Inhibition by Omeprazole of Proguanil Metabolism: Mechanism of the Interaction In Vitro and Prediction of In Vivo Results from the In Vitro Experiments

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ABSTRACT

Both the antimalarial prodrug proguanil and the gastric proton pump inhibitor omeprazole are substrates for cytochrome P450 (CYP)2C19 and CYP3A. However, the relative contribution of each enzyme to proguanil bioactivation to cycloguanil and to the metabolism of omeprazole, as well as their potential to interact, remains to be examined. The bioactivation of proguanil to its active metabolite cycloguanil was studied in vitro in human liver microsomes and in vivo in 12 healthy subjects, in the absence and in the presence of omeprazole. The formation of cycloguanil from proguanil exhibited biphasic kinetic behavior in four of six human livers, indicating that at least two enzymes are responsible for this metabolic step. Cycloguanil formation activity did not correlate with immunoreactive CYP3A4 content or with CYP3A4 activity, as measured by testosterone 6β-hydroxylation, suggesting that CYP3A4 plays a limited role in cycloguanil formation. Furthermore, troleandomycin (10 μM) inhibited only 10 to 17% of cycloguanil formation at proguanil concentrations of 100 and 500 μM. At a proguanil concentration of 20 μM, omeprazole at 10 μM inhibited cycloguanil formation in vitro by 47 ± 59%. These in vitro results were consistent with the results of our in vivo study in healthy subjects, which showed a 32 ± 11% decrease in proguanil apparent oral clearance and a 65 ± 8% decrease in proguanil partial metabolic clearance to cycloguanil in the presence of omeprazole (both P < .001). We conclude that in vitro studies of proguanil metabolism and interactions are predictive of in vivo situations, that CYP2C19 is the main enzyme responsible for proguanil bioactivation to cycloguanil and that omeprazole inhibits this biotransformation in vitro and in vivo by inhibiting this enzyme.

Proguanil is an antimalarial prodrug that must be activated to its main metabolite cycloguanil to exert its effects (Watkins et al., 1984; Yeo et al., 1994). This bioactivation is largely controlled by the genetically determined P450 activity that is responsible for the polymorphic 4’-hydroxylation of (S)-mephenytoin (CYP2C19) (Funck-Brentano et al., 1992; Helsby et al., 1990a,b; Wright et al., 1995). This observation could be clinically important, because up to 20% of some Asian populations and 4 to 6% of the Caucasian population share the (S)-mephenytoin poor metabolizer phenotype (Jurima et al., 1985; Watkins et al., 1990; Wilkinson et al., 1989) and thus could be inefficiently or insufficiently protected during malaria prophylaxis with proguanil (Skjelbo et al., 1996). The importance of the CYP2C19-mediated (Goldstein et al., 1994) polymorphic oxidation of (S)-mephenytoin in modulating the clinical response to proguanil administration has been recently debated, because it was suggested that the CYP3A isoforms may account for as much as 70% of the hepatic biotransformation of proguanil into cycloguanil in human liver microsomes in vitro (Birkett et al., 1994). However, whether these in vitro results apply to clinical situations remains to be determined.

Omeprazole is a gastric proton pump inhibitor used for the treatment of gastric and duodenal ulcers; it appears to be one of the most widely used drugs in the world. Similarly to that of proguanil, the metabolism of omeprazole cosegregates with the CYP2C19-mediated polymorphic oxidation of (S)-mephenytoin (Andersson et al., 1990b; Balian et al., 1995; Chiba et al., 1993; Sohn et al., 1992). In vitro studies have shown that the metabolism of omeprazole is also controlled in part by CYP3A isoforms (Andersson et al., 1993, 1994).

ABBREVIATIONS: Ae, amount excreted in urine; AUC, area under the plasma concentration vs. time curve; 4-CPB, 4-chlorophenylbiguanide; CYP or P450, cytochrome P450.
Thus, current knowledge indicates that both proguanil and omeprazole are metabolized by CYP2C19 in vivo and in vitro. The contribution of CYP3A isoforms to their metabolism has been clearly shown in vitro and could be relevant in vivo, in light of the good predictability from in vitro inhibition studies to in vivo situations (Kroemer et al., 1992; Miners et al., 1994). Theoretically, therefore, omeprazole and proguanil have several reasons to interact when they are administered simultaneously to humans. If omeprazole could inhibit the formation of the active cycloguanil metabolite in humans, this could possibly have important clinical implications for the prophylaxis of malaria in subjects treated with proguanil.

In a recent preliminary study in healthy subjects, we showed that simultaneous administration of omeprazole and proguanil was associated with a 2.5-fold increase in the proguanil to cycloguanil urinary ratio (Partovian et al., 1995). This preliminary result suggests that omeprazole is able to inhibit the biotransformation of proguanil into cycloguanil in vivo. However, only urinary data were obtained in that study and, because inferences drawn from urinary data in studies of drug metabolism are uncertain (Miners and Birkett, 1993; Schellens et al., 1989), definitive demonstration of the interaction requires measurements of plasma clearances.

The aim of the present study was to examine the contribution of CYP3A4 and CYP2C19 to the bioactivation of proguanil into cycloguanil in vitro and the influence of omeprazole on this metabolic step in vitro and in vivo. Our approach was to demonstrate the reality of the interaction in vitro in human liver microsomes, to identify the nature of the enzyme(s) involved and then to demonstrate the relevance of our in vitro findings by showing that they could predict the results observed in vivo.

