Comparison of (S)-mephenytoin and proguanil oxidation *in vitro*: contribution of several CYP isoforms

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Aims To compare the oxidative metabolism of (S)-mephenytoin and proguanil *in vitro* and to determine the involvement of various cytochrome P450 isoforms.

Methods The kinetics of the formation of 4'-hydroxymephenytoin and cycloguanil in human liver microsomes from 10 liver samples were determined, and inhibition of formation was studied using specific chemical inhibitors and monoclonal antibodies directed towards specific CYP450 isoforms. Expressed CYP450 enzymes were used to characterize further CYP isoform contribution *in vitro*. Livers were genotyped for CYP2C19 using PCR amplification of genomic DNA followed by restriction endonuclease digestion.

Results All livers were wildtype with respect to CYP2C19, except HLS#5 whose genotype was CYP2C19*1/CYP2C19*2. The K_m , V_{max} and CL_{int} values for the formation of 4'-hydroxymephenytoin from (S)-mephenytoin and the formation of cycloguanil from proguanil ranged from 50.8 to 51.6 and 43–380 μM , 1.0–13.9 and 0.5–2.5 $nmol\ mg^{-1}\ h^{-1}$, and 20.2–273.8 and 2.7–38.9 $\mu l\ h^{-1}\ mg^{-1}$, respectively. There was a significant association between the V_{max} values of cycloguanil and 4'-hydroxymephenytoin formation ($r_s=0.95$, $P=0.0004$). Cycloguanil formation was inhibited significantly by omeprazole (CYP2C19/3A), troleandomycin (CYP3A), diethyldithiocarbamate (CYP2E1/3A), furafylline (CYP1A2), and (S)-mephenytoin. 4'-Hydroxymephenytoin formation was inhibited significantly by omeprazole, diethyldithiocarbamate, proguanil, furafylline, diazepam, troleandomycin, and sulphaphenazole (CYP2C9). Human CYP2E1 and CYP3A4 monoclonal antibodies did not inhibit the formation of cycloguanil or 4'-hydroxymephenytoin, and cycloguanil was formed by expressed CYP3A4 and CYP2C19 supersomes. However, only expressed CYP2C19 and CYP2C19 supersomes formed 4'-hydroxymephenytoin.

Conclusions The oxidative metabolism of (S)-mephenytoin and proguanil *in vitro* is catalysed by CYPs 2C19 and 1A2, with the significant association between V_{max} values suggesting that the predominant enzymes involved in both reactions are similar. However the degree of selectivity of both drugs for CYP isoforms needs further investigation, particularly the involvement of CYP3A4 in the metabolism of proguanil. We assert that proguanil may not be a suitable alternative to (S)-mephenytoin as a probe drug for the CYP2C19 genetic polymorphism.

Keywords: (S)-mephenytoin, CYP1A2, CYP2C19, CYP3 A4, genotype, human liver microsomes, proguanil

Introduction

The hepatic oxidative metabolism of (S)-mephenytoin to 4'-hydroxymephenytoin is controlled by a genetic poly-

morphism affecting the cytochrome P450 isozyme, CYP2C19 [1]. Poor metabolisers (PM_m) represent ~3.5% of Caucasians [2] and 18–25% of Orientals [3, 4]. This deficiency is inherited in an autosomal recessive fashion [5], and currently five genetic defects within the gene coding for CYP2C19 have been identified (CYP2C19*2 A and 2B, CYP2C19*3, CYP2C19*4, CYP2C19*5 A and 5B, and CYP2C19*6) [1, 6–9]. The

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combination of *CYP2C19*2 A and B* and *CYP2C19*3* account for 100% of mutant alleles in PM_m Oriental subjects [1]. All of the genetic defects are found in the Caucasian population (percentage of defective alleles represented by *CYP2C19*2 A and B*, *CYP2C19*3*, *CYP2C19*4*, *CYP2C19*5 A and B*, and *CYP2C19*6*, respectively, are 86.5%, 1.4%, 2.7%, 1.4% and 1.4%) [8, 9].

Rac-mephenytoin is used by many investigators as a probe for the *CYP2C19* polymorphism. However, due to the occurrence of idiosyncratic reactions, including fatal blood dyscrasias, it is no longer approved for marketing in Australia and some other countries. Therefore, its accessibility is limited and much interest has focused on finding a suitable replacement phenotyping probe [10]. Proguanil is an antimalarial prodrug, whose conversion to the active triazine metabolite cycloguanil is also under genetic control and thus 3–10% of Caucasians [11, 12], and 18% of Orientals [13] are poor metabolisers.

Chemical inhibition studies using human liver microsomes have indicated that the polymorphisms in 4'-hydroxymephenytoin and cycloguanil formation cosegregate. Meier and associates reported competitive inhibition of 4'-hydroxymephenytoin formation by proguanil [14], and Helsby and colleagues found that (S)-mephenytoin was also a competitive inhibitor of cycloguanil formation *in vitro* with a K_i of 22 μM [11], a value similar to its K_m ($38 \pm 10 \mu\text{M}$) [14]. However, in human liver microsomes the formation of cycloguanil was shown to correlate with hepatic *CYP3A* content, benzo(a)pyrene hydroxylation, caffeine 8-oxidation, and hydroxymeprazole formation [10]. These reactions are wholly or substantially catalysed by *CYP3A4*. Thus proguanil activation to cycloguanil is likely to be mediated by *CYP2C19* and *CYP3A* isoforms.

