IN VITRO REVERSAL OF CHLOROQUINE RESISTANCE IN PLASMODIUM FALCIPARUM WITH DIHYDROETHANOANTHRACENE DERIVATIVES

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Abstract. The effects of combining four dihydroethanoanthracenic (DEA) derivatives and chloroquine were assessed in vitro against Plasmodium falciparum chloroquine resistant parasites W2, Palo Alto, FCR3, and Bres1. Like verapamil or promethazine, the four dihydroethanoanthracenic derivatives tested can be added to the growing list of agents that show capability in enhancing the activity of chloroquine against resistant parasites. The structurally related tricyclic antihistaminic compounds examined in this study exerted different intrinsic antimalarial activity, but the same chloroquine-potentiating activity as verapamil or promethazine. They may act both on the rate of chloroquine accumulation and on its access to ferrirrthroporphyrin IX. The reversal mechanism would be assumed to result from competition between DEA derivatives and chloroquine for efflux translocation sites, thus causing an increase in steady-state accumulation of chloroquine and a return to susceptibility. Restoration of therapeutic efficacy of chloroquine against resistant parasites by the administration of an additional drug available at relatively low cost may be a more effective strategy than the introduction of another antimalarial drug at the national level.

INTRODUCTION

The current options for reducing the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. During the past 20 years there has been an emergence of strains of Plasmodium falciparum resistant to chloroquine and other antimalarial drugs.1,2 Failures of antimalarial prophylaxis and chemotherapy are chemoprophylaxis and chemotherapy for drug design and therapy to limit this emergence and spreading of parasite resistance to currently used antimalarial drugs. First, novel compounds need to be discovered and developed by identification of novel chemotherapeutic targets.8 The second approach, the reversal of chloroquine resistance by compounds with little intrinsic antimalarial activity is a well-established chemotherapeutic alternative.9 Several compounds such as verapamil,10–13 desipramine,14–17 and antihistaminic drugs18–20 have demonstrated in the past decade promising capability to reverse the chloroquine resistance in parasite isolates in vitro, in animal models21,22 and human malaria.23,24

The aim of the present study was to determine in vitro intrinsic antimalarial activity of four dihydroethanoanthracenic (DEA) derivatives against Plasmodium falciparum chloroquine resistant parasites W2, Palo Alto, FCR3 and Bres1, and to assess their synergistic interaction in reversing chloroquine resistance.

MATERIALS AND METHODS

Strains of P. falciparum. Four chloroquine resistant parasites (W2 [Indochina], Palo Alto [Uganda], FCR3 [Gambia], and Bres1 [Brazil]) were maintained in culture. When required for drug assays, cultures were synchronized by lysis with sorbitol.25 Susceptibilities to chloroquine and DEA derivatives were determined after suspension in RPMI 1640 medium (Life Technologies, Paisley, United Kingdom), supplemented with 10% human serum (pooled from different A+ or AB sera from non-immune donors who did not reside in an area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM NaHCO3 (hematocrit = 1.5%, parasitemia = 0.5%).

Drugs. The synthesis of BG 920 and BG 958 was described previously.26,27 For BG 996, the starting compound is the corresponding 11-carboethoxy-9,10-dihydro-9,10-ethanoanthracene, which was saponified with NaOH/H2O/methanol. The corresponding acid chloride was then treated with methyamine and the product obtained was reduced with LiAlH4. For BG 1001 the starting compound is the corresponding 11-carboxymethoxy-12-carboxy-9,10-dihydro-9,10-ethanoanthracene, which was treated in the same way as BG 996. The chemical structures of these agents are shown in Figure 1.

Chloroquine was obtained from Sigma (St. Louis, MO). Stock solutions were prepared in sterile distilled water for chloroquine and in ethanol-water (2:23 [v/v]) for the DEA derivatives promethazine and verapamil. Two-fold serial dilutions were prepared in RPMI 1640 medium for all of these drugs. Final concentrations distributed for the evaluation of drug interaction ranged from 25 nM to 3,200 nM for chloroquine, 25 μM to 0.025 μM for verapamil, 50 μM to 0.01 μM for promethazine, 25 μM to 0.01 μM for BG 920, 25 μM to 0.025 μM for BG 958, 50 μM to 0.01 μM for BG 996, and 100 μM to 0.05 μM for BG 1001.

