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HYDROXYLATION OF THE ANTIMALARIAL DRUG 58C80 BY CYP2C9 IN HUMAN LIVER MICROSOMES: COMPARISON WITH MEPHENYTOIN AND TOLBUTAMIDE HYDROXYLATIONS

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Abstract—58C80 [2-(4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone] is an experimental naphthoquinone antimalarial drug which undergoes extensive alkyl hydroxylation to a single *t*-butylhydroxy metabolite in man *in vivo* and also in human liver microsomes, where this is catalysed primarily by a 54 kDa CYP2C9 form of cytochrome P450, P450hB$_{20-27}$. Microsomal 58C80 hydroxylation (58OH) activity showed a marked inter-individual variation in a bank of 39 individual human livers but did not correlate with the immunoquantified levels of either of two microsomal proteins (54 and 50 kDa, respectively) recognised by a polyclonal antibody against CYP2C9 (P450hB$_{20-27}$). Neither 58OH activity nor the concentrations of the CYP2C9-immunorelated proteins showed any relationship with the individuals' age, sex, cigarette smoking habit, alcohol consumption or clinical drug treatment, including long term antiepileptic therapy with phenobarbitone or phenytoin. 58OH activity did not correlate with either TBOH (tolbutamide hydroxylation) or MPOH (S-mephenytoin 4′-hydroxylation) activities, while 58C80 inhibited both TBOH and MPOH in human liver microsomes non-competitively (K_i = 30 and 175 μM for TBOH and MPOH, respectively). 58C80 could be a useful model substrate for measuring human CYP2C activity *in vitro*.

Key words: cytochrome P450; drug metabolism; enzyme specificity; polymorphism

The experimental antimalarial compound 58C80,‡ is extensively metabolized in man by monohydroxylation of the *t*-butyl side-chain to the compound 298C84, with approximately 50% of the dose being excreted as 298C84 and its further metabolites in urine over 24 hr [1]. The P450 form primarily responsible for the hydroxylation of 58C80 in human liver has been purified (P450hB$_{20-27}$) and identified as a member of the CYP2C9 subfamily (CYP2C9 comprises several forms which differ slightly in their amino acid sequence) [2]. Compared to the plethora of substrates for the human CYP2D and CYP3A P450 subfamilies, there are few known substrates for human CYP2C [3]. The characterization of a new human CYP2C reaction is therefore especially valuable. In order to further understand 58C80 hydroxylation in man, we have studied its inter-individual variability in a bank of human livers and compared it with the well-known human CYP2C-dependent reactions, MPOH [4] and TBOH [5].

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‡ Abbreviations: CsA-met, cyclosporin A metabolism; COH, coumarin 7-hydroxylation; EROD, ethoxyresorufin O-deethylation; MPOH, S-mephenytoin 4′-hydroxylation; TBOH, tolbutamide hydroxylation; 58C80, 2-(4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone; 298C84, 2-[4-(1,1-dimethyl-2-hydroxyethyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone; 58OH, 58C80 hydroxylation.

MATERIALS AND METHODS

Materials. The drug 58C80, its authentic hydroxylated metabolite (298C84) and [^{14}C]58C80 were synthesized by the Wellcome Research Laboratories (Beckenham, U.K.). Several compounds were generous gifts: *S*-mephenytoin and 4-hydroxymephenytoin from Dr M. Lennard, The Royal Hallamshire Hospital, Sheffield, U.K.; tolbutamide, hydroxytolbutamide and chlorpropamide from Hoechst AG (Frankfurt-am-Main, Germany); sulfaphenazole from Ciba-Geigy AG, (Basel, Switzerland). Lumax scintillation cocktail was purchased from Rhône-Poulenc Laboratory Products (Eccles, U.K.). All other materials were obtained from the usual commercial suppliers.

Liver microsome preparation. Human livers were removed with permission from renal transplant donors at Aberdeen Royal Infirmary within 30 min of circulatory arrest into ice-cold isotonic saline, chopped into approximately 1–2 cm cubes, frozen at −80° within 1 hr and stored at −80° until use. Microsomes were prepared as described previously [2].

