In vitro proguanil activation to cycloguanil by human liver microsomes is mediated by CYP3A isoforms as well as by S-mephenytoin hydroxylase

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- 1 The activation of proguanil to cycloguanil by human liver microsomes was studied to define the cytochrome P450 (CYP) isoforms involved in this reaction.
- 2 Apparent K_m values for proguanil ranged from 35 μM to 183 μM with microsomes from four human livers.
- 3 There was a 6.3-fold range of activity with microsomes from seventeen human livers. Rates of proguanil activation correlated significantly with CYP3A activities (benzo[a]pyrene metabolism, caffeine 8-oxidation and omeprazole sulphone formation) and CYP3A immunoreactive content. There was also a highly significant correlation with rates of hydroxyomeprazole formation. Correlations with activities selective for CYP1A2, CYP2C9/10 and CYP2E1, and with immunoreactive CYP1A2 content were not significant.
- 4 Proguanil activation was inhibited by R,S-mephenytoin, troleandomycin and by inhibitory anti-CYP3A antiserum and anti-CYP2C IgG and was activated by α -naphthoflavone. Inhibitors selective for CYP1A2, CYP2E1, CYP2A6 or CYP2C9/10 had little or no effect on proguanil activation. The extents of inhibition by R,S-mephenytoin, troleandomycin and the two antibodies varied with the immunoreactive CYP3A content of the microsomes used.
- 5 It is concluded that proguanil activation to cycloguanil by human liver microsomes is mediated both by S-mephenytoin hydroxylase and isoforms of the CYP3A subfamily. This has implications for the use of proguanil as an *in vivo* probe for the S-mephenytoin poor metaboliser phenotype.

Keywords proguanil cycloguanil human microsomal metabolism
CYP isoforms

Introduction

The genetic polymorphism affecting the 4-hydroxylation of S-mephenytoin was first reported by Kupfer & Preisig [1]. Family studies in both Caucasian [1, 2] and Asian [3] populations showed that S-mephenytoin 4-hydroxylation was under monogenic control with deficient metabolism representing an autosomal homozygous recessive trait. Meier *et al.* [4] further showed that deficient metabolism *in vivo* was due to partial or complete absence or inactivity of a cytochrome P450 isoform with high affinity for S-mephenytoin. The S-mephenytoin poor metaboliser

phenotype occurs with a frequency of about 1 to 3% in Caucasian populations but has a much higher frequency (up to 20%) in some Asian populations [5]. Unlike the CYP2D6 (debrisoquine/sparteine) polymorphism which affects the metabolism of numerous clinically used drugs, the metabolism of only a few drugs cosegregate with that of S-mephenytoin hydroxylase polymorphism [5].

The CYP isoform involved in the S-mephenytoin hydroxylase genetic polymorphism has not been definitely identified, although it seems likely to be a

member of the complex and microheterogeneous CYP2C subfamily [6]. In particular, the role of CYP2C18 remains unclear [7, 8].

There has been considerable interest in developing a more suitable and accessible probe for the S-mephenytoin polymorphism both *in vivo* and *in vitro*. Mephenytoin is available commercially only as the racemate and is only marketed as an approved drug in a few countries. Its use as an *in vivo* probe requires measurement of very low urinary concentrations of the S- and R-enantiomers usually by chiral g.l.c. analysis. Alternatively, an 'hydroxylation index' is used which expresses the amount of 4-hydroxymephenytoin as a fraction of the dose of S,R-mephenytoin administered. Recently, two widely used drugs, omeprazole and proguanil, have been shown to have metabolic pathways affected by the S-mephenytoin polymorphism. The hydroxylation of omeprazole has been shown *in vivo* to be deficient in poor metabolisers of S-mephenytoin [9, 10], but we have recently found with *in vitro* human liver microsomal studies that CYP isoforms in the CYP3A subfamily can play a significant but variable role in this reaction [11]. The activation of the biguanide antimalarial proguanil to cycloguanil has also been shown to be deficient in S-mephenytoin poor metabolisers [12, 13]. Helsby *et al.* [14] also showed that proguanil activation by human liver microsomes was inhibited by S,R-mephenytoin. It therefore seemed that proguanil may be a convenient *in vitro* and *in vivo* probe for the activity of the polymorphic S-mephenytoin hydroxylase.