Materials and Methods

Drugs, Chemicals and Reagents

Omeprazole sodium for in vitro studies was a generous gift from Astra France (Nanterre, France). Omeprazole tablets used in the clinical part of the study were purchased from the hospital pharmacy as commercially available 20-mg tablets (Mopral; Astra France). Proguanil hydrochloride for in vitro studies and drug assays and cycloguanil and 4-CPB for drug assays were obtained from Zeneca (Macclesfield, UK). Proguanil tablets used in the clinical part of the study were purchased from the hospital pharmacy as commercially available 100-mg tablets of the hydrochloride salt (Paludrine; Zeneca Pharma, Cergy, France). Troleandomycin was purchased from Sigma Chemical Co. (Saint-Quentin Fallavier, France). Glucose-6-phosphate dehydrogenase, glucose-6-phosphate and NADP were purchased from Boehringer Mannheim (Meylan, France). Stock solutions of proguanil and omeprazole were prepared in water immediately before in vitro incubations. Troleandomycin was dissolved in methanol.

Preparation of Human Liver Microsomes

Microsomes were prepared from liver samples of six human cadaveric donors collected and stored as previously described (Kremers et al., 1981). The P450 concentration was measured as described by Schoene et al. (1972), and the total protein concentration was assayed by the bicinchoninic acid method (Pierce, Rockford, IL) according to the supplier’s recommendation, using bovine serum albumin as the standard.

Determination of the Relative CYP3A4 Monooxygenase Levels in Human Liver Microsomes by Western Blotting and Testosterone 6β-Hydroxylation

For the six livers used in this study, the immunoreactive content of CYP3A4 was determined by immunoblotting. Anti-human CYP3A4 against pure human CYP3A4 expressed in bacteria (Belloch et al., 1996) was produced in rabbit. This antibody recognized a single band in human liver microsomes and did not cross-react with human CYP1A1, CYP1A2, CYP2D6, CYP2E1, CYP2C8, CYP2C9 and CYP2C18 expressed in yeast.

Microsomal proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to the method of Laemmli (1970), and were then electrotransferred onto nitrocellulose sheets. CYP3A4 was detected by the primary polyclonal rabbit anti-CYP3A4 antibody and peroxidase-conjugated secondary antibodies. Staining was performed with 4-chloro-1-naphthol as described previously (Walker et al., 1994). CYP3A4 was quantified by densitometry using a Hewlett Packard Scan Jet II, and results were expressed in arbitrary units per milligram of protein. Linearity as a function of P450 content was checked. CYP3A4 activity was measured in these livers as testosterone 6β-hydroxylation activity, which was assessed as previously described (Botsch et al., 1993).

Biotransformation of Proguanil to Cycloguanil In Vitro

The kinetics of cycloguanil formation were studied in microsomes obtained from the six human livers used for immunoblots. Each incubation (1-ml final volume) contained 2 mg of liver microsomes in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, and an NADPH-generating system consisting of 0.15 mM NADP, 2.5 mM glucose-6-phosphate and 1.7 U/ml glucose-6-phosphate dehydrogenase. Proguanil was used at 10 different concentrations ranging from 5 to 1000 μM. Each reaction was carried out at 37°C and was stopped after 30 min by addition of 2 ml of iced ethanol. The preparation was then centrifuged for 15 min at 3000 × g to remove the protein pellet, and the supernatant was collected and stored at −80°C until analysis. The cycloguanil formation rate was shown to be linear with time up to 30 min and microsomal protein concentration up to 2 mg/ml.

Inhibition of Proguanil Bioactivation to Cycloguanil In Vitro

Inhibition studies with proguanil were performed using three human liver microsomes (1006, 11A and A12) in the presence of progressively increasing concentrations of proguanil (0, 10, 100 and 500 μM). Inhibition studies with troleandomycin, a prototypic CYP3A inhibitor (Newton et al., 1995), were performed using three human liver microsomes (1006, A10 and A12) chosen for their different immunoreactive contents of CYP3A4. Troleandomycin (10 μM final concentration in 0.1% methanol) was preincubated for 10 min in the presence of the complete incubation mixture without the substrate. This concentration of troleandomycin was previously shown to inhibit CYP3A4-dependent cycloguanil formation (Birkett et al., 1994). Proguanil, at a final concentration of 100 or 500 μM, was then added for another 30 min. The results of the troleandomycin inhibition study were compared with controls without troleandomycin, in the presence of the same volume of methanol (0.1% final volume).