Analytical procedures. Microsomal protein and P450 were measured by the methods of Lowry *et al.* [6] and Omura and Sato [7], respectively. Hepatic microsomal monooxygenase activities were measured at 37° as follows; EROD and COH activities were measured fluorimetrically as described by Burke *et al.* [8] and Miles *et al.* [9], respectively. CsA-met was measured using HPLC with radiometric detection

as described previously [10]. 58OH activity was measured as described previously [2], except that analysis was by HPLC as follows: a 30 μL aliquot of the final extract dissolved in methanol was injected onto a Hypersil 5μ SAS column (25 cm × 4.6 mm) and eluted at room temperature using a linear mobile phase gradient of sodium acetate–methanol–acetonitrile (30:10:60 changing to 10:10:80 over 5 min then remaining isocratic for a further 3 min), with an on-line radioisotope detector (Reeve Analytical, Glasgow, U.K.) operating in homogeneous mode using scintillant (Xylene-Lumax, 3:1) mixed with column effluent in a ratio of 3:1. Retention times of 58C80 and its hydroxylated metabolite (298C84) were 7.0 and 4.3 min, respectively. MPOH activity was measured by HPLC with UV detection using a modification of the method of Shimada *et al.* [4] as described previously [2], except that a 0.5 mL incubation was used (containing 50 μL of 2 mM *S*-mephenytoin stock solution in 2% ethanol–1.15% KCl), with termination by the addition of 100 μL 6% perchloric acid. TBOH activity was measured with 100 μM tolbutamide using a modification of the method of Miners *et al.* [11] as follows: a standard NADPH-generating system was employed and initial velocity reaction conditions were ensured by using 45-min incubations and 1–1.5 mg microsomal protein. The reaction (1.0 mL total volume containing 50 μL of a freshly made 2 mM tolbutamide stock solution in 2% ethanol–1.15% KCl) was stopped with 200 μL 1 M orthophosphoric acid, 200 μL of the internal standard, chlorpropamide (2% in water) added, the mixture extracted for 40 min with 11 mL methyl *tert* butyl ether, evaporated to dryness under vacuum at 40° and the residue finally redissolved in 25 μL 25% aqueous acetonitrile. A 100 μL aliquot was analysed by HPLC, using a Waters Novapack C_{18} radial compression cartridge column at room temperature and an isocratic mobile phase (26% acetonitrile in water), with detection at 230 nm. Base-line separations were achieved, with retention times of 4.6, 16.5 and 25.6 min for hydroxytolbutamide, chlorpropamide and tolbutamide, respectively. In all the HPLC analyses, metabolites and parent compounds were quantified by computer-integration of peak areas (Dynamax Method Manager from Rainin Inc., Woburn, MA, U.S.A., running on a Macintosh SE30 computer).

Immunoinhibition. Rabbit polyclonal antibodies against purified human CYP2C9 ($P450hB_{20-27}$) and CYP3A4 (P450hA7) and rat CYP1A1 and CYP2B1 (purified P450 contents 16.6–19.3 nmol P450/mg protein) were raised, characterized and used for immunoinhibition as described previously [2]. Pre-immune rabbit serum globulin was prepared and used similarly as a control for non-specific effects. Microsomal protein (approximately 20 mg protein/mL) and antibody or pre-immune globulin were incubated on ice for 20 min in 50 μL reaction buffer before adding the remaining constituents of the reaction.

SDS–PAGE, immunoblotting and immunoquantitation. Proteins were resolved using SDS PAGE, electroblotted onto nitrocellulose and immunostained with the antibodies described above using horseradish peroxidase and diaminobenzidine as described previously [2]. P450 was immunoquantified on stained immunoblots by image analysis using a Joyce-Loebl Magiscan in macroscopic mode with Gemini densitometric software (vs 4.2). An integrated area × density value was obtained for each immunostained band. Every blot included six separate aliquots of a human liver microsomal sample loaded over the range 1–12 μg protein, to furnish a standard curve (integrated area × density versus amount of protein loaded). The P450 concentration of each band in the test samples was expressed as "μg equivalent P450 protein" by comparison of its integrated area × density value with the standard curve.