In setting up the *in vitro* proguanil to cycloguanil assay, we were initially unable to reproduce the inhibition by R,S- or S-mephenytoin. This prompted us to examine in more detail the CYP isoform selectivity of proguanil activation. This paper reports the results of correlation and inhibition (chemical and antibody) studies of proguanil activation by human liver microsomes.

Methods

Chemicals and reagents

NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, troleandomycin, diethyldithiocarbamate and coumarin were purchased from Sigma Chemical Co. (St Louis, MO) and α -naphthoflavone from Aldrich Chemical Co (Milwaukee, WI). Other drugs and metabolites were obtained from the following sources: proguanil (PG), cycloguanil (CG) and chlorocycloguanil (CCG) from ICI (Melbourne, Australia); sulphaphenazole from Ciba-Geigy (Sydney, Australia), and R,S-mephenytoin from Sandoz Ltd (Basel, Switzerland). Furaflavone and dextromethorphan were kind gifts from Dr R. Gasser, Hoffmann La Roche (Basel, Switzerland) and Dr Ieva Stupans, University of South Australia (Adelaide, Australia), respectively. All other chemicals and reagents were of analytical reagent quality.

Human liver microsomes

Microsomal fractions of human liver were obtained as described previously from renal transport donors. The use of such tissue in these studies had local Research Ethics Committee approval and, where appropriate, the Coroner's permission. The clinical details of the donors of the liver samples (H5-15, 17, 18, 20-23) used in this study have been published elsewhere [15].

In vitro proguanil metabolism

The assay conditions to measure the microsomal metabolism of PG were initially based on the method described by Helsby *et al.* [15]. However, in our hands several modifications had to be made as follows. Incubations (1.0 ml) contained human liver microsomes (0.5-1.0 mg) in phosphate buffer (0.1 M, pH 7.4), NADPH generating system (consisting of 1 mM NADP, 10 mM glucose 6-phosphate, two units glucose 6-phosphate dehydrogenase, and 5 mM $MgCl_2$) and PG (10-500 μM). After 60 min, reactions were terminated by placing tubes on ice and adding 5 ml hexane and the internal standard, CCG (165 ng/50 μl water). Following rotary extraction for 30 min the organic phase was discarded and the aqueous phase re-extracted with 5 ml isopropanol-dichloromethane (1:5) after the addition of 150 μl 1 M NaOH. The organic phase was transferred to a conical tube and evaporated to dryness under nitrogen. The residue was reconstituted in 150 μl mobile phase and 50 μl aliquots used for h.p.l.c. analysis. The chromatograph was fitted with a Spherisorb S5 (5 micron) Octyl column (25 cm $\times$ 4.6 mm i.d., ICI Instruments, Melbourne, Australia). The mobile phase was triethylamine-acetonitrile-water (0.5:14:85.5, the pH adjusted to 3.0 with orthophosphoric acid) at a flow rate of 2 ml min⁻¹. CG and the internal standard, CCG, were monitored by u.v. absorbance at 238 nm. Under these conditions the retention times for CG and CCG were 8.2 min and 18.2 min, respectively. Unknown concentrations of CG were determined by comparison of peak height ratios with those of a calibration curve prepared using CG concentrations in the range 50-1000 nM. Using this procedure the mean recovery of CG, calculated by comparing the peak height for extracted compound with that of an equal amount injected directly into the chromatograph, was $91 \pm 3.9\%$ for six samples in the concentration range 50-1000 nM. Imprecision, both between day and within day was < 5% at a concentration of 250 nM CG.

