Intrinsic Efficacy of Proguanil against Falciparum and Vivax Malaria Independent of the Metabolite Cycloguanil

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Mutations in human CYP2C19 and parasite dihydrofolate reductase (dhfr) genes, related to poor metabolism of proguanil and resistance to cycloguanil, respectively, have both been assumed to be associated with poor antimalarial effect by proguanil. To study this, 95 subjects with uncomplicated Plasmodium falciparum or Plasmodium vivax infections in Vanuatu received proguanil treatment for 3 days (adult relative dose of 300–500 mg/day) and were followed up for 28 days. A similarly high antimalarial efficacy against both infections was observed in 62 patients with CYP2C19-related poor metabolizer genotype and in 33 with extensive metabolizer genotype, even though blood cycloguanil was significantly more often detected in those with extensive metabolizer genotype than in those with poor metabolizer genotype. All 28 P. falciparum isolates had two dhfr mutations (residues 59 and 108), suggesting moderate resistance to cycloguanil. The results suggest that the parent compound proguanil has significant intrinsic efficacy against falciparum and vivax malaria independent of the metabolite cycloguanil.

Malaria is a pandemic disease drawing increasing attention globally. The increasing resistance of Plasmodium falciparum to common antimalarial drugs, such as chloroquine and sulfadoxine-pyrimethamine, has resulted in a renewed interest in alternative drug regimens.

Proguanil, a biguanide, was synthesized and found to have antimalarial activity in 1945 [1]. Proguanil is metabolized in the liver to the active metabolite, cycloguanil [2], which has shown much higher in vitro activity against P. falciparum blood stages than has the prodrug or the second metabolite, 4-chlороphenylbiguanide [3, 4]. Cycloguanil and pyrimethamine, another antifolate compound, act by selectively inhibiting dihydrofolate reductase (DHFR) in the Plasmodium parasites [5]. P. falciparum resistance to the compounds has been associated with specific point mutations in the dhfr gene [6, 7]. Recent findings of a high synergistic activity between proguanil and an electron transport inhibitor atovaquone for malaria treatment [8] and prophylaxis [9], despite widespread resistance to DHFR inhibitors, have suggested a possible non–DHFR-related intrinsic antimalarial activity of the prodrug.

Proguanil and mephenytoin metabolism is mediated via the cytochrome P-450 isoenzyme CYP2C19 [10]. A poor ability to metabolize S-mephenytoin (poor metabolizer [PM] phenotype) has been observed in only 3%–5% of Caucasians [11], Ethiopians [12], and Zimbabweans [13] but in 13%–23% of Oriental populations [11]. The impaired metabolism results from defects in the CYP2C19 gene. Two genetic defects, m1 and m2, have been identified [14]. At least one wild type (wt) allele is considered necessary to be an extensive metabolizer (EM). We have recently found a uniquely high frequency (71%) of CYP2C19-related PM genotype status in two islands in which malaria is endemic (Tanna and Malakula) of Vanuatu [15]. Whether the
poor metabolism of proguanil, resulting in low plasma concentrations of cycloguanil, would lead to the poor therapeutic efficacy against malaria has not properly been studied.

We have, therefore, studied the antimalarial efficacy of proguanil treatment in patients with uncomplicated malaria in Vanuatu in relation to human CYP2C19 genotypes, capillary blood concentrations of proguanil-cycloguanil, and parasite dhfr genotypes.

Methods

Study area and patient selection. Vanuatu consists of 80 islands in Melanesia, in many of which malaria is endemic [16]. The first-line drug for malaria treatment in children aged <5 years old was changed in 1991 from chloroquine to sulfadoxine-pyrimethamine because of increasing resistance of P. falciparum to chloroquine.

In February–March 1996, malariometric surveys were conducted in Levampa Village and surrounding primary schools on the central island of Malakula. Subjects found to have malaria were selected to be included in the study. Exclusion criteria were mixed infections, moderate or severe symptoms, intake of antimalarial(s) within the previous 3 weeks, significant concomitant diseases, <1 year of age, pregnancy, or breast-feeding.

Microscopic examinations. Parasitologic diagnosis of malaria was made microscopically on thick and thin blood smears, which were stained with 10% Giemsa solution and examined for 10 min. Parasite density was estimated by counting asexual parasites against 100–400 leukocytes in a thick film, assuming a leukocyte count of 8000/µL of blood.