Study in Healthy Subjects

The clinical part of the study consisted of a two-period crossover trial, which was performed with 12 healthy male volunteers. Clinical examination and standard laboratory tests were performed before inclusion of the subjects, to ensure that they were normal. The subjects’ status with respect to CYP2C19 phenotype was unknown before their inclusion. In the first study period, subjects received two tablets of 100 mg of proguanil hydrochloride, taken orally with 150 ml of tap water after an overnight fast, and blood samples were collected from an antecubital vein immediately before proguanil administration and 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 34 and 48 hr
thereafter. Subjects emptied their bladders immediately before proguanil administration, and urine was collected during time intervals of 0–12 hr, 12–24 hr and 24–48 hr after proguanil intake. Blood samples were drawn onto lithium heparin in glass tubes and centrifuged at 3000 × g and +4°C within 15 min; plasma was collected and stored at −28°C until further analysis. The volume of each urine collection was measured, and 20-ml samples were stored at −28°C until analysis. Subjects were hospitalized during the first 24 hr and remained in a fasting state until 3 hr after proguanil administration. In the second study period, which started 1 week after the first administration of proguanil, subjects were asked to take two tablets of 20 mg of omeprazole orally each morning for 7 days. On the morning of the seventh day, subjects were hospitalized, the last dose of omeprazole was taken together with 200 mg of proguanil and procedures implemented during the first study period were repeated in exactly the same way. The study protocol was approved by the Committee for the Protection of Human Subjects in Biomedical Research of Paris–Pitié-Salpêtrière. Subjects gave their written informed consent to participate, and the study was performed according to French regulations.

**Determination of Proguanil and Its Metabolites in Plasma and Urine and of Cycloguanil in Liver Microsomes**

**In vivo study.** Plasma and urine concentrations were measured according to the method of Taylor et al. (1987), as slightly modified. After addition of chlorocycloguanil as internal standard, solid extraction was performed on C18 cartridges (100 mg), with methanol/perchloric acid (99:1, v/v). After dilution, the eluate was injected via a loop column into the chromatographic ion-pairing system. An ASPEC automated system (Gibson France, Villiers-le-Bel, France) performed all steps from extraction to injection. Quantification limits were 2 ng/ml (0.5 nmol/ml) for cycloguanil and 4-CPB and 2.5 ng/ml (10 nmol/ml) for proguanil base in plasma. Interexperimental variability was 2.1 to 10.7% from 3 to 450 ng/ml for proguanil, 1.6 to 17.9% from 5 to 180 ng/ml for cycloguanil and 2.3 to 15.5% from 2.5 to 100 ng/ml for 4-CPB.

**In vitro studies.** The same method was used for the assay in human liver microsomes, but the cartridges were 500 mg and omeprazole was eluted with methanol/triethylamine (99:1) before elution of proguanil and metabolites, to avoid interference with the acid degradation products. Calibration and quality control solutions always contained 0.1 mM proguanil. Under these conditions, omeprazole and troleandomycin did not interfere with the assay. The quantification limit was 2 ng/ml, and between-run variability was always <6%.

**Data Analysis**

**In vitro experiments.** Kinetic analyses of the formation of cycloguanil were initially evaluated by visual examination of Eadie-Hofstee plots to assess whether one or two enzymatic sites were involved in this metabolic step. The estimates of the kinetic parameters from this evaluation were then used as the initial estimates for nonlinear regression analysis. The latter analysis fitted the parameters of the Michaelis-Menten equation for one (eq. 1) and two (eq. 2) enzymatic sites to the data (Fig.P version 2.5; Biosoft, Cambridge, UK).

\[
V = \frac{V_{\text{max}} \cdot S}{K_m + S} \quad (1)
\]

\[
V = \frac{V_{\text{max}} \cdot S}{K_{m1} + S} + \frac{V_{\text{max}} \cdot S}{K_{m2} + S} \quad (2)
\]

where \( V \) is the velocity of cycloguanil formation, \( S \) is the concentration of proguanil in the incubation mixture, \( K_m \) is the apparent affinity constant, \( V_{\text{max}} \) is the maximum initial enzyme velocity and the subscripts 1 and 2 represent the high- and low-affinity sites, respectively.

The model providing the best fit of parameters to the actual data was chosen by examination of the S.E. of parameter estimates, of the sum of squares of the residuals and of the distribution of residuals. Where necessary to choose between models, an F test was performed, as previously described (Motulsky and Ransnas, 1987).

**Quantitative estimation of omeprazole-induced inhibition of cycloguanil formation in vitro.** Despite the fact that visual examination of Eadie-Hofstee plots clearly indicated that four of our six livers exhibited two-enzyme kinetics, non linear regression analysis was unable to identify two sets of \( V_{\text{max}} \) and \( K_m \) values (see “Results”). Thus, the following equation was used to calculate the expected percentage inhibition of cycloguanil formation in the presence of omeprazole, assuming competitive inhibition for one enzymatic site:

\[
V = \frac{V_{\text{max}} \cdot S}{K_m \cdot \left(1 + \frac{I}{K_i}\right)} \quad (3)
\]

where \( V, S, V_{\text{max}} \) and \( K_m \) are as above, \( I \) is the concentration of omeprazole and \( K_i \) is the dissociation constant of the enzyme-inhibitor (omeprazole) complex. The expected inhibition was estimated by calculating the percentage change of \( V \) in equation 3 relative to \( V \) in equation 1.

For this analysis, mean \( V_{\text{max}} \) and \( K_m \) parameters previously obtained in our experiments were fixed in equations 1 and 3 and calculations were made twice with different \( K_i \) values taken from the literature to reflect dissociation constants of the CYP2C19-omeprazole complex (3 µM) (Chiha et al., 1993; VandenBranden et al., 1996) and of the CYP3A4-omeprazole complex (44 µM) (VandenBranden et al., 1996). Calculations were then performed for \( I \) (omeprazole) values of 1, 5 and 10 µM and \( S \) (proguanil) values ranging from 0.1 to 100 µM.