Statistics. Correlations were calculated using the Statview program for Macintosh computers, with $P \leq 0.01$ as the criterion for significance.

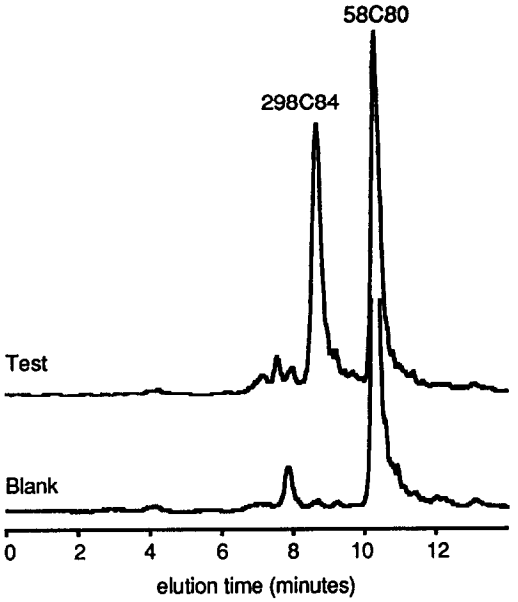

Fig. 1. HPLC chromatogram of 58C80 metabolized by human liver microsomes. [^{14}C]58C80 was incubated with human liver microsomes (id. No. 49) either with NADPH (test) or without (blank) and analysed by HPLC with radiometric detection as described in Materials and Methods. 298C84 is the CYP2C9-dependent metabolite of 58C80.

RESULTS

The incubation of 58C80 with human liver microsomes produced a single metabolite with the same retention time as the authentic *t*-butyl-monohydroxy derivative, 298C84 (Fig. 1; see Ref. 2 for chemical structures). Microsomal 58OH activity showed a marked (11-fold) inter-individual variation among 39 different human livers (Fig. 2). 58OH activity did not inter-relate with the age, sex, cigarette smoking habit, alcohol consumption or clinical drug treatment of the individuals from whom the livers were obtained. A polyclonal antibody

