Dihydroethanoanthracene Derivatives as In Vitro Malarial Chloroquine Resistance Reversal Agents

Julie Millet,1,2 Marylin Torrentino-Madamet,1,2 Sandrine Alibert,2,3 Christophe Rogier,1,2 Christiane Santelli-Rouvier,2,3 Joel Mosnier,1,2 Eric Baret,1,2 Jacques Barbe,2,3 Daniel Parzy,1,2 and Bruno Pradines1,2*

Unité de Parasitologie, Institut de Médecine Tropicale du Service de Santé des Armées,1 GERCTOP-UMR CNRS 6009, Faculté de Pharmacie,2 and Institut Fédératef de la Recherche 48,3 Marseille, France

Received 15 December 2003/Returned for modification 28 January 2004/Accepted 10 March 2004

The ability of four 9,10-dihydroethanoanthracene derivatives (BG920, BG932, BG958, and BG996), as well as verapamil and promethazine, to reverse chloroquine resistance was assessed against 24 chloroquine-resistant and 10 chloroquine-susceptible strains of Plasmodium falciparum from different countries. The 9,10-dihydroethanoanthracene derivatives clearly increase chloroquine susceptibility only in chloroquine-resistant isolates.

The current option for reducing the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. Until recently, chloroquine (CQ) has been a key weapon in the fight against this disease. During the past 20 years, there has been an emergence of strains of Plasmodium falciparum resistant to CQ and other antimalarial drugs (27, 29). Failures of antimalarial prophylaxis with CQ, the combination of CQ and proguanil (4), and mefloquine (15, 23) and clinical failures with halofantrine (8) and quinine (S. Molinier, P. Imbert, D. Verrot, M. Morillon, D. Parzy, and J. E. Touze, Letter, Presse Med. 23:1484, 1994) have been observed in Africa. The spread of antimalarial drug-resistant strains increased the prevalence of malaria (29), which affects 40% of the world’s population and kills more than 1 million people each year. One strategy that can be pursued to reduce the prevalence of malaria is to “reverse” CQ resistance chemically. In the past decade, several compounds, such as verapamil (VER) (1, 17, 24), desipramine (6, 7, 10–12), and antihistaminic drugs (5, 16, 19–21, 26), have demonstrated promising capability to reverse the CQ resistance in parasite isolates in vitro, in animal models, and in human malaria.

The aim of this study is to determine the capacity of four 9,10-dihydroethanoanthracenes (DEAs), BG920, BG932, BG958, and BG996 (Fig. 1), to reverse the resistance of several isolates of P. falciparum from different countries to CQ. These DEAs were selected from about 60 compounds. They exert more than 80% of in vitro reversal of CQ resistance at 1 μM (2). Their reversal activity 50% inhibitory concentrations (IC50s), i.e., the drug concentration of a modulator which induces an increase of 50% of the parasite susceptibility to CQ, are statistically better than those of VER or promethazine (PRM) (2). These chemosensitizers are less hemotoxic and cytotoxic in vitro than VER or PRM (2). In addition, it has been reported in previous studies that these DEAs increase the CQ accumulation in CQ-resistant (CQR) parasites 5 to 8 times more than PRM (2, 22).

Isolates of P. falciparum. Thirty-four strains of Plasmodium falciparum, 27 isolates and 7 clones from several countries, were used for this study. Twenty-five isolates were African strains originating from nine countries, including Cameroon (two strains), Comoros (seven strains), Ivory Coast (four strains), Djibouti (two strains), Gabon (one strain), Gambia (one strain), Niger (two strains), Uganda (one strain), Senegal (five strains), and an undetermined African country (one strain). Six isolates came from three Asian countries, including Cambodia (four strains), Indochina (one strain), and Thailand (one strain). Additionally, one strain came from Brazil, and another was a Caribbean strain. Among the 34 parasites, 24 were CQR and 10 were CQ susceptible (CQS). All parasites were maintained in culture in RPMI 1640 medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% human serum and buffered with 25 mM HEPES and 25 mM NaCl (hematocrit, 1.5%; parasitemia, 0.5%).
Drugs. The synthesis of BG920, BG932, BG958, and BG996 was described previously (2, 3, 14). CQ, VER, and PRM were obtained from Sigma Chemical (St. Louis, Mo.). CQ was diluted in sterile distilled water. DEA derivatives, VER, and PRM were diluted in methanol. Final concentrations of CQ distributed for the evaluation of drug interaction ranged from 25 to 3,210 nM for resistant strains and from 3 to 200 nM for susceptible strains. VER, PRM, and DEAs were used at the following concentrations, corresponding to 1/5 and 1/20, respectively, of their intrinsic IC50s against the CQR clone W2: 2,600 and 650 nM for VER, 3,700 and 925 nM for PRM, 860 and 215 nM for BG920, 420 and 105 nM for BG932, 2,350 and 585 nM for BG958, and 1,600 and 400 nM for BG996. At these concentrations, none of the molecules has detectable antimalarial activity.

In vitro assay. For in vitro isotopic microtests to assess synergy between CQ and DEA compounds, 25 μl of CQ, 25 μl of the drug tested, and 200 μl of the suspension of parasitized red blood cells (final parasitemia, 0.5%; final hematocrit, 1.5%) were distributed in 96-well plates. Parasite growth was assessed by adding 1 μCi of [3H]hypoxanthine with a specific activity of 14.1 Ci/mmol (Amersham, Buckinghamshire, United Kingdom) to each well at time zero. The plates were then incubated for 48 h at 37°C in an atmosphere of 10% O2, 5% CO2, and 85% N2, with a humidity of 95%. Immediately after incubation, the plates were frozen and then thawed to lyse...
erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter GF/B; Perkin-Elmer, Courtaboeuf, France) and washed by using a cell harvester (FilterMate Cell Harvester; Perkin-Elmer). Filter microplates were dried, and 25 μl of scintillation cocktail (Microscint O; Perkin-Elmer) was placed in each well. Radioactivity incorporated by the parasites was measured with a scintillation counter (Top Count; Perkin-Elmer).