It has been suggested that proguanil may provide a convenient *in vivo* phenotypic probe to replace (S)-mephenytoin in large scale population studies [15–17]. To assume this role, a close relationship between (S)-mephenytoin and proguanil oxidative metabolism would have to be established, and the relative contribution of the *CYP3A* isoform to cycloguanil formation evaluated. In addition, there are few data on the involvement of other CYP isoforms in (S)-mephenytoin hydroxylation and the precise relationship between the formation rates of 4'-hydroxymephenytoin and cycloguanil in human liver microsomes is unclear.

The aims of this study were to determine (i) the CYP isoforms involved in the formation of 4'-hydroxymephenytoin and cycloguanil, and (ii) the relation between their respective formation rates, using the same human liver microsomes.

Methods

Chemicals and reagents

Furafylline, ($\pm$)-4'-hydroxymephenytoin and S(+)-mephenytoin were purchased from Ultrafine Chemicals (Manchester, England). Chlorcycloguanil, cycloguanil and proguanil were obtained from ICI Pharmaceuticals (Macclesfield, England). Bovine serum albumin (fraction V), butylated hydroxytoluene (BHT), diethyldithiocarbamate (DDC), ($\pm$)-isocitric acid Na₃, folin-ciocalteu reagent, isocitrate dehydrogenase (NADP, type IV), sulphaphenazole, triethylamine (TEA) and troleandomycin (TAO) were obtained from Sigma Chemical Company (St Louis, Missouri, USA). Phenobarbitone sodium was supplied by F.H. Faulding Ltd (Adelaide, Australia), and omeprazole was obtained from Astra Pharmaceuticals Pty Ltd (Sydney, Australia). Diazepam was a gift from Professor J. Miners (Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, Adelaide, Australia) and dextromethorphan hydrobromide was obtained from Roche Pty. Ltd. (Sydney, Australia). Human *CYP2E1* and *CYP3A4* monoclonal antibodies and microsomes from human lymphoblastoid cells containing expressed *CYP2D6*, *CYP2C19* and *CYP3A4*, and *CYP2C19* supersomes were obtained from Gentest Corporation (Woburn, MA, USA). All other chemicals and reagents were purchased from commercial sources and were of analytical grade quality.

Human liver microsomes

Liver tissue was obtained from 10 patients undergoing partial hepatectomy for hepatic tumours. This procurement was approved by the Committee on the Ethics of Human Experimentation of the University of Adelaide and the Human Ethics Committee of the Royal Adelaide Hospital. Microsomes were prepared by differential centrifugation of liver homogenate based on the method of Zanger *et al.* [18] and liver and microsomes in buffer were stored at -80°C . The patients' characteristics were as follows: their ages ranged from 25 to 72 years, six were male and four were female, they had normal clinical chemistry and haematology prior to surgery, except that some hepatic enzyme concentrations were above the normal range: patient 15—high serum alkaline phosphatase ($5 \times$ upper limit of normal [ULN]) and alanine transaminase (ALT, $2.7 \times$ ULN); patient 19—high gamma glutamyl transaminase ($3.4 \times$ ULN); patient 20—high lactate dehydrogenase (LD, $3.4 \times$ ULN), serum aspartate transaminase (AST, $7.9 \times$ ULN), and ALT ($7.5 \times$ ULN); patient 32—high LD ($5.5 \times$ ULN) and AST ($16.2 \times$ ULN). Total protein content and total cytochrome P450 content of the microsomes were determined using

the methods of Lowry *et al.* [19], and Omura & Sato [20], respectively. Despite the enzyme abnormalities, the total cytochrome P450 and protein content in livers #15, 19, 20 and 32 were similar to the remaining livers.

Genotyping procedure

Liver samples were genotyped for the two major genetic mutations of the *CYP2C19* gene, namely *CYP2C19**2 and *CYP2C19**3. Genomic DNA was isolated from liver tissue using a QIAamp[®] tissue kit (QIAGEN Pty Ltd, Victoria, Australia). Genotyping was then performed via polymerase chain reaction (PCR) amplifications and restriction endonuclease digestions of PCR products as previously described [16].

Kinetic studies

Cycloguanil formation All incubations were performed in duplicate at 37°C in a shaking water bath for 45 min. Since cycloguanil formation from proguanil was linear up to 60 min and 1 mg ml⁻¹ microsomal protein, the incubates contained 25 µl NADPH generating system, proguanil (50, 100, 200, 400, 750, 900, 1000 and 1200 µM) and 800 µg ml⁻¹ microsomal protein. The total incubation volume of 250 µl was made up with microsomal incubation buffer (0.1 M sodium phosphate, pH 7.40). The formation of cycloguanil was stopped by the addition of acetonitrile (150 µl) containing the internal standard chlorcycloguanil (15 µg ml⁻¹). Samples were then centrifuged and aliquots analysed for cycloguanil by h.p.l.c.

4'-Hydroxymephenytoin formation 4'-hydroxymephenytoin (4'-OH-M) formation from (S)-mephenytoin was linear up to 150 min and 1.5 mg ml⁻¹ microsomal protein. All incubations were performed in duplicate at 37°C for 120 min. The incubates of final volume 250 µl contained 25 µl NADPH generating system (S)-mephenytoin (10, 20, 40, 70, 100, 150, 200, and 300 µM), 1 mg ml⁻¹ microsomal protein and the microsomal incubation buffer. The formation of 4'-OH-M was stopped by the addition of 150 µl acetonitrile containing the internal standard phenobarbitone (5 µM). Samples were then centrifuged and aliquots analysed for 4'-hydroxymephenytoin by h.p.l.c.