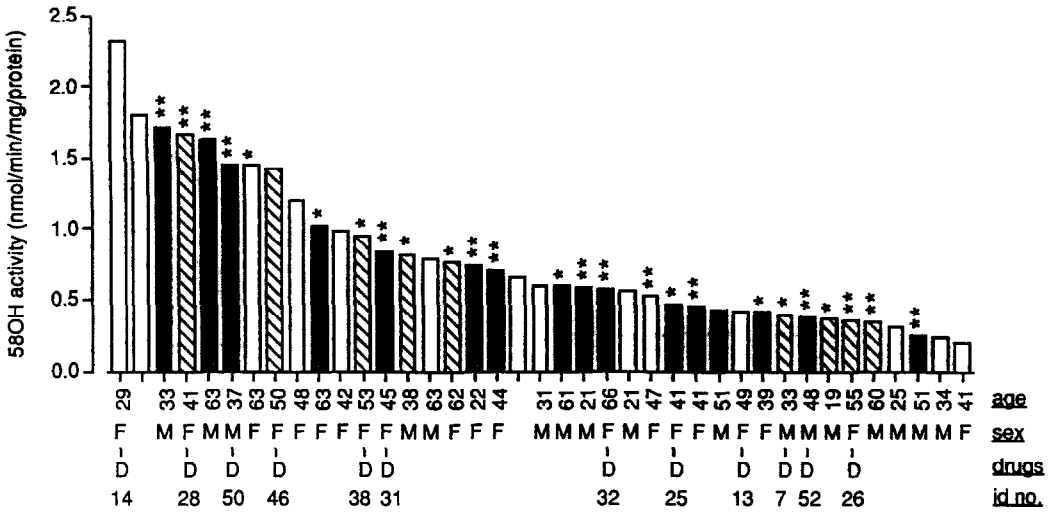

Fig. 2. Microsomal 58C80 hydroxylation activity in a bank of 39 human livers. [^{14}C]58C80 was incubated with human liver microsomes and analysed by HPLC as described in Materials and Methods. Each bar shows the activity (mean of duplicate determinations) for an individual liver, with the individuals' age and sex shown below. Identification (id.) numbers are shown only for those individuals who had been receiving therapeutic drugs (indicated by the letter D), as follows: No. 7—long-term phenobarbitone, phenytoin, carbamazepine and valproate for epilepsy; No. 13—amitryptaline and chlorpromazine; No. 14—terminal betamethasone and methylprednisolone; No. 25—long-term phenytoin, prednisolone, salbutamol and theophylline for epilepsy and asthma; No. 26—long-term phenytoin for epilepsy; No. 28—cyclopenthiazide; No. 31—clorazepate; No. 32—diazepam; No. 38—atenolol; No. 46—nifedipine and metoprolol; No. 50—thyroxine; No. 52—terminal phenytoin (2 × 500 mg on 1 day). (▨) Non-smoker; (▩) medium smoker (6–20 cigarettes/day); (■) heavy smoker (>20/day); (□) no information on smoking. (*) Non- or occasional drinker; (⁂) frequent or heavy drinker; the absence of an asterisk indicates no information on drinking.

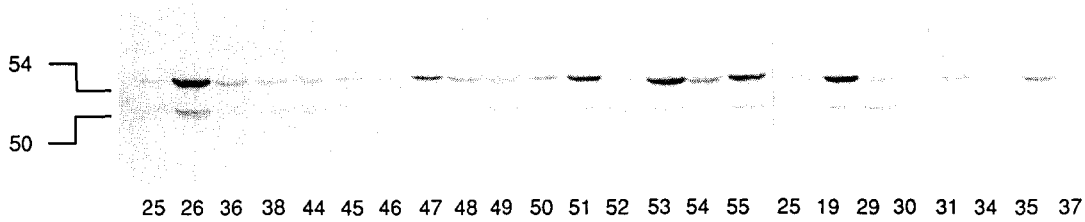

Fig. 3. Immunoblot of CYP2C9-immunoreactive microsomal proteins in a bank of human livers. Hepatic microsomal proteins (loaded at 52 μg protein, one liver per lane) were resolved by SDS–PAGE and immunoblotted with a polyclonal antibody against CYP2C9 (P450hB$_{20-27}$) as described in Materials and Methods. Individual liver id. numbers are shown below each lane. Molecular weights (kDa) of the two immunoreactive protein bands are shown to the left. Because the figure is a photomontage of two blots, liver id. No. 25 was included in both blots for comparison. The microsomes used were a subset of those listed with information in Fig. 2.

raised against a purified sample of the P450 form responsible for most of the 58OH activity in human liver, P450hB$_{20-27}$ (CYP2C9) [2], recognised 2 protein bands, at 54 and 50 kDa, respectively, on immunoblots of human liver microsomes (Fig. 3). The immunoquantified levels of the 54 and 50 kDa protein bands showed a marked inter-individual variation among 24 different livers and were highly intercorrelated ($r = 0.73$, $P = 0.0001$, Fig. 4), but neither band correlated individually with the microsomal 58OH activity ($r = 0.01$, $P = 0.96$).

The correlation of 58OH activity with the two "archetypal" human CYP2C activities, TBOH and MPOH, was analysed. 58OH activity in 19–22 livers did not correlate with either TBOH ($r = 0.023$, $P = 0.92$, $N = 22$) or MPOH ($r = 0.23$, $P = 0.34$, $N = 19$) (Fig. 5). Similarly, the TBOH and MPOH activities in 20 livers did not intercorrelate ($r = 0.24$, $P = 0.32$). (The different N values arose because either MPOH or TBOH was not measured in 4 of the 23 livers assayed for 58OH activity.) Two of the livers showed particularly high MPOH activity, but

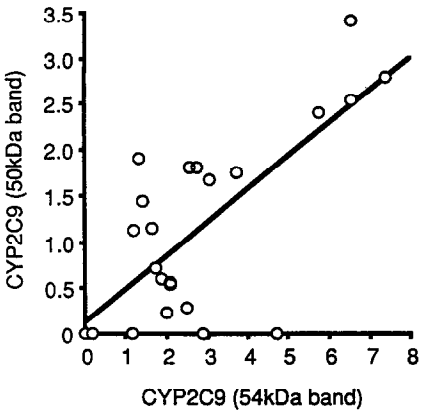

Fig. 4. Correlation between the immunoquantified microsomal concentrations of 54 and 50 kDa CYP2C9-immunoreactive protein bands in a bank of human livers. The immunoblotted bands shown in Fig. 3 were quantified by image analysis as described in Materials and Methods and the results plotted as the "μg equivalent P450 protein" value for each band using the Statview Macintosh program for linear regression. The microsomes used were a subset of those listed with information in Fig. 2.

