Letter to the Editor

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

Proveblue (international patent no 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 IC$_{50}$s 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$_{50}$ and polymorphisms in the genes that are involved in quinoline resistance, such as pfcr1, pfmdr1, pfmdr2, pfmrp and pfhhe-1; furthermore, there was no significant association between Proveblue IC$_{50}$ and the copy numbers of pfmdr1 and pfmdr2 (11).

In the present study, we tested the effects of Proveblue in combination with standard antimalarial drugs, such as, chloroquine (CQ), monodesethylamodiaquine (MDAQ), the active metabolite of amodiaquine, quinine (QN), mefloquine (MQ), 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 P. falciparum strains that had different phenotypic profiles: 3D7, W2, Palo Alto, FCR3, FCM29, IntVol, IntK2, IntL1 and Int10500 (3). Each strain was assessed once in triplicate for 8 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 presented exhibited noticeable synergistic effects in combination with MQ and QN.
but high synergistic effects in combination with DHA and AVA. CQ IC₅₀s were not
significantly reduced in combination with Proveblue (Table 1). MQ and DHA IC₅₀s were
significantly reduced from 12.6% to 31.54% and 18.9% to 48%, respectively when adding
Proveblue at concentrations ranging from 0.04 to 0.63 nM (9 to 140 fold-less than Proveblue
IC₅₀ mean on the 9 strains and 0 to 2% of growth inhibition when used alone).

These results were in agreement with the previous data on methylene blue non compliant
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 and with 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 with artemether, respectively; and synergy in
combination with QN (5). Artemisinin induces synergistic interaction with MB: artemisinin
re-oxidizes leucomethylene blue, produced by reduction of MB in parasite by the NADPH-
flavin reductase system, in MB which both together oxidize FADH₂ (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 IC₅₀ (r² = 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 association of Neph MB and CQ is not sufficiently effective in vivo in
malaria (8).
Proveblue demonstrated synergistic effects in combination with AVA, a synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A. AVA IC₅₀ values were significantly reduced from 24.6% to 63.1% when adding Proveblue at concentrations ranging from 0.04 to 0.63 nM. Like Proveblue, AVA improved the in vitro activity of MQ (14), QN (10) or DHA (13), and the IC₅₀ values for 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 impurities and heavy metals of recognized toxicity and could be integrated into new, low-cost, antimalarial combination therapies.

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REFERENCES


TABLE 1. Diminution of the in vitro IC₅₀ of chloroquine (CQ), monodesethylamodiaquine (MDAQ), quinine (QN), mefloquine (MQ), dihydroartemisinin (DHA) and atorvastatin in combination with Proveblue

<table>
<thead>
<tr>
<th>Antimalarial</th>
<th>+ Proveblue at 0.04 nM (IC₅₀ mean/140)</th>
<th>+ Proveblue at 0.08 nM (IC₅₀ mean/70)</th>
<th>+ Proveblue at 0.16 nM (IC₅₀ mean/35)</th>
<th>+ Proveblue at 0.31 nM (IC₅₀ mean/18)</th>
<th>+ Proveblue at 0.63 nM (IC₅₀ mean/9)</th>
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<tr>
<td>CQ</td>
<td>4.3% [0.9-7.7] (P = 0.250)</td>
<td>4.1% [0.6-7.6] (P = 0.441)</td>
<td>8.8% [2.9-14.7] (P = 0.130)</td>
<td>9.2% [1.1-17.4] (P = 0.054)</td>
<td>11.8% [2.2-21.3] (P = 0.054)</td>
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<td>MDAQ</td>
<td>6.2% [0.040] (P = 0.075)</td>
<td>15.1% [6.1-24.0] (P = 0.044)</td>
<td>15.4% [7.4-23.2] (P = 0.044)</td>
<td>19.3% [8.3-30.3] (P = 0.039)</td>
<td>17.4% [4.3-30.6] (P = 0.008)</td>
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<td>QN</td>
<td>3.0% [0.0-6.3] (P = 0.820)</td>
<td>7.5% [0.0-17.4] (P = 0.383)</td>
<td>8.3% [1.8-15.7] (P = 0.074)</td>
<td>15.3% [5.6-24.9] (P = 0.004)</td>
<td>20.6% [12.1-29.0] (P = 0.009)</td>
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<td>MQ</td>
<td>12.6% [5.0-20.1] (P = 0.027)</td>
<td>15.1% [5.3-25.0] (P = 0.020)</td>
<td>20.9% [8.4-33.5] (P = 0.004)</td>
<td>25.6% [14.0-37.3] (P = 0.004)</td>
<td>31.5% [22.7-40.3] (P = 0.004)</td>
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<td>DHA</td>
<td>18.9% [8.3-29.4] (P = 0.012)</td>
<td>23.7% [11.8-35.5] (P = 0.008)</td>
<td>33.0% [19.3-46.7] (P = 0.008)</td>
<td>41.2% [27.9-54.5] (P = 0.004)</td>
<td>48.0% [32.6-63.3] (P = 0.009)</td>
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<td>AVA</td>
<td>24.6% [13.8-35.4] (P = 0.020)</td>
<td>37.0% [18.1-56.0] (P = 0.020)</td>
<td>43.6% [28.9-58.2] (P = 0.008)</td>
<td>56.3% [40.8-71.8] (P = 0.020)</td>
<td>63.1% [51.7-74.4] (P = 0.020)</td>
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FIG. 1. *In vitro* effects of Proveblue in combination with dihydroartemisinin, atorvastatin, mefloquine, quinine, chloroquine and monodesethylamodiaquine against nine strains of *P. falciparum*.

Strains with antagonistic effects were above the line of additivity; strains with synergistic effects were below the line of additivity and strains with additive effects were on the line.