Assays

Reversed phase h.p.l.c. was used to quantify cycloguanil and 4'-hydroxymephenytoin. The equipment comprised a LC-6 A pump (Shimadzu Corporation, Kyoto, Japan), a Wisp 710B autoinjector (Waters Associates, Milford, MA, USA), a 4.6-mm × 15 cm stainless-steel column

packed with Spherisorb 5 µm C₁₈ (S5ODS2) packing material (Phase Separations Ltd, Deeside, UK), an ultraviolet detector (875-UV/VIS, Jasco Spectroscopic Company Ltd, Tokyo, Japan) and a C-R6 A Chromatopac integrator (Shimadzu). U.v. detection was monitored at a wavelength of 210 nm and 238 nm for 4'-hydroxymephenytoin and cycloguanil, respectively, and optimal separation of the substrates from their metabolites was achieved with mobile phases flowing at 1.5 ml min⁻¹ comprising 20% acetonitrile, 10 mM triethylamine, 5.5 mM NaH₂PO₄, at a final pH of 2.2 (cycloguanil assay) and 20% acetonitrile, 10 mM triethylamine, 5.5 mM NaH₂PO₄, at a final pH of 5.50 (4'-hydroxymephenytoin assay). The intra- and interassay precision as represented by the relative standard deviation (CV = (s.d./mean) × 100) was 14.1% (*n* = 6) and 12.2% (*n* = 12), respectively, at the 200 nm limit of quantification for cycloguanil, and 7.8% (*n* = 6) and 10.5% (*n* = 12), respectively, at the 500 nm limit of quantification for 4'-hydroxymephenytoin. Quality control samples (low, medium and high) were also used to evaluate inter- and intra-assay precision and accuracy and were ≤ 10% (*n* = 12) for both precision and accuracy for the two assays.

Chemical inhibition studies

Microsomes from four of the human liver samples (HLS #5, 16, 22, 31) were used to examine the inhibition of cycloguanil and 4'-hydroxymephenytoin formation. Fixed concentrations of proguanil and (S)-mephenytoin based on their *K_m* values were incubated in a total volume of 250 µl with the NADPH generating system, microsomal protein, microsomal incubation buffer and 100 µM of inhibitors. The chemical inhibitors considered to be specific for various isoforms were: furafylline (25 and 100 µM, CYP1A2), diethyldithiocarbamate (100 µM, CYP2E1/3A4), sulphaphenazole (100 µM, CYP2C9), and troleandomycin (10 and 100 µM, CYP3A4) [21, 22]. Omeprazole (100 µM, CYP2C19/3A4) and diazepam (100 µM, CYP2C19/3A4) were used as positive controls [23, 24], and inhibition by dextromethorphan (10 µM, CYP2D6) was studied as a negative control [25]. Additionally, the inhibition of cycloguanil formation by (S)-mephenytoin and 4'-hydroxymephenytoin formation by proguanil was assessed by incubation of either proguanil or (S)-mephenytoin in the presence of the other, and combination inhibition by troleandomycin (10 µM), furafylline (25 µM) and (S)-mephenytoin (100 µM) of cycloguanil formation was also evaluated. Conditions of incubations did not alter from the kinetic study, except that furafylline, diethyldithiocarbamate and troleandomycin required 15 min preincubation prior to the addition of the substrates. Inhibitor stocks were made in varying solvents due to differences in solubility. Of

those not made up in water, acetonitrile (ACN) (2% in water, 0.5% final incubation concentration) was used to dissolve furafylline, dimethyl sulphoxide (DMSO) (2% in water, 0.5% final incubation concentration) was used to dissolve sulphaphenazole, and methanol (2% in water, 0.5% final incubation concentration) was used to dissolve troleandomycin and diazepam, whilst methanol (1% in water, 0.25% final incubation concentration) was also used to dissolve (S)-mephenytoin. Omeprazole was dissolved in a 2% solution of methanol in pH 10.5 water to prevent degradation, as its stability is highly pH dependent. Incubations containing equivalent amounts of ACN, DMSO and methanol were used as controls. Of the solvents used, 2% DMSO caused 38% activation and 48% inhibition, 2% methanol caused 9% and 15% inhibition, 2% methanol pH 10.5 caused 5% activation and 22% inhibition of formation of cycloguanil and 4'-hydroxymephenytoin, respectively, and 2% ACN caused 35% inhibition of formation of 4'-hydroxymephenytoin, when compared to aqueous control.

Inhibition with monoclonal antibodies

Cycloguanil formation Microsomes from three livers (HLS #5, 16, and 21) were used to examine the inhibition of cycloguanil formation by human CYP3A4 monoclonal antibodies (8 μ l 100 μ g⁻¹ microsomal protein, in duplicate). Additionally, microsomes from one liver (HLS #5) were used to examine the inhibition by human CYP2E1 monoclonal antibodies (2 μ l 100 μ g⁻¹ and 8 μ l 100 μ g⁻¹ microsomal protein, in duplicate). A fixed concentration of proguanil (based on the K_m values for the livers) was incubated in a total volume of 250 μ l with the NADPH regenerating system, microsomal protein, microsomal incubation buffer, in the presence of human CYP2E1 and CYP3A4 monoclonal antibodies.