Treatment regimens and in vivo follow up. Patients received proguanil for 3 days in daily doses corresponding to adult doses of 500 mg (initially) or 300 mg. Doses for children were calculated as a fraction of the adult dose based on the age as follows: one-third (1–4 years), one-half (5–8 years), two-thirds (9–15 years), and full (>15 years). The proguanil tablets (Paludrine, 100 mg tablet; Zeneca Pharmaceuticals, Wilmington, DE) were administered between meals under close supervision.

Symptoms and clinical signs were recorded daily from day 0 (day of treatment) to day 3 in addition to parasitologic examinations. Subjects were again similarly examined on days 7, 14, 21, and 28. The parasitologic responses were graded according to the modified World Health Organization criteria (S, no parasites on day 7 and no recurrence days 14–28; R1, no parasites on day 7 and with recurrence day 14–28; R2, day 2 parasite density ≤25% of that on day 0 but recurrence by day 7; R3, day 2 parasite density >25% of that on day 0). Therapeutic efficacy was assessed mainly by the S + R1 rate.

Whenever treatment was interrupted because of side effects, or the response was defined as R3, R2, or R1, the follow-up was ended and an alternative treatment with sulfadoxine-pyrimethamine was provided.

Filter paper blood sampling. Fingerprick blood samples were drawn into one or two capillary tubes (100 µL, heparinized; Drummond Scientific, Broomall, PA) and transferred on to chromatography filter paper (ET31CHR, Whatman, Maidstone, UK) both before treatment (day 0) and 3 and 24 h after the first and third doses, respectively. The dried filter paper samples were stored in small plastic bags at −20°C before analysis by polymerase chain reaction (PCR) and high-performance liquid chromatography (HPLC).

Human and parasite genotyping. DNA was first extracted from one-fourth of a dried blood spot (25 µL) on the day 0 filter paper as described by Uchida et al. [17].

The PCRs for the human CYP2C19 gene were then conducted as described by de Morais et al. [14], by amplification of exon 5 followed by SmaI digestion (CYP2C19m1) and amplification of exon 4 followed by BamHI digestion (CYP2C19m2).

The nested PCRs for the P. falciparum dhfr gene were done as described by Reeder et al. [18]. The purified fragment from the dhfr amplification was directly sequenced by a dideoxy chain-terminating method (DNA Sequencing Kit; Perkin Elmer, Norwalk, CT). Data were collected on an automated sequencer (ABI 377; Perkin Elmer), and complementary sequences from both strands were examined.

HPLC assay. Proguanil, cycloguanil, and 4-chlorophenylbiguanide were determined in the 100-µL capillary blood paper samples by an adapted method by use of a solid-phase extraction technique and HPLC. The coefficients of variation for proguanil and cycloguanil were <15% at all observed concentration ranges. The limits of determination were 100 nmol/L for proguanil and 50 nmol/L for cycloguanil and 4-chlorophenylbiguanide.

Statistical analyses. The numeric data are given as mean ± SE. Standard statistical methods, including Student’s t test, χ2 test, and Fisher’s exact test, were used where appropriate. All reported confidence intervals are 95% and P values are two-sided.

Results

Parasitologic findings. Of 1521 persons surveyed, 110 (7.2%) were found to have malaria, 46 due to P. falciparum, 63 due to Plasmodium vivax, and 1 with a mixed infection. Five subjects did not meet the inclusion criteria. An additional 10 were lost to follow-up because of inaccessibility of their residences. Of the remaining 95 patients, 40 were males and 55 females. The median age was 7 years, with the age distribution as follows: 1–4 years, 16 subjects; 5–8 years, 45; 9–15 years, 30; and >15 years, 4. The geometric mean parasite density of the 38 P. falciparum infections was 1401 (range, 160–19,840) parasites/µL of blood and that of the 57 P. vivax infections was 250 (range, 80–5040).

Human CYP2C19 genotypes. The CYP2C19 genotyping of the 95 study subjects revealed 5 wt/wt, 22 wt/mt1, 6 mt1/m2, 29 mt1/m1, 26 mt1/m2, and 7 m2/m2. The frequency of the CYP2C19mt1 allele was 0.56, whereas that of CYP2C19m2 was 0.24 and that of CYP2C19mt2 was 0.20. The distribution of individual genotypes followed the Hardy-Weinberg equilibrium.