**In vivo study in healthy subjects.** Proguanil pharmacokinetics were analyzed by using noncompartmental techniques. Proguanil apparent oral clearance was calculated as dose/AUCPG, where dose is the proguanil dose administered as the base (175 mg) and AUCPG is the area under the proguanil plasma concentration vs. time curve. For this calculation, AUCPG was calculated from zero time to infinity. Partial metabolic clearance of proguanil to cycloguanil was calculated as \( AEC_{CG}/AUC_{PG} \), where \( AEC_{CG} \) is the amount of cycloguanil excreted in urine and AUCPG is as described above (Walle et al., 1986). For this calculation, terms were expressed in molar units and the ratio was calculated from data measured over 48 hr. The proguanil apparent plasma elimination half-life was calculated as 0.693\( k_e \), where \( k_e \) is the slope of the log (proguanil plasma concentration) vs. time line after least-squares regression analysis of the terminal portion of this relationship. The first point in time corresponding to this terminal portion was identified as the data point for which least-squares linear regression had the best coefficient of determination when the data point was included in the regression. Renal clearances of proguanil and cycloguanil were calculated as \( AEC_{CG}/AUC_{PG} \) and \( AEC_{CG}/AUC_{CG} \), respectively, using data measured over 48 hr. Finally, to examine whether the change in proguanil clearance (oral or partial metabolic clearance) during omeprazole coadministration was influenced by the level of this clearance in the absence of omeprazole, we plotted each parameter (oral or partial metabolic clearance) measured during omeprazole administration as a function of its value in the absence of omeprazole and compared the slope of the regression line with unity, as previously described (Funck-Brentano et al., 1994; MacGregor et al., 1985; Sumner et al., 1988). In such an analysis, a slope significantly different from unity indicates that, on average, the change in proguanil clearance during omeprazole coadministration depends on its base-line value.

Statistical analyses were performed by using PCINFO computer software (Retriever Data System, Seattle, WA). Parameters obtained in the absence and in the presence of omeprazole coadministration were compared by Student’s paired t test. Standard least-squares
linear regression techniques were used for correlation analyses in healthy subjects. Spearman rank correlation was performed to analyze the relation between immunoreactive CYP3A4 content and testosterone 6β-hydroxylation activity and total enzymatic activity in the livers used for in vitro studies. A difference was considered statistically significant if the probability of erroneously rejecting the null hypothesis of no difference was <5%.

Results

Proguanil Biotransformation to Cycloguanil in Human Liver Microsomes

One of the six livers used for these experiments (liver A12) had a 5- to 17-fold higher V\textsubscript{max} than the other livers. Detection of cycloguanil was not always possible for substrate concentrations below 50 μM. Eadie-Hofstee plots were biphasic in four livers (A2, 1006, 11A and 1002) and linear in two (A10 and A12). Figure 1 shows Eadie-Hofstee plots representative of each situation. However, nonlinear regression analysis of the data could identify only one set of V\textsubscript{max} and K\textsubscript{m} values for all livers. Table 1 shows the kinetic parameters derived from Michaelis-Menten equations for one-enzyme kinetics.

Relationship between CYP3A4 and Cycloguanil Formation In Vitro

Despite the wide range of immunoreactive CYP3A4 contents found in the livers (table 1), there was no correlation between CYP3A4 content and V\textsubscript{max} in the six livers tested (Spearman rank correlation r = 0.09, P = not significant, n = 6). Similarly, there was no correlation between CYP3A4 activity, measured as testosterone 6β-hydroxylation, and V\textsubscript{max} in the six livers tested (Spearman rank correlation r = 0.09, P = not significant, n = 6). For example, liver A2, which had the second highest total enzyme activity, had the lowest CYP3A4 content and the lowest CYP3A4 activity. To further examine the role of CYP3A4 in the biotransformation of proguanil into cycloguanil, we incubated proguanil in the presence of 10 μM levels of the CYP3A4 inhibitor troleandomycin, using three livers chosen for their different CYP3A4 contents (see “Materials and Methods”). Troleandomycin inhibited cycloguanil formation by 10 to 17% regardless of immunoreactive CYP3A4 content, CYP3A4 activity or proguanil concentration (table 2).

Interaction between Omeprazole and Proguanil in Human Liver Microsomes

Omeprazole inhibited cycloguanil formation in the three livers tested (1006, 11A and A12) (fig. 2). For example, omeprazole at 10 μM inhibited cycloguanil formation in vitro by 47 ± 59% at a proguanil concentration of 20 μM.

Calculated estimates of omeprazole-induced inhibition of cycloguanil formation for the two K\textsubscript{i} values taken from the literature to reflect inhibition of CYP2C19 (K\textsubscript{i} = 3 μM) and CYP3A4 (K\textsubscript{i} = 44 μM) are shown in table 3. The expected percentage inhibition of cycloguanil formation in the presence of 5 μM omeprazole with theoretical proguanil concentrations ranging from 0.1 to 100 μM was estimated to be 53 to 62% for inhibition of CYP2C19 and 7 to 10% for inhibition of CYP3A4.