4'-Hydroxymephenytoin formation Microsomes from one liver (HLS #5) were used to examine the inhibition of 4'-hydroxymephenytoin formation by human CYP2E1 (2 μ l 100 μ g⁻¹ and 8 μ l 100 μ g⁻¹ microsomal protein, in duplicate) and human CYP3A4 (2 μ l 100 μ g⁻¹ and 8 μ l 100 μ g⁻¹ microsomal protein, in duplicate) monoclonal antibodies. A fixed concentration of (S)-mephenytoin (based on the K_m values for the liver) was incubated in a total volume of 250 μ l with the NADPH regenerating system, microsomal protein, microsomal incubation buffer, in the presence of human CYP2E1 and CYP3A4 monoclonal antibodies.

Antibodies were preincubated with microsomal protein for 15 min on ice prior to the addition of remaining incubate constituents. Condition of incubations and

sample processing did not alter from each of the kinetic studies.

Metabolism by expressed CYP450 isoforms

The metabolism of proguanil to cycloguanil and (S)-mephenytoin to 4'-hydroxymephenytoin was studied with the use of expressed CYP isoforms. Microsomes from human lymphoblastoid cells containing expressed CYP2D6, CYP2C19, and CYP3A4, and CYP2C19 supersomes (baculovirus infected insect cells) were incubated with 300 μ M proguanil or 50 μ M (S)-mephenytoin in a total volume of 250 μ l with the NADPH regenerating system, and microsomal incubation buffer. Additionally, a kinetic study of cycloguanil and 4'-hydroxymephenytoin formation by CYP2C19 supersomes was conducted. CYP2C19 supersomes were incubated with varying concentrations of proguanil (5, 10, 15, 25, 50, 75, 100, 200 and 300 μ M) or (S)-mephenytoin (5, 10, 25, 50, 75 and 200 μ M), in a total volume of 250 μ l with the NADPH regenerating system, and microsomal incubation buffer. Following this, the remainder of the experiment was identical to that for human liver microsomes kinetic study.

Data analysis

The rates of metabolite formation were expressed as nmol mg⁻¹ protein h⁻¹. Interpretation of the Eadie-Hofstee plots (V vs V/S) resulted in a single-enzyme Michaelis-Menten equation being fitted to the data using nonlinear least-squares regression analysis (Regression; Blackwell Scientific Software, Blackwell Scientific Publications, Oxford, UK) which provided estimates of K_m and V_{max} . A two enzyme equation was also evaluated, however, the single enzyme equation produced a substantially better fit to the data. Intrinsic clearance (CL_{int}) was calculated as V_{max}/K_m . The relationships between V_{max} of cycloguanil and 4'-hydroxymephenytoin formations, and between CL_{int} of proguanil to cycloguanil and (S)-mephenytoin to 4'-hydroxymephenytoin were evaluated using the Spearman Rank correlation. Inhibition data were expressed as a percentage of the corresponding controls and the one-tailed t -test was used to determine if inhibition was significantly different from controls. All data are tabulated as mean $\pm$ s.d.

Results

CYP2C19 genotyping

The genotyping assay revealed the presence of CYP2C19*1 (wildtype) and CYP2C19*2 alleles, but no CYP2C19*3 alleles were found. Overall genotyping indicated that all livers were wildtype, except HLS #5

which was heterozygote *CYP2C19**1/*CYP2C19**2. Genotyping of HLS #16 was not possible because of insufficient tissue.

Kinetic studies

Cycloguanil and 4'-hydroxymephenytoin formations The derived Michaelis-Menten kinetic parameters are summarized in Table 1. In two livers, HLS #20 and #22, cycloguanil formation was extremely low and Michaelis-Menten kinetics, and consequently the CL_{int} could not be quantified. However, at 400 μ M proguanil, the cycloguanil formation rate was 0.4 ± 0.002 nmol $mg^{-1} h^{-1}$ in HLS #20 ($n=2$) and 0.5 ± 0.1 nmol $mg^{-1} h^{-1}$ in HLS #22 ($n=2$). The formation rate in the next lowest activity liver (HLS #31) at 400 μ M proguanil was 0.5 ± 0.005 nmol $mg^{-1} h^{-1}$. For association analysis, the formation rates of cycloguanil at 400 μ M proguanil for HLS #20 and #22 were assumed to be the V_{max} . For 4'-hydroxymephenytoin there was virtually no variability in the K_m values but V_{max} showed a 14-fold range between the 9 livers. In one liver (HLS #23) both the cycloguanil and 4'-hydroxymephenytoin formation was

too low and the Michaelis-Menten kinetics could not be accurately estimated using the single enzyme model.

The V_{max} of cycloguanil formation was significantly associated with V_{max} of 4'-hydroxymephenytoin formation ($r_s=0.95$, $P=0.0004$, $n=9$, Figure 1). However, no association was observed between the CL_{int} of proguanil and the CL_{int} of (S)-mephenytoin ($r_s=0.60$, $P=0.167$, $n=7$, Figure 2).

Chemical inhibition studies

Cycloguanil formation was significantly inhibited by omeprazole, 10 and 100 μ M troleandomycin, diethyldithiocarbamate, 25 and 100 μ M furafylline, and (S)-mephenytoin with mean differences from control of $-48 \pm 9\%$ ($P<0.01$), $-23 \pm 4\%$ ($P<0.05$), $-41 \pm 8\%$ ($P<0.001$), $-44 \pm 5\%$ ($P<0.0005$), $-36 \pm 9\%$ ($P<0.005$), $-31 \pm 13\%$ ($P<0.01$), and $-21 \pm 10\%$ ($P=0.01$), respectively (Figure 3). Inhibition of cycloguanil formation by 10 μ M troleandomycin plus 25 μ M furafylline plus 100 μ M (S)-mephenytoin was not 100%, with mean difference from control of $-46 \pm 10\%$ ($P<0.02$). This was not significantly different from the mean difference