Kinetic and inhibitor studies

Reaction rate was shown to be linear with time to 90 min and for microsomal protein to at least 1.25 mg ml⁻¹. In experiments to determine the Michaelis-Menten parameters for CG formation, activity was measured for six PG concentrations over the range of 12.5-500 μM using microsomes prepared from five human livers. The effects of a range of P450 isoform selective inhibitors/activators on PG activation were

determined at two inhibitor concentrations in microsomes from two livers (H9 and H23) using substrate concentrations approximating the apparent K_m value for PG activation determined previously in these livers. With the exception of diethyldithiocarbamate, which was dissolved in water, compounds were dissolved in dimethylsulphoxide (DMSO) [final solvent concentration 0.5% v/v]. At this concentration of DMSO, proguanil activation was inhibited by about 20–25%. The suitability of a number of solvents for solubilizing mephenytoin was investigated because of previous reports of solubility problems [16; Helsby & Ward, personal communication]. Propylene glycol, the solvent used by Gut *et al.* [16], and DMSO had similar effects on proguanil activation causing relatively minor and acceptable inhibition of proguanil activation. DMSO at a final concentration of 0.5% v/v was chosen as it was also used to solubilize other inhibitors. However, the use of propylene glycol rather than DMSO to dissolve mephenytoin gave similar inhibition characteristics in preliminary experiments (data not shown). By contrast, Tween (0.28% w/v final incubation concentration) as used (personal communication) in the studies by Helsby & Ward [14] in our hands inhibited PG activation by approximately 90%. Reactions containing troleanomycin were preincubated at 37° C for 10 min with the NADPH generating system prior to adding PG. Furaflavine was not preincubated, but we have found, under similar conditions to those used here for proguanil, that furaflavine 5 μM inhibits phenacetin *O*-deethylation by expressed human CYP1A2 or human liver microsomes by greater than 80%. Proguanil activation rates in the presence of inhibitor/activator were compared with appropriate controls (no preincubation, 10 min preincubation, or addition of 0.5% DMSO v/v), and results expressed as a percentage of the uninhibited/unactivated rate.

Immunoinhibition studies with an anti-rabbit CYP2C3 antibody [17] and anti-rat CYP3A1 antisera [18] were carried out by preincubating antibody/antisera with microsomes at room temperature for 15 min, before initiating reactions by the addition of substrate and NADPH generating system. The total amount of protein (in the form of antibody/antisera) added to each incubation was kept constant by the addition of preimmune antibody or antisera.

Correlation studies

The rates of proguanil activation to cycloguanil with microsomes from seventeen human livers from the Flinders Medical Centre liver bank were correlated with activities towards the metabolism of other isoform selective substrates. The data for tolbutamide hydroxylation and phenytoin 4-hydroxylation [17], benzo(a)pyrene metabolism [15] and hydroxyomeprazole and omeprazole sulphone formation [11] have been published previously. Phenacetin *O*-deethylation (high affinity component) was determined by the method of Tassaneeyakul *et al.* [19] and 4-nitrophenol hydroxylation by the method of Tassaneeyakul *et al.* [20]. Caffeine 3-demethylation (high affinity

component) and 8-oxidation were determined by the method of Tassaneeyakul *et al.* [21]. Proguanil activation rates were also correlated with immunoreactive CYP1A2 and CYP3A protein contents determined previously by Western blot analysis [15].

Analysis of results

Where appropriate, results are presented as mean $\pm$ s.d. Initial estimates of the Michaelis-Menten parameters, apparent K_m and $V_{\max}$ values for PG activation were determined by linear regression analysis of Eadie-Hofstee plots. These values were then used as first estimates for MKMODEL, an extended least squares modelling program [22].

Results

The kinetics of proguanil activation were studied with microsomes from five human livers. In each case, the Eadie-Hofstee plots were linear over the range of substrate concentration from 12.5 to 500 μM (Figure 1). The apparent K_m values ranged from 35 μM to 183 μM and there was a 7.4-fold range in apparent $V_{\max}$ values (Table 1).

Figure 1 Substrate vs velocity and Eadie-Hofstee plots for formation of cycloguanil using microsomes from H22.