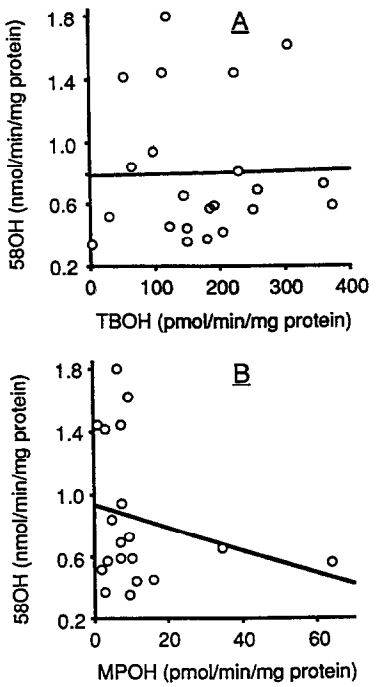

Fig. 5. Correlations of 58OH with TBOH and MPOH activities in microsomes from a bank of human livers. A: 58OH vs TBOH; B: 58OH vs MPOH. Activities were measured as described in Materials and Methods, using a subset of the microsomes listed with information in Fig. 2. The results were plotted using the Statview Macintosh program for linear regression.

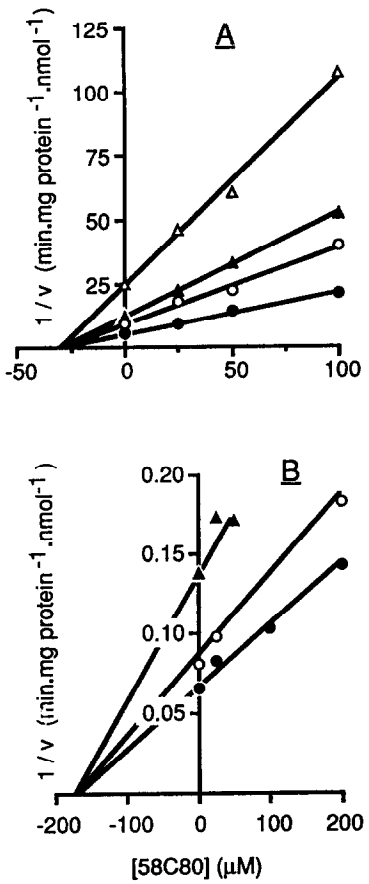

Fig. 6. Dixon plots of the inhibition of TBOH and MPOH activities in human liver microsomes by 58C50. A: TBOH; B: MPOH. Activities using either (A) 12.5 (△), 25 (▲), 50 (○) and 100 μM (●) tolbutamide or (B) 50 (▲), 100 (○) and 200 (●) μM *S*-mephenytoin substrate concentrations and livers id. No. 47 and 38, respectively, were measured as described in Materials and Methods in the presence of 0–100 μM and 0–200 μM 58C80, respectively.

there was still no correlation of MPOH with either 58OH or TBOH if these individuals were omitted from the regression analysis. Dixon plots showed that 58C80 inhibited both TBOH and MPOH non-competitively, with K_i = 30 μM (TBOH) and 175 μM (MPOH), respectively (Fig. 6).