In vitro assay. For in vitro isotopic microtests to determine intrinsic activity, 25 μL/well of antimalarial agents and 175 μL/well of the suspension of parasitized erythrocytes (final parasitemia = 0.5%, final hematocrit = 1.5%) was distributed in 96-well plates. To assess synergy between chloroquine and DEA compounds, 25 μL of chloroquine, 25 μL of subinhibitory fixed concentrations of the drugs tested, and 150 μL of the suspension of parasitized red blood cells (final parasitemia = 0.5%, final hematocrit = 1.5%) were distributed in each well. Parasite growth was assessed by adding 1 μCi of 3H-hypoxanthine with a specific activity of 14.1 Ci/mmol (New England Nuclear Products, Dreieich, Germany) to each well at t0. The plates were then incubated for 48 hours at 37°C in an atmosphere of 10% O2, 6% CO2, 84% N2, and a hu-
midity 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 (Uni-fil™ GF/B; Packard Instrument Company, Meriden, CT) and washed using a cell harvester (FilterMate™ Cell Harvester; Packard Instrument Company). Filter microplates were dried and 25 μL of scintillation cocktail (Microscint™ O; Packard) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count™; Packard Instrument Company).

The 50% inhibitory concentration (IC50), i.e., the drug concentration corresponding to 50% of the uptake of 3H-hypoxanthine by the parasites in drug-free control wells, was determined by non-linear regression analysis of log-dose/response curves. Data were analyzed after logarithmic transformation and expressed as the geometric mean IC50 and 95% confidence intervals (CIs) were calculated.

**Evaluation of drug interactions.** To evaluate modulation of chloroquine resistance by DEA compounds, isobolograms were constructed by plotting a pair of fractional IC50 values for each combination of chloroquine and the different compounds and for both parasite strains. The different DEA derivatives fractional IC50 was calculated by dividing their fixed concentrations by the IC50 of tested drugs alone and plotted on the horizontal axis. The corresponding chloroquine fractional IC50 was calculated by dividing the IC50 of chloroquine combined with fixed concentrations of DEA derivatives and plotted on the vertical axis. Points lying above the straight diagonal line (corresponding to the points where there is no interaction between the drugs) are antagonistic, points below the straight diagonal line are considered to be synergistic.28

**RESULTS**

Average parameter estimates for intrinsic antimalarial activity of DEA compounds against the chloroquine resistant parasites W2, FCR3, Bres1, and Palo alto are shown in Table 1. Isobolograms of the interactions between chloroquine and the four DEA derivatives, verapamil, and promethazine in the four chloroquine-resistant strains are shown in Figure 2. The six drugs enhanced chloroquine susceptibility of these parasite strains. The IC50 value for chloroquine against W2 parasites was found to decrease by 50% using verapamil at a concentration of 0.8 μM, promethazine at 0.5 μM, BG 1001 at 0.6 μM, BG 920 at 0.4 μM, BG 996 at 0.3 μM, and BG 958 at 0.2 μM.

**DISCUSSION**

These results indicate that the four DEA derivatives tested can be added to the growing list of agents that show the capability of enhancing the activity of chloroquine against resistant parasites. Structurally related tricyclic antihistaminic compounds examined in this study exerted different intrinsic activity but the same chloroquine-potentiating activity. The IC50 value for chloroquine against W2 parasites was found to decrease by 50% using verapamil at a concentration of 0.8 μM, promethazine at 0.5 μM, BG 1001 at 0.6 μM, BG 920 at 0.4 μM, BG 996 at 0.3 μM, and BG 958 at 0.2 μM.
TABLE 1

Intrinsic in vitro antimalarial activity of dihydroethanoanthracenic compounds and verapamil and promethazine against the chloroquine-resistant strains W2, FCR3, Bres1, and Palo Alto of Plasmodium falciparum