HLS #	K_m (μ M)	V_{max} (nmol $mg^{-1} h^{-1}$)	CL_{int} (μ L $h^{-1} mg^{-1}$)	Coefficient of determination (r^2)
<i>Cycloguanil</i>				
5	76	1.2	16	0.92
15	48	1.1	23	0.96
16	43	0.8	19	0.75
19	380	1.0	2.6	0.75
21	51	0.6	12	0.72
31	52	0.6	12	0.78
32	63	2.5	40	0.96
Mean	102	1.1	18	0.83
s.d.	123	0.7	12	0.11
CV(%) ^a	121	64	67	13
<i>4'-hydroxymephenytoin</i>				
5	51	4.8	95	0.98
15	51	10	199	0.98
16	51	3.5	69	0.87
19	51	5.3	104	0.68
20	51	1.3	26	0.90
21	51	2.4	47	0.63
22	51	1.4	28	0.89
31	51	3.0	59	0.96
32	51	14	274	0.99
Mean	51	5.1	100	0.88
s.d.	0.0	4.3	84	0.13
CV(%)	0.0	84	84	15

HLS—human liver sample.

a—CV=s.d./mean.

Table 1 Michaelis-Menten kinetic parameters for cycloguanil and 4'-hydroxymephenytoin formation from proguanil (a) and (S)-mephenytoin (b), respectively, in human liver microsomes.

Figure 1 Relationship between the maximum formation rates ($V_{\max}$) of 4'-hydroxymephenytoin and cycloguanil in human liver microsomes ($r_s = 0.95$; $P = 0.0004$; $n = 9$).

Figure 2 Relationship between the intrinsic clearance of (S)-mephenytoin and progvanil in human liver microsomes ($r_s = 0.60$; $P = 0.167$; $n = 7$). Intrinsic clearance = $V_{\max}/K_m$.

observed in the presence of 10 μM troleandomycin alone ($P = 0.114$) or 25 μM furafylline alone ($P = 1.000$). Diazepam, dextromethorphan and sulphaphenazole did not significantly inhibit cycloguanil formation (Figure 3).

4'-hydroxymephenytoin formation was significantly inhibited by omeprazole, diethyldithiocarbamate, progvanil, 25 and 100 μM furafylline, diazepam, 100 μM troleandomycin, and sulphaphenazole with mean differences from control of -98% ($P < 0.0001$), $-90 \pm 10\%$ ($P < 0.0005$), $-36 \pm 9\%$ ($P < 0.005$), $-33 \pm 4\%$ ($P < 0.001$), $-36 \pm 6\%$ ($P = 0.005$), $-25 \pm 2\%$ ($P < 0.0001$), $-20 \pm 12\%$ ($P < 0.05$), and $-12 \pm 2\%$ ($P < 0.001$), respectively (Figure 3); 10 μM troleandomy-

cin and 10 μM dextromethorphan did not significantly inhibit 4'-hydroxymephenytoin formation (Figure 3).

Inhibition with monoclonal antibodies

Human CYP2E1 and CYP3A4 monoclonal antibodies did not inhibit the formation of cycloguanil from progvanil. The mean differences from control values in the presence of 2 μl and 8 μl CYP2E1 100 μg^{-1} microsomal protein were $27 \pm 10\%$ and $8 \pm 10\%$, respectively, and in the presence of 2 μl and 8 μl CYP3A4/100 μg microsomal protein were $27 \pm 2\%$ and $-9 \pm 8\%$, respectively. Human CYP2E1 and CYP3A4 monoclonal antibodies did not inhibit the formation of 4'-hydroxymephenytoin from (S)-mephenytoin. The mean differences from control values in the presence of 2 μl and 8 μl CYP2E1 100 μg^{-1} microsomal protein were $1 \pm 15\%$ and $4 \pm 14\%$, respectively, and in the presence of 2 μl and 8 μl CYP3A4 100 μg^{-1} microsomal protein were $10 \pm 4\%$ and $24 \pm 5\%$, respectively.

Metabolism by expressed CYP450 isoforms

Microsomes from human lymphoblastoid cells containing expressed CYP2D6 did not form cycloguanil and 4'-hydroxymephenytoin from progvanil or (S)-mephenytoin, respectively. Conversely, microsomes from human lymphoblastoid cells containing expressed CYP3A4 and CYP2C19 supersomes formed cycloguanil, but the formation of cycloguanil by expressed CYP2C19 only was below the limit of quantification of the assay. The formation rates of cycloguanil by the expressed enzymes were compared with those in microsomes from a human liver sample (HLS #16) (Table 2). Only microsomes from human lymphoblastoid cells containing expressed CYP2C19, and CYP2C19 supersomes were able to form 4'-hydroxymephenytoin. The formation rates of 4'-hydroxymephenytoin by the expressed enzymes were compared to those in microsomes from a human liver sample (HLS #5) (Table 2).

The formation kinetics of cycloguanil and 4'-hydroxymephenytoin by CYP2C19 supersomes revealed linear Eadie-Hofstee plots, and kinetic estimates were obtained from a single Michaelis-Menten enzyme model. The K_m , $V_{\max}$ and coefficient of determination (r^2) for the fit of the model to the data for cycloguanil and 4'-hydroxymephenytoin were, respectively: 7.7 μM , 2.5×10^{-2} nmol pmol⁻¹ P450 h⁻¹ and 0.95 and, 70 μM , 55×10^{-2} nmol pmol⁻¹ P450 h⁻¹ and 0.99.