NADH was previously shown to be unusually effective compared with NADPH in supporting human hepatic microsomal 58OH activity [2]. In the current study TBOH activity (with liver id. No. 53) was 72% as high in the presence of NADH as with NADPH. TBOH activity in the presence of the normal NADPH-generating system (0.25 mM NADP, 2.5 mM D,L-isocitric acid, 0.6 U isocitrate dehydrogenase per mL incubate and 15 mM $MgSO_4$) was 200 pmol/min/mg microsomal protein; with NADPH or NADH (0.25 mM) instead of the generating system TBOH activity was 155 and 111 pmol/min/mg protein, respectively. The presence of $MgSO_4$ (15 mM) increased the activity with

Table 1. Effects of anti-P450 antibodies on 58OH and other monooxygenase activities in human liver microsomes

Activity	Inhibition by antibody (% inhibition of control activity)			
	CYP2C9	CYP1A1*	CYP2B1*	CYP3A4
MPOH	96	4	10	0
TBOH	100	50	28	0
EROD	10	98	6	0
COH	33	0	91	0
CsA-met	4	0	6	100
58OH	93	25	2	6

Antibodies, designated by the P450 form against which they were raised, were added at a concentration of 20 mg antibody preparation per mg microsomal protein. *Anti-CYP1A1 and anti-CYP2B1 anitbodies recognize human CYP1A2 and CYP2A6, respectively. Control activities, measured in the presence of an equivalent concentration of pre-immune serum preparation in lieu of antibody, were (nmol/min/mg microsomal protein): 58OH (measured using liver No. 19) = 1.480; MPOH (liver No. 32) = 0.027; TBOH (liver No. 19) = 0.067; EROD (liver No. 30) = 0.041; COH (liver No. 52) = 1.022; CsA-met (liver No. 25) = 0.030. The presence of pre-immune serum globulin alone caused less than 10% change in any activity (data not shown). Human livers were chosen to show high control activity for the reaction being measured, in order to facilitate the measurement of inhibition by antibody. Results are means of duplicate determinations, with replicates showing no more than 5% variation from the mean.

NADPH 1.5-fold to 233 but had little effect (a 7% decrease) on the activity with NADH (results are means of duplicates).

The relative involvements of different P450 forms in 58OH activity in human liver microsomes were identified using immunoinhibition. First, the P450 selectivity of the antibodies to CYP1A1, CYP2B1, CYP2C9 and CYP3A for immunoinhibition of human liver microsomal monooxygenation was established, using widely recognised P450-selective model reactions (Table 1). Immunoinhibition, of 58OH and other reactions, was measured using 20 mg antibody protein per mg microsomal protein. On immunoblots the anti-(rat) CYP1A1 and CYP2B1 antibodies recognised human CYP1A2 and CYP2A6, respectively (cDNA expressed in lymphoblastama cells, kindly provided by Dr C. L. Crespi, Gentest Corporation, Woburn, MA, U.S.A.) but did not recognise purified human CYP2C9 or CYP3A4 [2] (and data not shown). Anti-(rat) CYP1A1 antibody completely inhibited EROD (CYP1A2-dependent in human liver [12]) but did not inhibit COH (CYP2A6-dependent [9]), MPOH (mainly CYP2C19-dependent [13, 14]) or CsA-met (CYP3A4-dependent [10]). Anti-(rat) CYP2B1 antibody almost totally inhibited COH but did not inhibit EROD, MPOH or CsA-met appreciably (≤10%). Anti-(human) CYP3A antibody completely inhibited CsA-met but did not inhibit EROD, COH, MPOH or TBOH (mainly CYP2C9-dependent [5]). Anti-CYP2C9 antibody, as would be expected, completely inhibited MPOH and TBOH but did not inhibit EROD or CsA-met. However, anti-CYP2C9 antibody also partially inhibited COH by 33% (although it did not recognise expressed human CYP2A6 on immunoblots—data not shown), whilst the anti-CYP1A1 and anti-CYP2B1 antibodies partially inhibited TBOH by 50% and 28%, respectively. 58OH activity was strongly inhibited (93%) by the antibody to CYP2C9 ($P450hB_{20-27}$), but not by the anti-CYP1A1, CYP2B1 or CYP3A antibodies (Table 1).