The subjects with at least one mt allele were classified as EMs and those without any mt allele as PMs. Of the 33 (35%) identified EMs, 13 had P. falciparum and 20 had P. vivax infections.
Of the 62 PMs, 25 had *P. falciparum* and 37 had *P. vivax* infections.

EMs and PMs were comparable with regard to age profiles and parasite densities of *P. falciparum* and *P. vivax*, respectively.

**Treatments and side effects.** The proguanil treatment dosage for the first 10 selected subjects was 500 mg daily (adult relative dose). Five of them complained of adverse events and 3 of these 5 would not continue the whole regimen. After the treatments, the most frequently reported side effects were gastrointestinal events: abdominal pain (37%) and vomiting (24%). This was an unusually high prevalence of these symptoms, but there was no difference in the types or the frequency of adverse events between PMs and EMs.

**Drug concentrations.** Proguanil and cycloguanil concentrations in whole capillary blood of the 72 patients who took 300 mg of proguanil daily for 3 days are summarized in table 1. Proguanil was detected in all samples but with relatively higher concentrations in PMs than in EMs. In contrast, cycloguanil was significantly more often detected in EMs than in PMs. Within PMs, cycloguanil was more often detected 3 h after the first dose than after the third dose ($P = .02$, $\chi^2$ test). The concentration-time profile of 4-chlorophenylbiguanide was similar to that of cycloguanil; that is, the subjects who poorly metabolized proguanil to cycloguanil also poorly metabolized the parent compound to 4-chlorophenylbiguanide (data not shown).

**Therapeutic efficacy and parasite dhfr genotypes.** A total of 79 patients (31 *P. falciparum*– and 48 *P. vivax*–infected) completed the 3-day proguanil treatment course. There was no significant difference in the therapeutic efficacy between PMs and EMs for either of the two parasite infections ($P > .5$, Fisher’s exact test) (table 2). Similarly, there was no correlation between cycloguanil concentrations and efficacy against either parasite infection. No significant correlation was found between initial parasite density and parasitologic response to treatment.

**dhfr genotypes** were determined for the isolates from 28 of the 31 *P. falciparum*–infected patients (table 3). All isolates, regardless of in vivo response, had the same two point mutations, normally associated with moderate resistance to DHFR-inhibiting drugs [19].

### Table 1. Drug concentrations in whole capillary blood after onset of 3-day proguanil treatment corresponding to adult doses of 300 mg at 0, 24, and 48 h in non-severe malaria patients in Vanuatu in relation to CYP2C19 genotypes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Postdose time (h)</th>
<th>Extensive metabolizer (EM): $\text{wt/wt, wt/m}_1, \text{wt/m}_2$ ($n = 24$)</th>
<th>Poor metabolizer (PM): $\text{m}_1\text{/m}_1, \text{m}_1\text{/m}_2, \text{m}_2\text{/m}_2$ ($n = 48$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detection rate (%)</td>
<td>Mean ± SE (nmol/L)</td>
<td>Detection rate (%)</td>
</tr>
<tr>
<td>Proguanil</td>
<td>3</td>
<td>22/22 (100)</td>
<td>3721 ± 458</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>23/23 (100)</td>
<td>578 ± 51</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>21/21 (100)</td>
<td>4179 ± 496</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>21/21 (100)</td>
<td>831 ± 117</td>
</tr>
<tr>
<td>Cycloguanil</td>
<td>3</td>
<td>20/22 (91)</td>
<td>171 ± 32</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>10/23 (44)</td>
<td>91 ± 7</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>20/21 (95)</td>
<td>215 ± 31</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>16/21 (76)</td>
<td>78 ± 5</td>
</tr>
</tbody>
</table>

* No. detected/no. measured. Detection limits = 100 nmol/L for proguanil and 50 nmol/L for cycloguanil in 100 µL of capillary blood spotted on filter papers.

* For subjects with detectable drug concentrations.

* Student’s $t$ test.

* $\chi^2$ test.