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Mean IC₅₀ in μM (95% confidence intervals)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W2</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.699 (0.590–0.829)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>13.2 (11.4–15.2)</td>
</tr>
<tr>
<td>Promethazine</td>
<td>18.5 (17.2–19.7)</td>
</tr>
<tr>
<td>BG 920</td>
<td>4.0 (3.8–4.1)</td>
</tr>
<tr>
<td>BG 958</td>
<td>10.1 (9.6–10.6)</td>
</tr>
<tr>
<td>BG 996</td>
<td>8.0 (7.4–8.6)</td>
</tr>
<tr>
<td>BG 1001</td>
<td>32.1 (26.7–38.7)</td>
</tr>
<tr>
<td></td>
<td>FCR3</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.716 (0.617–0.831)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>11.5 (10.2–12.8)</td>
</tr>
<tr>
<td>Promethazine</td>
<td>43.5 (37.9–49.8)</td>
</tr>
<tr>
<td>BG 920</td>
<td>11.5 (9.1–14.5)</td>
</tr>
<tr>
<td>BG 958</td>
<td>48.8 (43.0–55.3)</td>
</tr>
<tr>
<td>BG 996</td>
<td>26.4 (23.4–29.6)</td>
</tr>
<tr>
<td>BG 1001</td>
<td>44.6 (39.6–50.8)</td>
</tr>
<tr>
<td></td>
<td>Bres1</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.740 (0.604–0.906)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>19.0 (15.8–22.9)</td>
</tr>
<tr>
<td>Promethazine</td>
<td>29.0 (25.4–33.3)</td>
</tr>
<tr>
<td>BG 920</td>
<td>17.6 (15.1–20.5)</td>
</tr>
<tr>
<td>BG 958</td>
<td>5.3 (3.6–7.9)</td>
</tr>
<tr>
<td>BG 996</td>
<td>39.6 (36.4–43.2)</td>
</tr>
<tr>
<td>BG 1001</td>
<td>38.7 (32.2–46.6)</td>
</tr>
<tr>
<td></td>
<td>Palo Alto</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.570 (0.463–0.702)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>11.4 (10.8–11.9)</td>
</tr>
<tr>
<td>Promethazine</td>
<td>30.8 (26.6–35.8)</td>
</tr>
<tr>
<td>BG 920</td>
<td>23.2 (19.6–27.6)</td>
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<tr>
<td>BG 958</td>
<td>35.2 (30.8–40.3)</td>
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<tr>
<td>BG 996</td>
<td>33.8 (30.2–37.8)</td>
</tr>
<tr>
<td>BG 1001</td>
<td>8.8 (6.3–12.1)</td>
</tr>
</tbody>
</table>

* Values are the geometric mean 50% inhibitory concentrations (IC₅₀) of 3–10 assays.

FIGURE 2. Isobolograms showing the in vitro synergistic interactions between BG 920, BG 958, BG 996, BG 1001, verapamil, promethazine, and chloroquine (CQ) in the chloroquine-resistant Plasmodium falciparum parasites Palo Alto (A), FCR3 (B), Bres1 (C), and W2 (D). IC₅₀ = 50% inhibitory concentration.
the chloroquine IC₅₀ at concentrations ranging from 0.2 µM to 0.6 µM. These data are close to those reported on W2 parasites for tricyclic antidepressants: 50% chloroquine reversion with desipramine at 0.15 µM and with imipramine at 0.19 µM; for antidepressants such as fluoxetine: 66% reversion with 0.5 µM; or for calcium antagonists such as chlorpromazine: 75% reversion at 0.62 µM.

Any discussion of the mechanism of resistance of malaria parasites to drugs should first deal with the mode of action of the drug. Chloroquine reaches the parasite food vacuole, where it accumulates due to the weak basic properties of the drug and the local acidic pH, which is higher in resistant parasites than in susceptible parasites. However, a recent report has shown that the pH values in chloroquine-resistant parasites appear to be lower than those in chloroquine-susceptible parasites. These findings are in direct contrast to the earlier idea that reduced vacuolar pH would lead to increased accumulation of chloroquine. In addition, it was initially suggested that the most convincing explanation of the chloroquine activity lies in its capacity to interfere with degradation of hemoglobin in the food vacuole by inhibition of the polymerization of the free heme by the formation of a toxic heme-chloroquine complex and/or by raising the vacuolar pH. Nevertheless, it has recently been found that short-term exposure to chloroquine has no significant effect in susceptible or resistant parasites and that long-term exposure seems to decrease vacuolar pH in susceptible parasites.

It has been suggested that the cellular uptake of chloroquine is dependent on binding to ferrisprotoporphyrin IX (FPIX) and is independent of the Na⁺/H⁺ exchanger activity. The acidification of the digestive vacuolar pH contributes to drug resistance via the profound effects that pH has on the solubility of the drug. Chloroquine reduces the solubility of FPIX, and the drug flux across the digestive vacuole membrane is directly linked with chloroquine resistance. The digestive vacuole acidification that DEA compounds might enhance chloroquine accumulation and acid flux across the digestive vacuole membrane is directly altered by a structural change in the chloroquine resistance P. falciparum. The PfCRT mutations appear to be associated with chloroquine resistance. The Pfcrt T76 allele was suggested to be associated with chloroquine resistance, but allelic modifications have ruled out a role for this gene in chloroquine resistance.

