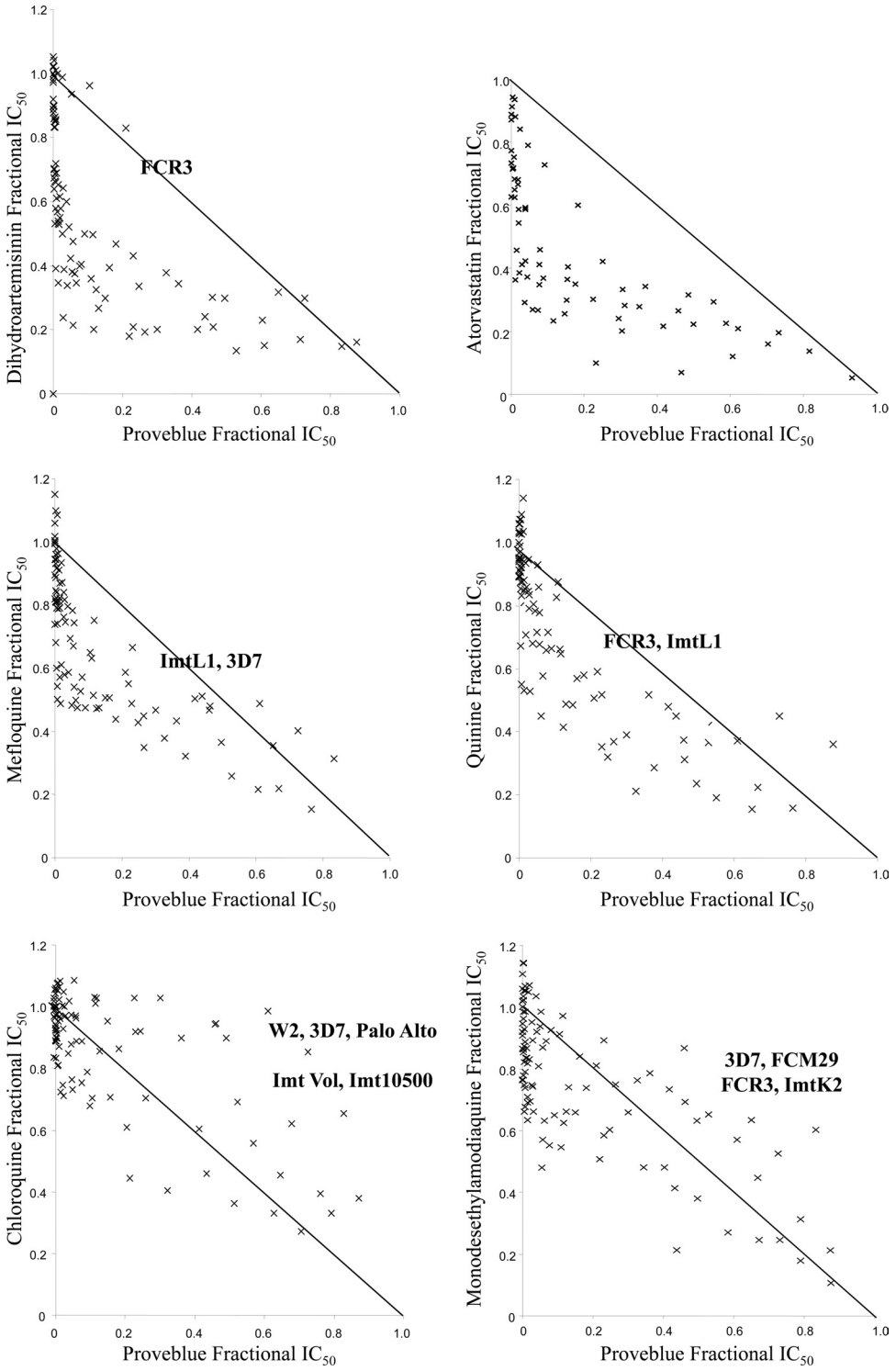

FIG 1 *In vitro* effects of Proveblue in combination with DHA, AVA, MQ, QN, CQ, and MDAQ against nine strains of *P. falciparum*. Strains with antagonistic effects are above the additivity line, strains with synergistic effects are below the line, and strains with additive effects are on the line.

0.04 to 0.63 nM. Like Proveblue, AVA improved the *in vitro* activity of MQ (14), QN (10), or DHA (13) and the IC_{50}s of AVA were unrelated to the mutations that occurred in the transport protein genes that are involved in quinoline resistance (9). The synergistic effect of AVA on MQ was significantly associated with the *pfmdr1* copy number (14). However, there was no association between Proveblue activity and the *pfmdr1* copy number (11). Even if we cannot explain the synergy between Proveblue and AVA, this observation supports the calls for *in vivo* evaluations in the murine malaria model.

These results confirm the therapeutic potential of Proveblue, which is a new methylene blue that contains limited organic im-

purities and heavy metals of recognized toxicity and could be integrated into new, low-cost antimalarial combination therapies.

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