### Table 2. Therapeutic efficacy of proguanil, 300–500 mg daily for 3 days, in non-severe malaria patients in Vanuatu in relation to human CYP2C19 genotypes, corresponding to extensive (EM) or poor (PM) metabolizers.

<table>
<thead>
<tr>
<th>In vivo response to treatmenta</th>
<th>Plasmodium falciparum</th>
<th>Plasmodium vivax</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EM ($n = 11$)</td>
<td>PM ($n = 20$)</td>
</tr>
<tr>
<td>S</td>
<td>6 (1)</td>
<td>14 (1)</td>
</tr>
<tr>
<td>R1</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>R2</td>
<td>3 (1)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>R3</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

S + R1 rate (95% confidence interval) 64% (35%–92%) 75% (50%–94%) 88% (71%–100%) 91% (81%–100%)

NOTE. Data are no. (no. who initiated treatment with daily proguanil dose of 500 mg).

* According to modified World Health Organization classification, based on 28-day parasitologic response as follows: S, no parasites day 7, no recurrence days 14–28; R1, no parasites day 7, recurrence day 14–28; R2, day 2 parasite density $\leq$ 25% of that on day 0 but recurrence on day 7; R3, day 2 parasite density $> 25%$ of that on day 0.
Table 3. Dihydrofolate reductase (dhfr) genotypes of Plasmodium falciparum isolates and in vivo responses to 3-day proguanil treatment in Vanuatu.

<table>
<thead>
<tr>
<th>Isolates by in vivo response&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid residue of dhfr domain&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
</tr>
<tr>
<td>S/R1 (n = 19)</td>
<td>Ala</td>
</tr>
<tr>
<td>R2/R3 (n = 9)</td>
<td>Ala</td>
</tr>
<tr>
<td>Reported wild type</td>
<td>Ala</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amino acid residues where point mutations associated with resistance have been previously reported [6, 7, 18, 19]. Underline indicates mutated residues.

<sup>b</sup> World Health Organisation classification system (explained in table 2).

Discussion

The therapeutic efficacy of an antimalarial drug depends on its bioavailability, the susceptibility of the parasites, and the antimalarial immune status of the human host. We have investigated the efficacy of proguanil treatment in malaria patients living in an area in which malaria is endemic in relation to the CYP2C19 pharmacogenetic and proguanil pharmacokinetic profiles of the human host and the dhfr genetic profile of the parasite.

The observed high frequency of CYP2C19 mutations confirms our previous findings on the same island [15], and the pharmacokinetic data confirmed that they were associated with poor metabolism of proguanil. The mean proguanil concentrations were somewhat higher in PMs than in EMs, whereas the cycloguanil concentrations were mostly below the detection limit of the assay in PMs, as expected.

The wide use of the drug for many years for chemoprophylaxis and its extremely rare reports of severe adverse events indicate a generally low toxicity of proguanil. Nevertheless, we more often observed abdominal pain and vomiting during the proguanil treatment than generally thought. In contrast to our findings, few side effects were observed in Thai patients receiving even higher doses of proguanil, up to 1 g/day [20]. However, when combined with chloroquine for chemoprophylaxis in Caucasians [21] or atovaquone for treatment of uncomplicated falciparum malaria in Gabon [8], abdominal side effects similar to those observed in our study have been reported. These differences in reported side effects are difficult to explain, but they may possibly be attributed to genetic differences that are even unrelated to the CYP2C19-related metabolic capacity.

Proguanil is normally not to be considered for monotherapy, but we believed it to be justified, because all selected malaria patients in this study were asymptomatic or had only mild symptoms. A control group without treatment would have provided information on the degree of spontaneous “self-cure” in the study group. However, the medical practice such that malaria patients do not undergo any treatment was not considered to be ethically justified. Some indirect evidence of relatively low spontaneous remission rates of asymptomatic P. falciparum infections is available from studies on pyrimethamine efficacy in schoolchildren with relatively high degrees of resistance in areas of Africa in which malaria is endemic. Such studies have shown up to 100% resistance (as shown by parasite prevalence) on day 7 [22, 23]. These are significantly higher day 7 parasite rates than those we observed in our Vanuatu study after treatment with proguanil, suggesting some “true” effect by proguanil that, as we have shown, does not differ between EMs and PMs.