Pharmacokinetics of Proguanil, Given Alone and Together with Omeprazole, in Healthy Subjects

Subjects had a mean (range) age of 25 (20 to 31) years and completed the study without side effects. Omeprazole com-

Table 1: Kinetics of proguanil activation to cycloguanil by human liver microsomes

<table>
<thead>
<tr>
<th>Liver</th>
<th>K\textsubscript{m} μM</th>
<th>V\textsubscript{max} pmol/mg/min</th>
<th>Immunoactive CYP3A4 Content</th>
<th>Testosterone 6β-Hydroxylation Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>231 ± 35</td>
<td>7.1 ± 0.3</td>
<td>arbitrary units</td>
<td>13</td>
</tr>
<tr>
<td>1006</td>
<td>256 ± 70</td>
<td>4.2 ± 0.4</td>
<td>45</td>
<td>2.1</td>
</tr>
<tr>
<td>11A</td>
<td>206 ± 69</td>
<td>4.7 ± 0.6</td>
<td>63</td>
<td>4.1</td>
</tr>
<tr>
<td>A10</td>
<td>156 ± 7</td>
<td>5.1 ± 0.1</td>
<td>100</td>
<td>5.8</td>
</tr>
<tr>
<td>1002</td>
<td>226 ± 61</td>
<td>2.1 ± 0.2</td>
<td>130</td>
<td>6.1</td>
</tr>
<tr>
<td>A12</td>
<td>107 ± 52</td>
<td>36.4 ± 5.4</td>
<td>200</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Mean ± S.D. = 197 ± 55 10.0 ± 13.1

Inhibition of proguanil activation to cycloguanil by troleandomycin (10 μM) in human liver microsomes

Experiments were performed in duplicate for each liver tested. Results are expressed as mean ± S.D. of percent inhibition of cycloguanil formation.

<table>
<thead>
<tr>
<th>Liver</th>
<th>Immunoactive CYP3A4 Content</th>
<th>Testosterone 6β-Hydroxylation Activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>arbitrary units</td>
<td>nmol/mg/min</td>
<td></td>
</tr>
<tr>
<td>1006</td>
<td>45</td>
<td>2.1</td>
<td>11 ± 22</td>
</tr>
<tr>
<td>A10</td>
<td>100</td>
<td>5.8</td>
<td>10 ± 12</td>
</tr>
<tr>
<td>A12</td>
<td>200</td>
<td>9.3</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

Fig. 1. Eadie-Hofstee plots for the formation of cycloguanil, using microsomes from human liver A10 (upper) and liver 11A (lower) incubated with proguanil.
Urinary recovery of proguanil and its metabolites in healthy subjects

TABLE 4

<table>
<thead>
<tr>
<th>Omeprazole Concentration (μM)</th>
<th>Inhibition of CYP2C19 (K&lt;sub&gt;i&lt;/sub&gt; = 3 μM)</th>
<th>Inhibition of CYP3A4 (K&lt;sub&gt;i&lt;/sub&gt; = 44 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>18–25</td>
<td>1–2</td>
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<tr>
<td>5</td>
<td>53–62</td>
<td>7–10</td>
</tr>
<tr>
<td>10</td>
<td>69–77</td>
<td>13–19</td>
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</tbody>
</table>

Fig. 2. Inhibition of cycloguanil formation by omeprazole, using microsomes from human livers. Mean ± S.D. of percent inhibition of cycloguanil formation in three livers (1006, 11A and A12) is shown.

TABLE 3

Calculated percent inhibition of cycloguanil formation by omeprazole

Calculations were performed as described in the text, using K<sub>i</sub> values of 3 μM and 44 μM in equation 3 to reflect omeprazole-induced CYP2C19 and CYP3A4 inhibition, respectively. Results are given for theoretical proguanil concentrations ranging from 0.1 to 100 μM.

<table>
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<tr>
<td>10</td>
<td>69–77</td>
<td>13–19</td>
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</table>

FIG. 2

Proguanil Concentration (μM)

Drainage was judged to be excellent, based on pill count. Based on proguanil to cycloguanil urinary metabolic ratios (Funck-Brentano et al., 1992; Helsby et al., 1990a), all subjects were extensive metabolizers of mephenytoin (table 4).

Urinary excretion of proguanil and its metabolites (table 4). Technical problems with urine processing precluded analysis of the 12–24 hr urine samples from the first study period in two subjects. Therefore, data could be analyzed using the 0–12 hr urine collection interval for all 12 subjects and the 0–48 hr interval for only 10 subjects. When omeprazole was administered together with proguanil, cycloguanil fractional urinary excretion decreased significantly, from 21 ± 4% to 10 ± 2% (P < .001). This fall in cycloguanil urinary excretion was associated with an increase in proguanil fractional urinary excretion from 32 ± 4% to 44 ± 4% (P < .001). Fractional urinary excretion of 4-CPB decreased from 10 ± 2% to 5 ± 1% (P < .001). Total urinary excretion of proguanil as the parent compound and its metabolites cycloguanil and 4-CPB decreased moderately but significantly, from 63 ± 7% to 59 ± 5% (P = .04), in the presence of omeprazole. As a result of these changes, the proguanil to cycloguanil urinary metabolic ratio was increased significantly during omeprazole coadministration (table 4; fig. 3). However, this ratio remained within the range of values found for extensive metabolizers of mephenytoin (Funck-Brentano et al., 1992; Helsby et al., 1990a), and subjects' CYP2C19 phenotype was not changed into a pseudo-poor metabolizer phenotype during omeprazole coadministration.