DISCUSSION

58C80 is a novel experimental antimalarial drug, which although pharmacologically potent against target parasites, is rapidly hydroxylated in human volunteers [1]. The hydroxylation of 58C80 in human liver is mainly due to the CYP2C subfamily of cytochromes P450, as shown by purification of the CYP2C9 form responsible [2] and selective inhibition by anti-CYP2C9 antibody (this study). There was, however, no correlation between 58OH activity and either TBOH (a mainly CYP2C9-dependent reaction [5]), or MPOH (catalysed mainly by CYP2C19 [13, 14] but also possibly by CYP2C9 [4] and CYP2C18 [15]), nor was there any correlation between 58OH activity and the microsomal immunoquantified CYP2C protein concentration. Although the anti-CYP2C9 antibody used here recognises more than one (possibly all) human CYP2C forms, the 54 kDa CYP2C-immunoreactive protein quantified in this study was probaby comprised to a significant extent of CYP2C9, since it had the same M_r on immunoblots as the purified CYP2C9 58C80-hydroxylase ($P450hB_{20-27}$) used to raise the antibody [2] and also virtually the same M_r (54.5 kDa) as reported for human liver microsomal CYP2C9 by other workers [16]. CYP2C9 protein levels correlate with CYP2C9-dependent *tetra*hydrocannabinol 7-hydroxylation and hexobarbital 3′-hydroxylation activities in human liver microsomes [17, 18], but have been reported either to correlate [18] or not correlate [4, 16, 19] with MPOH or TBOH. A probable explanation for our results is that 58OH, TBOH and MPOH are together catalysed by a number of similar CYP2C forms, each of which has a different reaction selectivity but cross-reacts immunologically with the polyclonal anti-CYP2C9 antibody; analogous findings with MPOH and TBOH alone have been explained similarly [20]. The observation that 58C80 inhibited MPOH and TBOH non-competitively is a further indication that 58C80 interacts with several forms of CYP2C that are responsible for MPOH and TBOH. In contrast, phenytoin and tolbutamide are both hydroxylated by the same CYP2C9 form and show both mutual competitive inhibition and a correlation between their microsomal hydroxylation activities [21]. Conversely, the absence of mutual inhibition between the CYP2C9 substrate, tienillic acid, and mephenytoin was taken to indicate that these two drugs are metabolized by entirely different CYP2C forms [22].

It should be noted that the implications of competitive inhibition in the case of TBOH activity are not straightforward, since both sulphaphenazole

(a selective inhibitor of CYP2C9) and nifedipine (a selective substrate of CYP3A4 [3]) are strong competitive inhibitors of TBOH in human liver microsomes (K_i = 0.12 and 15 μM, respectively) [11]. Moreover, α-naphthoflavone, which is a selective inhibitor of human CYP1A1 and CYP1A2 [23] but also inhibits some CYP2A6-dependent [24] and CYP3A4-dependent [25] reactions, is also a powerful inhibitor of TBOH in human liver microsomes (IC_{50} = 3 μM) [26]. This might indicate that competitive inhibition is not a reliable guide to P450 specificity, or alternatively that TBOH is not such a selective CYP2C9 substrate probe as often assumed. Antibody inhibition results in the present study support the idea that a proportion of TBOH activity in human liver microsomes is catalysed by CYP1A forms and also indicate the involvement of CYP2A6 (recognised by an anti-CYP2B1 antibody). The antibody inhibition data also suggest that a CYP2C form contributes to COH activity, although this is catalysed mainly by CYP2A6 [9].

The mean microsomal MPOH and TBOH activities recorded here (0.011 and 0.17 nmol/min/mg protein, respectively) were remarkably similar to those reported in a recent study of 12 human livers (0.04 and 0.18 nmol/min/mg protein for MPOH and TBOH, respectively) [16]. The mean 58OH activity (0.80 nmol/min/mg protein) was almost 5-fold higher than TBOH and over 70-fold higher than MPOH in this study. For comparison, the mean specific activities reported for other CYP2C-dependent activities in human liver microsomes are (nmol/min/mg protein): tetrahydrocannabinol 7-hydroxylation, 0.91 [17]; hexobarbital 3′-hydroxylation, 0.71 [18]; tienillic acid 5-hydroxylation, 0.12 [22]; S-warfarin 7-hydroxylation, 0.009 (V_{max}) [27]; phenytoin 4-hydroxylation, 0.001 [26]. Mean V_{max} values for human liver microsomal CYP2C activities are (nmol/min/mg protein): tenoxicam 5′-hydroxylation, 5.4 [28]; 58OH, 2.4 [2]; diclofenac 4′-hydroxylation, 1.0 [29]; TBOH, 0.27 [11]. Thus, 58OH is one of the most active hepatic microsomal CYP2C9 reactions reported.