In our study, only about one-third of the P. falciparum and one-tenth of the P. vivax patients were parasite-positive on day 7 after a total 3-day dose corresponding to 900 mg of proguanil in an adult. Recently, in Thailand, after an adult 3-day dose of 3000 mg of proguanil, 8 of 11 P. falciparum patients were parasite-positive on day 7 [24]. This may reflect a higher degree of parasite resistance to proguanil-cycloguanil in Thailand, although a higher degree of malaria immunity in the patients in Vanuatu may also account for this difference.

We observed no correlation between therapeutic efficacy and CYP2C19 genotypes. There was even a trend toward less efficacy in EM patients (table 2). This was unexpected, since cycloguanil has been reported to be much more active against P. falciparum and is assumed to be critical for the antimalarial efficacy of proguanil prophylaxis or treatment [2–4]. Similarly to our findings, in vitro activity of proguanil-atovaquone in Thai patients’ plasma samples did not depend on the metabolic phenotype of proguanil, although this may also be explained by the specific synergism between atovaquone and cycloguanil [25]. The causal prophylactic effect of proguanil alone appears to also be unrelated to the metabolic phenotype according to studies on malaria breakthroughs among Swedish travelers [26] or Japanese volunteers in Africa [27].

The high efficacy of proguanil treatment in PMs could possibly be explained by a high activity of an alternative metabolite through another metabolic pathway. However, the second known metabolite, 4-chlorophenylbiguanide, cannot be used to explain such an effect, since it follows the same pathway as cycloguanil and, in addition, has shown a poor antimalarial activity in vitro [3, 4].

All P. falciparum isolates in our study, regardless of in vivo response, had the same dhfr genotype, suggesting moderate resistance to both pyrimethamine and cycloguanil [19]. In a previous in vitro study on P. falciparum isolates from Papua New Guinea with the same dhfr genotype as in our study, cycloguanil IC<sub>50</sub>s were 80–320 nmol/L in the resistant parasites, whereas the IC<sub>50</sub>s were ≤5.0 mmol/L in the sensitive parasites with wt dhfr [18]. In early studies, the corresponding values for proguanil were ~10<sup>–10</sup> mmol/L for both cycloguanil-sensitive and -resistant strains [3, 4]. This suggests that in our study, the inhibitory blood levels of cycloguanil were attained only in EMs, whereas those of proguanil were mostly attained in both EMs and PMs (table 1).

Our results in the PMs thus suggest that the parent compound proguanil may be more active than previously believed.
and that it may have a significant intrinsic efficacy independent of the metabolite cycloguanil. Interestingly, a recent in vitro study of P. falciparum transformed with human DHFR has also suggested an intrinsic activity of proguanil against a target other than DHFR [28].

The therapeutic efficacy against P. vivax in our study was higher than might have been expected in view of the reduced sensitivity of P. vivax to sulfadoxine-pyrimethamine observed in Thailand [29] and also confirmed in Vanuatu (unpublished data). The efficacy of proguanil was, however, documented in the 1940s in a Hong Kong strain of P. vivax [30]. The possible intrinsic efficacy of the parent compound proguanil also against P. vivax suggests that proguanil-atovaquone, the recently developed antimalarial combination, may be used for the treatment of chloroquine-resistant P. vivax, which may represent a growing problem in Southeast Asia and the Pacific region [31] and more generally for malaria without species identification. A basis for resistance of P. vivax to pyrimethamine was recently suggested following the isolation of the gene encoding the DHFR of P. vivax [32], and there are PCR-based methods for assessing the mutations in that gene [33], which might also be associated with poor sensitivity to cycloguanil. We thus consider pursuing a further study to assess the P. vivax dhfr.

The efficacy of proguanil for treatment of or prophylaxis against malaria has been considered to be due to a DHFR inhibition by mainly cycloguanil and, therefore, to depend on the bioavailability of the metabolite cycloguanil, that is, the CYP2C19 pharmacogenetic profile of the human host and the dhfr genetic profile of the parasite. However, we observed a high antimalarial efficacy of proguanil in a population with high frequencies of the CYP2C19-related PM genotype and the parasite genotype suggesting a moderate resistance to DHFR-inhibiting drugs. We thus conclude that proguanil should receive more attention as an important antimalarial and that its therapeutic effectiveness may be more related to the parent compound than to the metabolite cycloguanil.

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References