Recently, pfmdr, a gene with 13 exons, was identified near cg2 on chromosome 7. This transmembrane protein localizes to the parasite digestive vacuole, the site of chloroquine action, where increased compartment acidification is associated with the Pfmdr1 point mutation. One mutation at the position 76 was present in all resistant isolates and absent from all susceptible isolates. The Pfmdr1 genotypes are strongly linked with chloroquine-resistant P. falciparum malaria. The Pfmdr mutations appear to be associated with changes in vacuolar pH. Models of chloroquine resistance can be envisaged that incorporate the effects of Pfmdr mutations in different ways: the decrease in the vacuolar pH associated with Pfmdr mutations reduces drug-heme interaction responsible for toxicity, and drug flux across the digestive vacuole membrane is directly altered by a structural change in the Pfmdr molecule itself or by an effect of PfCRT on the function of other molecules involved in the digestive vacuole physiology. Changes in pH are reported to have a steep effect on the conversion of soluble heme to insoluble aggregates. The aggregate form of FPIX dimers promoted by lower pH is still capable of crystallization to hemozoin but does not bind chloroquine as avidly as freely soluble FPIX. The pH midpoint of this conversion is close to the vacuolar pH values of chloroquine-resistant parasites. The formation of insoluble heme is much more efficient at the more acidic vacuolar pH values of chloroquine-resistant parasites. The digestive vacuole acidification would leave significantly less free heme available for the formation of toxic complexes with chloroquine. In addition, antimalarial drugs can influence the pH-dependent solubility of heme via apparent nucleation phenomena. The presence of chloroquine reduces the solubility of FPIX.

Some interesting observations suggest that the mechanism of malarial chloroquine resistance may be similar to the mechanism of mammalian multidrug resistance (MDR) in cancer cell lines. Resistant parasites actively expel chloroquine, probably by means of a transporter encoded by a multidrug resistance gene. Two genes, pfmdr1 and pfmdr2, which are homologs of the mammalian MDR genes, were cloned from P. falciparum. Pgh-1, the product of pfmdr1, is localized in the food vacuole membrane, suggesting that it could be involved in drug transport across this membrane. It was initially suggested that Pgh-1 pumps chloroquine out of the food vacuole and is expressed in chloroquine-resistant parasites. Another possibility could be that Pgh-1 pumps chloroquine into the food vacuole. This notion would agree with the idea that drug resistance may be due to mutations in pfmdr1. The presence of a mutation on the tyrosine-86 allele was suggested to be associated with chloroquine resistance. However, the idea that Pgh-1 acts as a drug pump seems not to be involved in resistance to chloroquine. Pgh-1 might act as a chloride channel or as a modulator of such a channel. As a chloride channel, Pgh-1 may be constitutively expressed in the vacuolar membrane to allow the maximal conversion of the proton motive force of the vacuolar H⁺-pump into acid pH. It has been recently shown that chloroquine resistance in a P. falciparum cross maps to a 36-kb segment of chromosome 7. This segment accommodates cg2, a gene encoding a unique protein, which has been detected in the parasite cytosol, the parasitophorous space, and the food vacuole in association with hemozoin. This cg2 molecule could therefore be implicated in chloroquine transport, and in the inhibition of FPIX polymerization. Polymorphisms in cg2 were highly associated with chloroquine resistance, but allelic modifications have ruled out a role for this gene in chloroquine resistance.
compete for a chloroquine-binding site on drug transporters involved in malaria resistance.

The DEA derivatives could lead effects on the digestive vacuolar pH of the malaria parasite. It has been found that verapamil normalizes vacuolar pH (increase in pH) for chloroquine-resistant parasites to a value close to that measured for chloroquine-susceptible parasites, without an effect on vacuolar pH in chloroquine-susceptible parasites.\textsuperscript{57} Verapamil has been shown to lower the $K_d$ of chloroquine binding in intact infected cells.\textsuperscript{36} The level of chloroquine set the $K_d$ of ferriprotoporphyrin/chloroquine complex formerly suggested\textsuperscript{58} for vacuolar pH in chloroquine-susceptible parasites.\textsuperscript{37} It has been found that verapamil-resistant parasites to a value close to that measured for the malaria parasite. It has been found that verapamil-resistant parasites to a value close to that measured for the malaria parasite.

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REFERENCES