Results obtained from plasma analyses. Figure 4 shows the proguanil and cycloguanil plasma concentration vs. time relationships during administration of proguanil alone and together with omeprazole. The apparent plasma elimination half-life of proguanil increased from 15 ± 3 hr when proguanil was administered alone to 19 ± 3 hr when it was administered together with omeprazole (P < .01). Proguanil AUC increased from 1767 ± 386 ng/ml/hr in the absence of omeprazole to 2634 ± 616 ng/ml/hr in its presence (P < .001). Cycloguanil AUC decreased from 1107 ± 222 ng/ml/hr in the absence of omeprazole to 589 ± 161 ng/ml/hr in its presence (P < .001). During concomitant administration of omeprazole, proguanil apparent oral clearance decreased significantly, and this fall was largely explained by a decrease in partial metabolic clearance of proguanil to cycloguanil (table 5). Proguanil apparent oral and partial metabolic clearances fell by 32 ± 11% and 65 ± 8%, respectively. Renal clearances of proguanil and cycloguanil were unaffected by omeprazole coadministration (table 5).

Correlation analyses. The 48 hr proguanil to cycloguanil urinary metabolic ratio strongly correlated with partial met-

TABLE 4

Urinary recovery of proguanil and its metabolites in healthy subjects

Values are mean ± S.D.

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<tr>
<th></th>
<th>Proguanil Alone</th>
<th>Proguanil with Omeprazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–12 hr urine collection (n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG&lt;sup&gt;*&lt;/sup&gt; recovery (% of PG dose administered)</td>
<td>20 ± 4</td>
<td>26 ± 4***</td>
</tr>
<tr>
<td>PG recovery (% of PG dose administered)</td>
<td>14 ± 3</td>
<td>6 ± 2***</td>
</tr>
<tr>
<td>4-CPB recovery (% of PG dose administered)</td>
<td>6 ± 2</td>
<td>2 ± 1***</td>
</tr>
<tr>
<td>Total urinary recovery (% of PG dose administered)</td>
<td>39 ± 6</td>
<td>34 ± 5**</td>
</tr>
<tr>
<td>PG/CG metabolic ratio</td>
<td>1.5 ± 0.5</td>
<td>5.0 ± 1.5***</td>
</tr>
<tr>
<td>0–48 hr urine collection (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG recovery (% of PG dose administered)</td>
<td>32 ± 4</td>
<td>44 ± 4***</td>
</tr>
<tr>
<td>CG recovery (% of PG dose administered)</td>
<td>21 ± 4</td>
<td>10 ± 2***</td>
</tr>
<tr>
<td>4-CPB recovery (% of PG dose administered)</td>
<td>10 ± 2</td>
<td>5 ± 1***</td>
</tr>
<tr>
<td>Total urinary recovery (% of PG dose administered)</td>
<td>63 ± 7</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>PG/CG metabolic ratio</td>
<td>1.6 ± 0.4</td>
<td>4.6 ± 1.1***</td>
</tr>
</tbody>
</table>

<sup>*</sup> PG, proguanil; CG, cycloguanil.

<sup>**</sup> P < .05.

<sup>***</sup> P < .001.

<sup>***P</sup> < .001 vs. proguanil alone.
abolic clearance of proguanil to cycloguanil during administration of proguanil alone ($r = -0.92$, $P < .001$, $n = 10$) and together with omeprazole ($r = -0.89$, $P < .001$, $n = 12$). Proguanil apparent oral clearance during omeprazole administration correlated with its value in the absence of omeprazole ($r = 0.73$, $P < .01$, $n = 12$) and the slope of this relationship was significantly smaller than unity (0.52; 95% confidence interval, 0.18–0.87), indicating that, on average, proguanil apparent oral clearance during omeprazole administration decreased more when its initial value was high (fig. 5). Similarly, partial metabolic clearance of proguanil to cycloguanil during omeprazole administration correlated with its value in the absence of omeprazole ($r = 0.83$, $P < .01$, $n = 10$) and the slope of this relationship was significantly smaller than unity (0.31; 95% confidence interval, 0.14–0.48), indicating that, on average, proguanil partial metabolic clearance to cycloguanil during omeprazole administration decreased more when its initial value was high (fig. 5).

**Discussion**

**Enzymes Responsible for the Formation of Cycloguanil in Human Liver Microsomes**

Results from our studies in human liver microsomes indicate that at least two enzymes are responsible for cycloguanil formation *in vitro*. Our study also shows that omeprazole is a potent inhibitor of proguanil biotransformation to its active metabolite cycloguanil in human liver microsomes *in vitro* and in healthy subjects *in vivo*. Previous studies have shown

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**TABLE 5**

Proguanil and cycloguanil clearance parameters in healthy subjects

<table>
<thead>
<tr>
<th></th>
<th>Proguanil Alone</th>
<th>Proguanil with Omeprazole</th>
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<tbody>
<tr>
<td>PG apparent oral clearance (liters/hr, $n = 12$)</td>
<td>$103 \pm 22$</td>
<td>$70 \pm 16^{***}$</td>
</tr>
<tr>
<td>Partial metabolic clearance of PG to CG (liters/hr, $n = 10$)</td>
<td>$23 \pm 8$</td>
<td>$8 \pm 3^{***}$</td>
</tr>
<tr>
<td>PG renal clearance (liters/hr, $n = 10$)</td>
<td>$34 \pm 5$</td>
<td>$34 \pm 7$</td>
</tr>
<tr>
<td>CG renal clearance (liters/hr, $n = 10$)</td>
<td>$35 \pm 5$</td>
<td>$33 \pm 8$</td>
</tr>
</tbody>
</table>

*PG, proguanil; CG, cycloguanil.