Although 58OH activity varied widely among 39 different human livers there were no obvious relationships of either this activity or the microsomal CYP2C9 (i.e. 54 kDa protein) concentration with the individuals' age, sex, cigarette smoking habit, alcohol consumption or clinical drug treatment, including long term antiepileptic therapy with phenobarbitone or phenytoin. In other studies human MPOH activity showed no sex-difference or age-relatedness *in vivo* or in liver microsomes [4, 30], while the microsomal concentration of CYP2C9 showed no relationship with alcohol drinking in human livers where CYP2E1 was modestly induced by "excessive drinking" [31]. There are several reports, however, that pentobarbital [32], prednisone [33] and rifampicin [34, 35] can induce MPOH, TBOH or hexobarbitone hydroxylation activities in man *in vivo*, while 5–10 days treatment with a barbiturate mixture (Atrium®) containing phenobarbitone (270 mg/day) induced hepatic microsomal CYP2C9 levels approximately 2-fold (compared to a 4-fold increase in CYP3A4) [31]. In a dissenting report, however, phenobarbitone therapy for 8 days (100 mg/day) did not affect human MPOH activity *in vivo* although it induced nifedipine metabolism (presumably CYP3A-dependent) [36].

So many P450 monooxygenation reactions are NADPH-dependent, with around a 90% drop in activity if supported by NADH instead, that NADPH-dependence is one of the standard tests used to verify the role of P450 in catalysing a reaction. It is therefore noteworthy that in human liver microsomes NADH was unusually effective in supporting both 58OH [2] and TBOH (this study), resulting in 35% and 72% of the NADPH-supported activity, respectively. NADH was even more competent in supporting 58OH with purified CYP2C9 (91% of the NADPH-supported activity), an effect which required, however, the presence of cytochrome b_5 [2]. NADH is similarly effective as an alternative to NADPH in supporting P450-catalysed *p*-nitroanisole O-demethylation in rabbit liver microsomes [37], and a P450 has been purified which shows an absolute requirement for cytochrome b_5 in order to catalyse this activity in the presence of a mixture of NADPH and NADH [38]. Another rare example is human foetal pentoxyresorufin *O*-dealkylase activity, which is even more active with NADH than with NADPH as the cofactor [39]. Cytochrome b_5 had been shown to stimulate the NADPH-dependent MPOH activity of purified human CYP2C9, but the effectiveness of NADH was not reported [4]. In contrast, cytochrome b_5 decreased the NADPH-dependent 58OH activity of purified CYP2C9 [2]. Cytochrome b_5 can, in fact, either stimulate or inhibit P450-mediated NADPH-dependent reactions, depending on both the P450 form and the substrate, with stimulation being particularly marked for rat CYP2C6 (P450RLM5a)-catalysed NADPH-supported *p*-nitroanisole O-demethylation [40]. Although most P450 reactions are absolutely dependent on NADPH, a synergistic stimulation by NADH of NADPH-supported activities has long been recognised and considered to illustrate the ability of cytochrome b_5 to transfer electrons to P450 from NADH [41]. A cytochrome b_5-dependent ability to utilize NADH as cofactor might, perhaps, be a characteristic of some human CYP2C reactions.

In conclusion, this study has shown that whilst the metabolism of the antimalarial drug, 58C80, is mainly CYP2C9-dependent in human liver microsomes, there is no relationship with the hydroxylations of either *S*-mephenytoin or tolbutamide. Several CYP2C forms are probably involved in 58C80 hydroxylation, but the exact identification of these will require more molecular studies. Nonetheless, in view of the rapidity and simple pattern of its metabolism and the ease of its assay compared with MPOH or TBOH, the hydroxylation of 58C80 could be a useful model reaction for the measurement of CYP2C activity in human liver microsomes.

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