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

Liver	Apparent K_m (μM)	Apparent V_{max} ($pmol\ mg^{-1}\ h^{-1}$)
9	62	833
20	122	589
21	35	241
22	183	1772
23	78	415
Mean	96	770
$\pm$ s.d.	(± 52)	(± 538)

Table 2 Correlations of proguanil activation to cycloguanil with activities of CYP isoform contents and CYP isoform selective activities

Activity or isoform	Correlation coefficient ¹
CYP1A2 content	0.456
Phenacetin <i>O</i> -deethylation	0.316
Caffeine 3-demethylation	0.397
Tolbutamide methylhydroxylation	0.034
Phenytoin 4-hydroxylation	0.039
Hydroxyomeprazole formation	0.882**
4-Nitrophenol hydroxylation	0.015
CYP3A content	0.643**
Benzo[a]pyrene hydroxylation	0.642**
Caffeine 8-oxidation	0.659**
Omeprazole sulphone formation	0.506*

¹Spearman rank correlation coefficient* $P < 0.05$, ** $P < 0.01$.

The activities at a proguanil concentration of 500 μM were measured with microsomes from seventeen human livers and there was a 6.3-fold range in activity across the livers. Table 2 shows the correlation of proguanil activation with activities characteristic of specific CYP isoforms. Correlations with CYP1A2 activities (phenacetin *O*-deethylation, caffeine 3-demethylation), CYP2C9/10 activities (tolbutamide methylhydroxylation and phenytoin 4-hydroxylation), and a CYP2E1 activity (4-nitrophenol hydroxylation) were not statistically significant. Significant correlations were found with the CYP3A activities, benzo[a]pyrene metabolism caffeine 8-oxidation, and omeprazole sulphone formation. A strong correlation was found with the rate of hydroxyomeprazole formation which is mediated mainly by S-mephenytoin hydroxylase but also involves CYP3A isoforms [11]. Proguanil activation also correlated significantly with immunoreactive CYP3A content but not with CYP1A2 content (Table 2).

Figure 2 shows the effects of isoform selective inhibitors on proguanil activation using microsomes two-fold different in immunoreactive CYP3A content (relatively high (H23) and relatively low (H9)). There was no or minimal inhibition in both cases by dextromethorphan (CYP2D6), furafylline or α -naphthoflavone (CYP1A2), or coumarin (CYP2A6).

At a concentration of 10 μM , diethyldithiocarbamate did not cause inhibition in either liver. At the higher concentration of 50 μM there was about 30% inhibition in liver H9, but selectivity for CYP2E1 is less at this higher concentration. Sulphaphenazole (CYP2C9/10) caused 10–15% inhibition in liver H23 but not in liver H9. R,S-Mephenytoin caused partial inhibition with H9 but not with H23, and troleandomycin (CYP3A) caused substantial inhibition with H23 (high CYP3A content) but only minor inhibition with H9 (lower CYP3A content). In agreement with this, activation by α -naphthoflavone (characteristic of CYP3A mediated activities) was more marked with H23 than with H9.

The extent of inhibition by troleandomycin was similar at 10 μM and 50 μM indicating that the residual activity was due to (a) CYP isoform(s) resistant to troleandomycin inhibition (presumably S-mephenytoin hydroxylase). The percent inhibition by 50 μM troleandomycin was used therefore as an index of the CYP3A component of proguanil activation in 13 sets of microsomes and varied from about 12% to 70%. Furthermore the percent inhibition by troleandomycin correlated significantly with immunoreactive CYP3A content ($r = 0.59$, $P < 0.05$) (Figure 3).

The effects of inhibitory anti-CYP3A1 antiserum and anti-CYP2C3 IgG on proguanil activation with microsomes from livers H9 and H23 are shown in Figure 4. Activity with H9 (relatively low immunoreactive CYP3A) was inhibited 25% by the anti-CYP3A antibody and 70% by the anti-CYP2C antibody. By contrast, activity with H23 (relatively high immunoreactive CYP3A) was inhibited 70% by the anti-CYP3A antibody and 25% by the anti-CYP2C antibody. The estimates of the CYP3A component in proguanil activation were similar using either anti-CYP3A antibody inhibition (25% and 70% for H9 and H23, respectively) or troleandomycin inhibition (21% for H9 and 69% for H23).