$^{***} P < .001$ vs. proguanil alone.
that both CYP2C19 and CYP3A4 contribute to the metabolism of omeprazole (Andersson et al., 1993) and proguanil (Birkett et al., 1994). However, our in vitro results indicate that the relative contributions of each enzyme to the biotransformation of proguanil to cycloguanil differ.

**Identification of two enzymatic sites responsible for cycloguanil formation.** We could identify low- and high-affinity enzymatic sites by visual examination of Eadie-Hofstee plots. These results are consistent with those of Birkett et al. (1994), who found that proguanil is metabolized by CYP2C19 and CYP3A4 but who could not visualize two-enzyme kinetics using Eadie-Hofstee plots. Those authors reported one-enzyme site $K_m$ values ranging from 35 to 183 $\mu$M. Unfortunately, we were unable to calculate $V_{max}$ and $K_m$ values for two-enzyme kinetics using nonlinear regression techniques. Distinction between one- and two-enzyme kinetic models in in vitro studies is often difficult, because one needs a sufficient number of data points measured with substrate concentrations bracketing the $K_m$ of each enzyme (Kato and Yamazoe, 1994). At low substrate concentrations nearing the $K_m$ of the high-affinity site, the amount of metabolite produced may be too small to allow accurate detection with the assay used. This may explain why we could not calculate two sets of $V_{max}$ and $K_m$ parameters in our experiments. Nevertheless, the identification of biphasic kinetics in four of our livers clearly indicates that at least two enzymatic sites are responsible for cycloguanil formation.

**Relative contribution of each enzymatic site to cycloguanil formation.** It is very likely that the two enzymic sites we have identified correspond to CYP2C19 and CYP3A4, because these enzymes are the only ones that have been shown to significantly contribute to the biotransformation of proguanil into cycloguanil, at least in vitro (Birkett et al., 1994; Helsby et al., 1990b; Wright et al., 1995). The relative contribution of each enzyme to this metabolic step remains uncertain but, in contrast to the findings of Birkett et al. (1994), our data strongly suggest that CYP3A4 plays a minor role within the range of proguanil concentrations observed during administration of therapeutic doses of this drug. Indeed, we found that 10 $\mu$M troleandomycin, a prototypic CYP3A4 inhibitor (Newton et al., 1995), could inhibit only 10 to 17% of cycloguanil formation in vitro and that inhibition was not clearly influenced by the immunoreactive CYP3A4 microsomal content. Also, CYP3A4 content and activity did not predict total enzyme activity responsible for cycloguanil formation. These results suggest that the low-affinity site we have identified corresponds to CYP3A4. In contrast to our results, Birkett et al. (1994) suggested that CYP3A could contribute as much as 70% to the biotransformation of proguanil into cycloguanil. Those authors found that cycloguanil formation correlated with various CYP3A activities and with immunoreactive CYP3A content in human liver microsomes. They also found that inhibition of cycloguanil formation by 10 $\mu$M troleandomycin correlated with CYP3A content and that cycloguanil formation was increased by the CYP3A activator a-naphthoflavone. Birkett et al. (1994) used 17 human livers, and this number may have increased their ability to find a correlation. However, the most likely explanation for the discrepancy between our results and those of Birkett et al. (1994) involves the high substrate concentration they used for their experiments. Indeed, their correlation analyses were based on experiments performed at a proguanil concentration of 500 $\mu$M. It is possible, as pointed out by Kato and Yamazoe (1994), that the high concentration of proguanil they used explored only the low-affinity site or a combination of the high- and low-affinity sites. Another argument that supports the small contribution of CYP3A to cycloguanil formation, compared with that of CYP2C19, comes from previous in vivo studies of poor metabolizers of (S)-mephenytoin. It was indeed shown that, in this CYP2C19-deficient population, approximately 1 to 3% of an oral dose of proguanil was recovered as cycloguanil in urine collected over 12 hr (Brøsen et al., 1993; Setiabudy et al., 1995). In those studies, urinary recovery of cycloguanil was 4- to 9-fold lower in poor metabolizers than in extensive metabolizers of (S)-mephenytoin. Overall, our results support the view that, at the low concentrations of proguanil observed during administration of therapeutic doses, the contribution of CYP3A4 to cycloguanil formation is limited in vivo.

**Inhibition by Omeprazole of Cycloguanil Formation**

We found that omeprazole inhibited the biotransformation of proguanil into cycloguanil in vitro and in vivo. This was expected, because these two compounds are metabolized by both CYP2C19 and CYP3A4 (Andersson et al., 1993, 1994; Birkett et al., 1994; Chiba et al., 1993; Helsby et al., 1990b). Also, in a previous study based on analysis of proguanil to cycloguanil metabolic ratios in urine, we showed that omeprazole was able to increase this ratio (Partovian et al., 1995), a result that was also found in the present study and that is consistent with inhibition of cycloguanil formation. Our re-
sults not only confirm the interaction but also help identify its potential mechanism and predictors.