Discussion

The kinetic parameters for the formation of 4'-hydroxymephenytoin from (S)-mephenytoin in the 10

Figure 3 Effect of chemical inhibitors on the formations (mean $\pm$ s.d.) of 4'-hydroxymephenytoin (open bars) and cycloguanil (shaded bars) in human liver microsomes ($n = 4$). *denotes statistically significant difference from control ($P < 0.05$).

	Cycloguanil formation (nmol pmol ⁻¹ P450 h ⁻¹)	4'-hydroxymephenytoin formation (nmol pmol ⁻¹ P450 h ⁻¹)
HLS #16 or #5	1.92×10^{-3}	1.37×10^{-2}
Expressed CYP3A4	1.33×10^{-3}	<L.O.Q.
Expressed CYP2C19	<L.O.Q.	8.56×10^{-2}
CYP2C19 supersomes	24.6×10^{-3}	18.8×10^{-2}

Table 2 Formation rates of cycloguanil and 4'-hydroxymephenytoin by expressed CYP2C19 and CYP3A4, and CYP2C19 supersomes in comparison with that of human liver microsomes (HLS #16 for cycloguanil or 5 for 4'-hydroxymephenytoin).

<L.O.Q. = less than limit of quantification.

livers were best estimated by a single Michaelis-Menten equation. The K_m values had an extremely small range (50.8–51.6 μM) which did not change when several different initial estimates of $V_{\max}$ and K_m were provided for data fitting purposes. These data agree with those of Meier and associates who reported a small range in K_m values ($37.8 \pm 9.6 \mu\text{M}$), in liver microsomes of 8 EM_m, and a four-fold increase in K_m in 2 PM_m [14]. Therefore, the small range observed in the present study is indicative of EM_m phenotype, consistent with the genotyping data, and the likely involvement of a single predominant enzyme. Similarly, Jurima and colleagues stated that the K_m for *p*-hydroxylation from racemic mephenytoin was relatively constant in human liver microsomes obtained from five livers (range: 59–143 μM) [26]. In contrast, Hall and colleagues observed a seven-fold variation (range: 31–212 μM) in the K_m values from five human

livers of unknown CYP2C19 pheno- and geno-type, indicating that the affinity of the enzyme(s) for (S)-mephenytoin varies widely [27]. The 14-fold range (1.0–13.9 nmol mg⁻¹ h⁻¹) in $V_{\max}$ values is similar to previous data reported by Meier and associates (range: 0.76–4.85 nmol mg⁻¹ h⁻¹) [14] and Jurima and associates (range: 0.12–1.24 nmol mg⁻¹ protein h⁻¹) [26]. The variation in $V_{\max}$ found in the present study resulted in a 10-fold variation in CL_{int} (range: 26–274 $\mu\text{l mg}^{-1} \text{h}^{-1}$), a similar range to that found for proguanil (see below).

Identification of CYP450 isoform(s) mediating the formation of 4'-hydroxymephenytoin was deduced from three observations. CYP2C19 and possibly 3A4 were concluded as being involved firstly by the use of chemical inhibition data, as significant inhibition of 4'-hydroxymephenytoin formation by omeprazole, diazepam

and 100 μM troleandomycin was observed. However, the small degree of inhibition by 100 μM troleandomycin, although statistically significant, suggests that the 4'-hydroxylation is not predominantly via CYP3A4, and that the inhibition by omeprazole and diazepam was predominantly via CYP2C19. This agrees with a study by Hall and colleagues who reported that diazepam was a partial competitive inhibitor ($K_i = 12 \mu\text{M}$) [27]. The role of CYP3A4 was further discounted due to the lack of inhibition by human CYP3A4 monoclonal antibodies, and the inability of expressed CYP3A4 to form 4'-hydroxymephenytoin. CYP2C19 was further confirmed as CYP2C19 supersomes formed the 4'-hydroxy metabolite. The K_m estimate obtained (70 μM) was similar to those obtained in human liver microsomes (51 μM), indicating that the CYP450 isoform mediating this reaction is predominantly CYP2C19. CYP1A2 was involved as furafylline significantly inhibited 4'-hydroxymephenytoin. This is consistent with the findings of Chiba and associates who showed that (S)-mephenytoin metabolism is inhibited in human liver microsomes by imipramine, whose *N*-demethylation pathway is mediated by CYP2C19, 1A2 and 3A [28]. Further investigations using expressed CYP1A2 are needed to confirm these results. CYP2C9 and CYP2D6 could be discounted due to lack of inhibition by sulphaphenazole and dextromethorphan. CYP2E1 was discounted as being involved due to lack of inhibition by human CYP2E1 monoclonal antibodies. This result again highlights the need for caution when drawing conclusions from inhibition studies utilizing diethyldithiocarbamate, which is a nonspecific inhibitor of CYP2E1 [22]. Therefore from these data it can be concluded that the CYP isoform mediating the formation of 4'-hydroxymephenytoin is predominantly CYP2C19, with the role of 1A2 yet to be confirmed.