Discussion

The results presented here strongly indicate that at least *in vitro*, proguanil activation to cycloguanil is mediated by CYP3A isoforms as well as by S-mephenytoin hydroxylase. The strongest correlation was with hydroxyomeprazole formation which is mediated mainly by S-mephenytoin hydroxylase but also by CYP3A isoform(s) [11]. Proguanil activation rate correlated significantly with benzo[a]pyrene hydroxylation, caffeine 8-oxidation and omeprazole sulphone formation, activities which are mediated wholly or substantially by CYP3A isoforms. There was also a significant correlation with immunoreactive CYP3A content. Correlations with substrates selective for other CYP isoforms were not significant.

The effects of isoform selective inhibitors were fully consistent with this. Extensive inhibition was seen only with R,S-mephenytoin (S-mephenytoin hydroxylase) and troleandomycin (CYP3A). Inhibitors selective for CYP1A2, CYP2A6, CYP2C9/10, CYP2D6

Figure 2 Effects of various CYP isoform selective inhibitors on proguanil activation in two human livers with a two-fold difference in immunoreactive CYP3A content. Relatively low CYP3A content, H9, upper panel; relatively high CYP3A content, H23, lower panel.

and CYP2E1 had no or minor effects on proguanil activation to cycloguanil. Furthermore, the observed activation rather than inhibition by α -naphthoflavone is characteristic of involvement of CYP3A isoforms in the reaction. Finally the reaction was inhibited by anti-CYP3A1 antisera previously shown to be inhibitory to human CYP3A isoforms 3A3, 3A4 and 3A5 [18].

The data indicate, however, that the extent of involvement of CYP3A isoforms varied widely across the individual livers studied from about 10% to as much as 70% of the total activity. An important determinant of this was the CYP3A content of individual livers as shown by the correlation between immunoreactive CYP3A content and percent inhibition by troleandomycin. The inhibition by anti-CYP3A antiserum or anti-CYP2C antibodies (Figure 4) shows the switch from predominant S-mephenytoin hydroxylase involvement (liver H9) to marked CYP3A involvement in a liver (H23) with relatively high CYP3A content. In both livers, the sum of inhibition

by the anti-CYP2C antibody plus the inhibition by the anti-CYP3A antibody is close to 100% (71% plus 29% respectively for liver H9, and 28% plus 75%, respectively for liver H23). This suggests that together S-mephenytoin hydroxylase and CYP3A isoforms account for essentially all the proguanil activation in human liver microsomes. The lack of correlation with substrates for other CYP isoforms and the lack of effect of inhibitors of other isoforms is consistent with this.

The apparent $V_{\max}$ values for proguanil activation in the present study (4.0 to 29.5 pmol min⁻¹ mg⁻¹) are in a similar range to those reported by Helsby *et al.* [14] (1.5–8.2 pmol min⁻¹ mg⁻¹), but our apparent K_m values are somewhat higher than those in the previous report. The apparent K_m values reported here (35 to 183 μ M) are, however, consistent with those (22 to 100 μ M) found by Helsby *et al.* in a range of livers wider than reported in the original publication (Helsby, personal communication, 1992). With all five livers studied, the Eadie-Hofstee plots were

Figure 3 Relationship between percentage inhibition of proguanil activation by troleandomycin and the immunoreactive CYP3A content determined in 13 human livers ($r = 0.59$, $P < 0.05$).

linear. This does not, however, exclude the involvement of two CYP isoforms if the apparent K_m values of the two forms are in the same range. Helsby *et al.* [14] also reported potent inhibition of proguanil activation by R,S-mephenytoin (K_i 3 to 49 μM), whereas 500 μM R,S-mephenytoin in our hands showed relatively weak inhibition (up to 40%). This discrepancy may be due in part to the use of different solvents to solubilize R,S-mephenytoin. Tween at a final concentration of 0.28% w/v as used by Helsby *et al.* (Helsby, personal communication, 1992) reduced proguanil activation by up to 90% in our hands, whereas DMSO 0.5% w/v used in the current work inhibited by only about 20%. The effect of Tween, although controlled for, may have altered the enzyme characteristics and the nature of the inhibition by R,S-mephenytoin.