**Enzymes involved in the inhibition of cycloguanil formation by omeprazole.** Several arguments suggest that CYP2C19, rather than CYP3A4, was the main enzyme involved in the interaction between omeprazole and proguanil. Firstly, omeprazole inhibited cycloguanil formation by 20 to 80% in vitro, whereas, as discussed above, the contribution of CYP3A4 to cycloguanil formation is limited. Secondly, omeprazole has been found to be a very potent inhibitor of CYP2C19 with a \( K_i \) of 2 to 4 \( \mu M \) (Chiba et al., 1993; VandenBranden et al., 1996), whereas the \( K_i \) of omeprazole for CYP3A4 is at least 10 times higher (VandenBranden et al., 1996). Because omeprazole is a substrate of both enzymes, the observed \( K_i \) values (obtained from competitive inhibition studies) represent the respective \( K_i \) values for the low- and high-affinity enzymes, thus suggesting that the low-affinity enzyme is not CYP2C19. Thirdly, omeprazole is a very weak inhibitor of CYP3A4 in vitro (Kerlan et al., 1992) and does not inhibit CYP3A4 activity in vivo (Galbraith and Michnovicz, 1993; Tateishi et al., 1995) or at low concentrations in vitro (Kerlan et al., 1992). Therefore, CYP2C19 appears to be the main enzyme responsible for the inhibition of cycloguanil formation by omeprazole. Based on previous studies involving drugs metabolized by CYP2D6 (Funck-Brentano et al., 1989a, b), one would expect that subjects with the CYP2C19 poor metabolizer phenotype would not be exposed to the interaction, because they lack the enzyme that is the target for the inhibition. Unfortunately, none of the subjects who participated in the clinical part of our study had the poor (S)-mephenytoin-metabolizer phenotype. Further studies are thus required to definitively demonstrate that omeprazole interacts with cycloguanil formation by selective inhibition of CYP2C19 activity.

**Other mechanisms for the omeprazole-proguanil interaction.** Inhibition of cycloguanil formation accounted for approximately 50% of the decrease in proguanil apparent oral clearance in our healthy subjects. Because renal clearance of proguanil was not altered by omeprazole coadministration, this indicates that omeprazole inhibited routes of proguanil elimination other than cycloguanil formation. The formation of 4-CPB, although a minor pathway, was also inhibited. The formation of this metabolite also depends, at least in part, on CYP2C19 activity (Brøsen et al., 1993; Setiabudy et al., 1995). It is thus likely that omeprazole also inhibited the CYP2C19-dependent formation of 4-CPB. Finally, total urinary recovery of proguanil as the parent compound and its main metabolites was slightly but significantly decreased in the presence of omeprazole. This suggests that omeprazole reduced proguanil intestinal absorption. Such a phenomenon would tend to further decrease the amount of cycloguanil formed after oral administration of proguanil in the presence of omeprazole.

**Predictors of the inhibition by omeprazole of cycloguanil formation.** Based on previous results from the literature (Andersson et al., 1990a; Chang et al., 1995), it may be estimated that the plasma concentration of omeprazole during the few hours after oral administration of 40 mg daily to our extensive metabolizer subjects was in the 0.3 to 0.5 \( \mu M \) range. Assuming a liver to plasma concentration ratio of 3 to 10, a range of ratios found in rats (Regårdh et al., 1985), the hepatic concentration of omeprazole in our subjects may be estimated to be in the 1 to 5 \( \mu M \) range. Our simulations of omeprazole inhibition based on the in vitro results of the present study and the literature predicted 53 to 62% inhibition of CYP2C19-dependent cycloguanil formation but only 7 to 10% inhibition of CYP3A4-dependent cycloguanil formation at an omeprazole concentration of 5 \( \mu M \). These expected changes for CYP2C19 inhibition, but not for CYP3A4 inhibition, are consistent with the results of our in vivo study, which showed a 65 ± 8% reduction in proguanil partial metabolic clearance to cycloguanil in the presence of omeprazole. However, it should be recognized that these simulations were based on many assumptions and should thus be interpreted with caution. Nevertheless, our in vitro experiments could reasonably well predict the results of our study in healthy subjects. Such predictability from in vitro drug metabolism studies has been reported by others (Kroemer et al., 1992; Miners et al., 1994).

Interestingly, the initial levels of total enzyme activity in vitro and of proguanil apparent oral and partial metabolic clearances in vivo were the best predictors of the extent of the omeprazole-proguanil interaction. Thus, the subjects with the highest proguanil clearances were those in whom omeprazole interacted with cycloguanil formation to the greatest extent. We documented a similar phenomenon with compounds interacting at the CYP2D6 level (Funck-Brentano et al., 1989a, b, 1994).

**Conclusion**

Our study shows that CYP2C19 is the main enzyme responsible for proguanil bioactivation to cycloguanil and that omeprazole inhibits this biotransformation by inhibiting this enzyme. The contribution of CYP3A4 to cycloguanil formation and to the interaction with omeprazole is comparatively limited at the plasma concentrations usually observed during administration of therapeutic doses of these compounds. The clinical consequences of the decrease in cycloguanil formation in the presence of omeprazole remain to be examined, but it is conceivable that protection against malaria may be decreased when omeprazole and proguanil are combined in subjects with the CYP2C19 extensive metabolizer phenotype. Finally, our study shows that in vitro drug interaction experiments performed in human liver microsomes allow good qualitative and quantitative predictions of the interaction in vivo.

**Acknowledgments**

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