Dose Responses of Proveblue Methylene Blue in an Experimental Murine Cerebral Malaria Model

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Proveblue, 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 (1). Proveblue exhibited noticeable synergistic effects in combination with mefloquine and quinine and high synergistic effects in combination with dihydroartemisinin, the active metabolite of artemisinin derivatives (2). Treatment with 10 mg/kg of body weight of Proveblue for 5 days significantly reduced or prevented cerebral malaria (CM) in mice (3, 4). However, this dose was too high to show synergistic effects in combination with dihydroartemisinin or atorvastatin in malaria cerebral prevention.

The objective of the present work was to evaluate the *in vivo* efficacy of Proveblue at several doses in a murine model of experimental CM and determine doses for future *in vivo* combination evaluation.

Fifty-six female C57Bl6/N mice (Charles Rivers, France) were infected on day 0 (D0) with *Plasmodium berghei* ANKA parasites by intraperitoneal inoculation with 10⁵ parasitized erythrocytes. All experiments adhered to French guidelines for animal research and were approved by the ethical committee of the Institut de Recherche Biomdicale des Armes (number 2012-04). The mice were treated when the parasitemia reached 0.1% by daily intraperitoneal (i.p.) injection with a normal saline solution (control) or Proveblue dissolved in a saline solution (3 and 10 mg/kg for 3 days; 1, 3, and 10 mg/kg for 5 days). The parasitemia, clinical signs, neurological symptoms, and weight were supervised as previously described (4).

In the control group, all the mice died between D10 and D14, with specific signs of CM and parasitemia ranging from 3.4 to 7.2% (Fig. 1). The observed symptoms for CM were consistent with those of previous publications (3–7). There was a significant difference between the control and treated mouse groups (0.00002 < *P* < 0.0028). In the mice treated with 10 mg/kg Proveblue for 5 days, one mouse (10%) died at D14 (10.5% parasitemia) with specific signs of CM and another died at D20 (85.9% parasitemia) with no specific signs of CM. Similar results were obtained for 10 mg/kg Proveblue for 5 days, with 78% of mice surviving after treatment (3, 4). In the mice treated with 3 mg/kg for 5 days, three mice (30%) died between D23 and D26 (parasitemia between 81.3 and 93.1%) with no specific signs of CM. In the mice treated with 1 mg/kg Proveblue for 5 days, two mice (20%) died at D8 (4.7% parasitemia) and D12 (1.8% parasitemia) with specific signs of CM and two others (20%) died at D20 (88.0% parasitemia) and D25 (93.1% parasitemia) with no specific signs of CM. In the mice treated with 10 mg/kg for 3 days, one mouse (11%) died at D11 (4.2% parasitemia) with specific signs of CM and six mice (66%) died between D26 and D35 (>86.9% parasitemia) with no specific signs of CM. In the mice treated with 3 mg/kg for 3 days, six mice

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FIG 1 Survival curve (Kaplan-Meier log-rank test) of the C57Bl6/N mice infected on day 0 (D0) with *P. berghei* ANKA parasites and treated daily with 10 mg/kg, 3 mg/kg, or 1 mg/kg Proveblue for 5 days or with 10 mg/kg or 3 mg/kg Proveblue for 3 days.

(60%) died between D23 and D36 ($>91.6\%$ parasitemia) with no specific signs of CM. At D39, the surviving mice had no detectable parasites in the five treated groups. The group treated with 10 mg/kg for 3 days was the only group that was significantly different from the group treated with 10 mg/kg for 5 days ($P = 0.0284$).

The treatment with Proveblue was significantly effective against CM ($P \leq 0.0028$). The dosage of 10 mg/kg for 5 days, the most effective in this study, has been shown to be safe in children treated with methylene blue that was noncompliant with the European Pharmacopoeia (8, 9). Daily treatment for 5 days was markedly more effective than the 3-day treatment (80% versus 23% for 10 mg/kg and 70% versus 40% for 3 mg/kg). However, the efficacy of Proveblue, and especially the low-dose regimen of 5 or 3 days, was able to be improved by the combination with antimalarial drugs, such as artemisinin derivatives, or drugs with a long elimination half-life, such as piperazine, thereby reducing the potential risk of toxicity. In addition, the oral route should be evaluated in the future.

The present work demonstrated the high efficacy of Proveblue in CM. There was a nonsignificant dose response with Proveblue in preventing CM (i.e., 60% at 1 mg/kg for 5 days, 70% at 3 mg/kg, and 80% at 10 mg/kg). Another advantage of its use is that methylene blue inhibits the maturation of gametocytes and their transmission (10, 11). These results confirm the therapeutic potential of Proveblue, which is a new methylene blue formulation that contains limited organic impurities and heavy metals of recognized toxicity.

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