The K_m values for proguanil cyclization (range: 43–76 μM) in the majority of human liver samples are in a range similar to those of Birkett and associates (range: 35–185 μM) [10]. This large range may indicate the involvement of several isoforms in this reaction. The degree of variability and absolute values of V_{max} were also similar to those previously reported [10]. CYP2C19 and CYP3A4 were confirmed as isoforms mediating cycloguanil formation due to the observation of significant inhibition by omeprazole and troleandomycin. It is unclear why diazepam did not produce significant inhibition in a similar manner to omeprazole. This may be due to diazepam inhibition being predominantly via CYP2C19, whilst omeprazole inhibits both CYP2C19 and CYP3A4, and may indicate that CYP2C19 plays a minor role in the metabolism of proguanil. This agrees with the observation that expressed CYP3A4 was able to form cycloguanil at a rate similar to that in human liver

microsomes, and expressed CYP2C19 formed cycloguanil at a much lower rate. The higher formation rate of cycloguanil by CYP2C19 supersomes than that seen with expressed CYP3A4 is likely to be due to reduced catalytic activity of CYP3A4 when compared with the CYP2C19 supersomes. Furthermore, the K_m estimate obtained when cycloguanil was formed by CYP2C19 supersomes was much lower than that obtained with human liver microsomes, indicating the involvement of more than one CYP isoform, and potentially a minor role for CYP2C19. It remains unknown why 100% inhibition was not obtained in the presence of 10 μM troleandomycin, 25 μM furafylline and 100 μM (S)-mephenytoin. It could be speculated from these data that other CYP isoforms, not inhibited by these chemicals, are involved in the formation of cycloguanil, although all the other CYP characterization data do not indicate this. CYP1A2 involvement was also evident by significant inhibition of cycloguanil by furafylline, in contrast with previous findings [10]. However, these investigators did not preincubate furafylline with NADPH and microsomes, which is essential as inhibition is mechanism-based [29]. This needs further investigation with expressed CYP1A2.

It has previously been proposed that an association between *in vitro* Michaelis-Menten kinetics for two drugs is evidence for a cosegregating route of metabolism [23]. A statistically significant high degree of association ($r_s = 0.95$) was found between the V_{max} of proguanil and the V_{max} of (S)-mephenytoin. Since V_{max} is a measure of the sum of the different enzymes present in both reactions, the data suggest that the enzyme(s) involved in both reactions are similar. In addition, significant inhibition of (S)-mephenytoin 4'-hydroxylation was observed in the presence of proguanil and *vice versa*. It is reasonable to conclude that this inhibition is not via CYP3A but CYP2C19, and possibly CYP1A2, as these isoforms were found in the present study to be involved in the metabolism of both substrates. The inhibition of proguanil metabolism by (S)-mephenytoin was small (although significant), which agrees with previous reports noting that the degree of inhibition is dependent on the CYP3A content of human liver microsomes [10]. Therefore if the livers used in the present study have a high CYP3A content, as evident by high degree of inhibition by troleandomycin, it is possible that a smaller degree of inhibition by (S)-mephenytoin would be evident.

It is interesting to note that in the two livers (HLS #20 and 22) whose kinetic parameters for the formation of cycloguanil could not be estimated, the K_m values for 4'-hydroxymephenytoin formation were comparable to the other livers. However, the maximum formation rates were at the lower end of activity. This was also the case in HLS #23, whose kinetic parameters for both 4'-hydroxymephenytoin and cycloguanil could not be

estimated using the single enzyme model. These data may suggest that the predominant isoform(s) mediating proguanil oxidation to cycloguanil (CYP3A4, 1A2) were deficient in these two livers, whilst metabolism of (S)-mephenytoin proceeded unimpaired as CYP2C19 is predominantly involved. This hypothesis would also explain the differences in the ranges for K_m of (S)-mephenytoin and proguanil. Additionally, the large variation in range of K_m for proguanil is influenced by the amount of each isoform in the liver, which in the case of CYP3A4 can vary up to 60-fold between individuals [30].

The genotyping results do not provide any further explanation for the differing kinetic estimates, as only one mutant allele (CYP2C19*2) was detected, and interestingly this liver (HLS #5) did not have the lowest intrinsic clearance of either proguanil or (S)-mephenytoin. It should be noted though that the genotyping in this study does not identify all mutant alleles of CYP2C19, since only $\approx 88\%$ could be tested.

Our data suggest that the oxidative metabolism of (S)-mephenytoin and proguanil *in vitro* is catalysed by two common CYP isoforms, CYP2C19 and 1A2, although the extent to which each contributes to the overall oxidation differs. CYP3A4 is also involved in the formation of proguanil. Population based phenotyping and genotyping may help in clarifying the role of proguanil as an *in vivo* phenotyping probe for the CYP2C19 genetic polymorphism.