The IC$_{50}$, i.e., the drug concentration corresponding to 50% of the uptake of $[^3H]$hypoxanthine by the parasite in drug-free control wells, was determined by nonlinear regression analysis of log dose-response curves. Data were analyzed after logarithmic transformation and expressed as the geometric mean IC$_{50}$.

**Evaluation of drug effect.** To evaluate the capacities of the four DEAs, VER, and PRM to potentiate CQ activity against isolates of *P. falciparum*, an activity enhancement index of CQ was defined as the difference between the IC$_{50}$ measured with CQ alone and the IC$_{50}$ measured for CQ associated with molecules of potentiation divided by the IC$_{50}$ measured for CQ alone.

**Results.** The CQ IC$_{50}$ ranged between 156 and 858 nM for isolates resistant to CQ and between 17 and 65 nM for parasites susceptible to CQ (Fig. 2).

The CQ IC$_{50}$ against CQ-resistant isolates and clones were not significantly modified by combination with DEAs, VER, or PRM (Fig. 2). CQ IC$_{50}$ against CQ-resistant isolates and clones were significantly reduced when combined with DEAs, VER, or PRM ($P < 0.001$). The CQ IC$_{50}$ estimated in the presence of CQ were significantly higher than the CQ IC$_{50}$ estimated in the presence of CQ associated with molecules of potentiation ($P < 0.001$).

The activity enhancement index of CQ by each molecule of potentiation at the studied concentration was significantly higher for CQ-resistant isolates compared to CQ-susceptible parasites ($P < 0.0001$). Potentiation of CQ activity on CQ-resistant parasites is dose dependent ($P < 0.001$) (Fig. 2).

The activity enhancement index of CQ in the presence of BG958 was significantly higher than the indices measured in the presence of the other DEAs ($P < 0.005$). The increase of CQ activity in the presence of BG958 was equivalent to that observed in the presence of PRM for CQR isolates. BG958 at 585 and 2,350 nM reversed in vitro the CQ resistance, i.e., decreased CQ IC$_{50}$ below the threshold of 100 nM, in 42% (10 out of 24) and 92% (22 out of 24) of CQR isolates, respectively (Table 1).

**Discussion.** Quinoline resistance in *P. falciparum* is frequently compared to multidrug resistance in mammalian cells, in part because of the observations that CQ resistance could be reversed by P-glycoprotein substrates such as VER and PRM. Our results suggest that the mechanism of action of the DEAs is associated with resistance properties; the DEA reversal of resistance in *P. falciparum* is specific for resistant parasites. DEAs clearly increase CQ susceptibility in CQR isolates, although not to the levels of the naturally susceptible strains; this result has been reported with very few isolates (one to three isolates) for VER (9), desipramine (7, 12), and PRM (19). These DEAs fully or partially reversed resistance in all CQR isolates from all different geographic regions (Africa, Asia, and South America).

**Table 1. Reversion of CQ strains by combinations of CQ and DEAs**

<table>
<thead>
<tr>
<th>Drug with CQ (concin µM)</th>
<th>Reversed strains for avg intrinsic IC$_{50}$ fractions of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/20</td>
</tr>
<tr>
<td>B6920 (4.3)</td>
<td>9/24</td>
</tr>
<tr>
<td>B6932 (2.1)</td>
<td>7/24</td>
</tr>
<tr>
<td>B6945 (4.5)</td>
<td>10/24</td>
</tr>
<tr>
<td>B6996 (8)</td>
<td>6/24</td>
</tr>
<tr>
<td>VER (13)</td>
<td>2/24</td>
</tr>
<tr>
<td>PRM (18.5)</td>
<td>8/24</td>
</tr>
</tbody>
</table>

*a* For CQR strains, IC$_{50} > 100$ nM.

CQ resistance can be either fully or partially sensitive to the presence of VER, and recent reports suggest that this behavior may be associated with the presence of specific sequences in the pfCRT gene, which encodes a vacuolar transport protein (13). While genetic differences in CQ resistance are known (13, 18, 28), the reverse phenotypic response may exist in all geographic regions, independent of the allelic variations. However, it has been reported that VER reversibility was more pronounced in clones expressing recombinant pfCRT or strains from the Old World carrying the pfCRT CVIET haplotype than in parasite isolates or clones expressing the recombinant New World allele carrying the SVMNT haplotype (18, 25). It would be interesting to know if these pfCRT polymorphisms were related to or were independent of the DEA CQ sensitization potential.

BG958 fully or partially reversed resistance in all CQR strains. The observation reported here suggests that BG958 is a good candidate for further studies. Evaluation of its in vivo reversal of CQ resistance and association with pfCRT and pfmdr1 polymorphisms is ongoing. Furthermore, the use of BG958 in combination with CQ, due to the low cost of BG958 synthesis, may be an economically viable proposition for developing countries and may be a more effective strategy than the introduction of another antimalarial drug at the national level.

This work is supported by the Délégation Générale pour l’Armement (DGA001 no. 010808/03-6). This work was carried out as a part of the COST B16 European Program.

**REFERENCES**