In vivo, proguanil activation to cycloguanil is a minor metabolic pathway with $11.2 \pm 4.2\%$ of the dose excreted in urine as cycloguanil [23]. Renal clearance of proguanil accounts for about 25% of total clearance [23, 24]. Clearance by *N*-dealkylation to 4-chlorophenylbiguanide is the other known metabolic pathway (Figure 5). It is apparent that a substantial proportion (up to 60%) of proguanil plasma clearance remains unaccounted for. In an initial population study Ward *et al.* [25] used the proguanil: cycloguanil ratios in spot plasma and urine samples as an index of proguanil metabolism to cycloguanil and found a non-normal distribution for both ratios. In a further study in 137 healthy subjects, Helsby *et al.* [26] found three individuals with urine proguanil: cycloguanil ratios greater than 20, whereas the others had ratios <10 . A formal pharmacokinetic study showed that maximum plasma cycloguanil concentrations were lower in the three subjects with urine ratios >20 than in seven subjects with ratios <10 . A panel study with poor and extensive metabolisers of mephenytoin and/or sparteine showed high urine proguanil: cycloguanil ratios were associated with poor metabolisers of mephenytoin but not sparteine [12]. The cosegregation of proguanil activation to cycloguanil with the

Figure 4 Effects of anti-CYP2C3 IgG (upper panel) and anti-CYP3A1 antiserum (lower panel) on proguanil activation in two human livers with a two-fold difference in immunoreactive CYP3A content. Relatively low CYP3A content, H9; relatively high CYP3A content, H23. The results are the mean of duplicate determinations at each point.

S-mephenytoin hydroxylase polymorphism was confirmed by Funck-Brentano *et al.* [13] in a study of 24 subjects two of whom were poor and one an intermediate metaboliser of S-mephenytoin.

The *in vivo* data discussed above are consistent with the *in vitro* results of this work which suggest a substantial role for S-mephenytoin hydroxylase in the activation of proguanil. The current *in vitro* results indicate, however, that in some individual livers, isoforms of the CYP3A subfamily may partly mediate proguanil metabolism to cycloguanil. *In vivo*, the poor S-mephenytoin metaboliser phenotype does not completely abolish the conversion of proguanil to cycloguanil. Plasma cycloguanil concentrations were 9 to 69 $\mu\text{g l}^{-1}$ in poor metabolisers and 20–545 $\mu\text{g l}^{-1}$ in extensive metabolisers in one study [25] and 54.2, 26.8 and 51.7 $\mu\text{g l}^{-1}$ in poor metabolisers compared with 52.6 to 197.4 $\mu\text{g l}^{-1}$ in extensive metabolisers in a second study [26]. In a study with healthy Kenyan adults, Watkins *et al.* [27] found measurable cycloguanil concentrations in plasma, but not in blood, of only two of eight poor metaboliser subjects. The residual formation of cycloguanil in S-mephenytoin poor metabolisers could be mediated by CYP3A isoforms as is suggested by our current *in vitro* results.

Figure 5 Scheme for proguanil metabolism in humans.

An alternative possibility is that cycloguanil is substantially further metabolised and that clearance as well as formation is deficient in poor metabolisers of S-mephenytoin.

Proguanil is the third substrate we have studied recently which is apparently metabolised by both S-mephenytoin hydroxylase and CYP3A isoforms, the others being omeprazole [11] and diazepam (unpublished data). The current work shows the importance of studying a number of individual livers in determining the CYP isoforms profile of a substrate. For example, we inadvertently chose liver H23 which has high CYP3A content for our initial work, and were unable to show significant inhibition by mephenytoin. The extent to which our current results affect the use of proguanil to phenotype for the S-mephenytoin hydroxylase polymorphism *in vivo* is uncertain. Investigation of the effects of CYP3A inducers and inhibitors *in vivo* is required to determine whether this CYP isoform can contribute significantly to cycloguanil formation *in vivo*.

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