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References

- de Morais SMF, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K, Goldstein JA. Identification of a new genetic defect responsible for the polymorphism of S-mephenytoin metabolism in Japanese. *Mol Pharmacol* 1994; **46**: 594–598.
- Reviriego J, Bertilsson L, Carrillo JA, Llerena A, Valdivielso MJ, Benitez J. Frequency of S-mephenytoin hydroxylation deficiency in 373 Spanish subjects compared to other Caucasian populations. *Eur J Clin Pharmacol* 1993; **44**: 593–595.
- Nakamura K, Goto F, Ray WA, et al. Interethnic differences in genetic polymorphism of debrisoquin and mephenytoin hydroxylation between Japanese and Caucasian populations. *Clin Pharmacol Ther* 1985; **38**: 402–408.
- Wilkinson GR, Guengerich FP, Branch RA. Genetic polymorphism of S-mephenytoin hydroxylation. *Pharmacol Ther* 1989; **43**: 53–76.
- Kupfer A, Preisig R. Pharmacogenetics of mephenytoin: a new drug hydroxylation polymorphism in man. *Eur J Clin Pharmacol* 1984; **26**: 753–759.
- de Morais SMF, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA, Goldstein JA. The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. *J Biol Chem* 1994; **269**: 15419–15422.
- Ferguson RJ, de Morais SMF, Benhamou S, et al. A new genetic defect in human CYP2C19: Mutation of the initiation codon is responsible for poor metabolism of S-mephenytoin. *J Pharmacol Exp Ther* 1998; **284**: 356–361.
- Ibeanu GC, Blaisdell J, Ghanayem BI, et al. An additional defective allele CYP2C19*5, contributes to the S-mephenytoin poor metaboliser phenotype in Caucasians. *Pharmacogenetics* 1998; **8**: 129–135.
- Ibeanu GC, Goldstein JA, Meyer U, et al. Identification of new human CYP2C19 alleles (CYP2C19*6 and CYP2C19*2B) in a Caucasian poor metaboliser of mephenytoin. *J Pharmacol Exp Ther* 1998; **286**: 1490–1495.
- Birkett DJ, Rees D, Andersson T, Gonzalez FJ, Miners JO, Veronese ME. *In vitro* proguanil activation to cycloguanil by human liver microsomes is mediated by CYP3A isoforms as well as by S-mephenytoin hydroxylase. *Br J Clin Pharmacol* 1994; **37**: 413–420.
- Helsby NA, Ward SA, Howells RE, Breckenridge AM. *In vitro* metabolism of the biguanide antimalarials in human liver microsomes: evidence for a role of the mephenytoin hydroxylase (P450MP) enzyme. *Br J Clin Pharmacol* 1990; **30**: 287–291.
- Ward SA, Watkins WM, Mberu E, et al. Inter-subject variability in the metabolism of proguanil to the active metabolite cycloguanil in man. *Br J Clin Pharmacol* 1989; **27**: 781–787.
- Edstein MD, Shanks GD, Teja-Isavadharm P, Rieckmann KH, Webster HK. Oxidative activation of proguanil and daspone acetylation in Thai soldiers. *Br J Clin Pharmacol* 1994; **37**: 67–70.
- Meier UT, Dayer P, Male P-J, Kronbach T, Meyer UA. Mephenytoin hydroxylation polymorphism: Characterization of the enzymatic deficiency on liver microsomes of poor metabolizers phenotyped *in vivo*. *Clin Pharmacol Ther* 1985; **38**: 488–494.
- Somogyi AA, Reinhard HA, Bochner F. Pharmacokinetic evaluation of proguanil: a probe phenotyping drug for the mephenytoin hydroxylase polymorphism. *Br J Clin Pharmacol* 1996; **41**: 175–179.
- Collier JK, Somogyi AA, Bochner F. Association between CYP2C19 genotype and proguanil oxidative polymorphism. *Br J Clin Pharmacol* 1997; **43**: 659–660.
- Hoskins JM, Shenfield GM, Gross AS. Relationship between proguanil metabolic ratio and CYP2C19 genotype in a Caucasian population. *Br J Clin Pharmacol* 1998; **46**: 499–504.
- Zanger UM, Vilbois F, Hardwick JP, Meyer UA. Absence of hepatic cytochrome P450b₁ causes genetically deficient debrisoquine oxidation in man. *Biochemistry* 1988; **27**: 5447–5454.
- Lowry OH, Roseburg NJ, Farr AL, Randall RJ. Measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.

- 20 Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes, II Solubilisation, purification and properties. *J Biol Chem* 1964; **234**: 2374–2385.
- 21 Newton DJ, Wang RW, Lu AYH. Cytochrome P450 inhibitors: Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metab Dispos* 1995; **23**: 154–158.
- 22 Eagling VA, Tjia JF, Back DJ. Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. *Br J Clin Pharmacol* 1998; **45**: 107–114.
- 23 Andersson T, Miners JO, Veronese ME, Tassaneeyakul W, Meyer UA, Birkett DJ. Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. *Br J Clin Pharmacol* 1993; **36**: 521–530.
- 24 Yasumori T, Qing-Hua L, Yamazoe Y, Ueda M, Tsuzuki T, Kato R. Lack of low Km diazepam n-demethylase in livers of poor metabolisers for S-mephenytoin 4'-hydroxylation. *Pharmacogenetics* 1994; **4**: 323–331.
- 25 Kerry NL, Somogyi AA, Bochner F, Mikus G. The role of CYP2D6 in primary and secondary oxidative metabolism of dextromethorphan: *in vitro* studies using human liver microsomes. *Br J Clin Pharmacol* 1994; **38**: 242–248.
- 26 Jurima M, Inaba T, Kalow W. Mephenytoin metabolism *in vitro* by human liver. *Drug Metab Dispos* 1985; **13**: 151–155.
- 27 Hall SD, Guengerich FP, Branch RA, Wilkinson GR. Characterization and inhibition of mephenytoin 4-hydroxylase activity in human liver microsomes. *J Pharmacol Exp Ther* 1987; **240**: 216–222.
- 28 Chiba K, Saitoh A, Koyama E, Tani M, Hayashi M, Ishizaki T. The role of S-mephenytoin 4'-hydroxylase in imipramine metabolism by human liver microsomes: a two-enzyme kinetic analysis of N-demethylation and 2-hydroxylation. *Br J Clin Pharmacol* 1994; **37**: 237–242.
- 29 Halpert JR, Guengerich FP, Bend JR, Correia MA. Contemporary issues in toxicology: Selective inhibitors of cytochromes P450. *Toxicol Appl Pharmacol* 1994; **125**: 163–175.
- 30 Forrester LM, Henderson CJ, Glancey MJ, *et al.* Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochem J* 1992; **281**: 359–368.