Site-directed mutation studies of human liver cytochrome *P*-450 isoenzymes in the CYP2C subfamily

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Evidence from human studies *in vivo* and *in vitro* strongly suggests that the methylhydroxylation of tolbutamide and the 4-hydroxylation of phenytoin, the major pathways in the elimination of these two drugs, are catalysed by the same cytochrome *P*-450 isoenzyme(s). In the present study we used site-directed mutagenesis and cDNA expression in COS cells to characterize in detail the kinetics of tolbutamide and phenytoin hydroxylations by seven CYP2C proteins (2C8, 2C9 and variants, and 2C10) in order to define the effects of small changes in amino acid sequences and the likely proteins responsible in the metabolism of these two drugs in man. Tolbutamide was hydroxylated to varying extents by all expressed cytochrome *P*-450 isoenzymes, although activity was much lower for the expressed 2C8 protein. While the apparent K_m values for the 2C9/10 isoenzymes (71.6–131.7 μM) were comparable with the range of apparent K_m values previously observed in human liver microsomes, the apparent K_m for 2C8 (650.5 μM) was appreciably higher. The 2C8 enzyme also showed quite different sulphaphenazole inhibition characteristics. The 4-hydroxylation of phenytoin was also more efficiently catalysed by the 2C9/10 enzymes. These enzymes showed similarities in kinetics of phenytoin hydroxylation and sulphaphenazole inhibition compared with human liver phenytoin hydroxylase. Also of interest was the observation that, among the 2C9 variants, small differences in amino acid composition could appreciably affect both tolbutamide and phenytoin hydroxylations. The amino acid substitution Cys-144→Arg increased both the rates of tolbutamide and phenytoin hydroxylations, while the Leu-359→Ile change had a greater effect on phenytoin hydroxylation. We conclude that: (1) although 2C8 and 2C9/10 proteins metabolize tolbutamide, only 2C9/10 proteins play a major role in human liver; (2) 2C9/10 proteins also appear to be chiefly responsible for phenytoin hydroxylation; and (3) subtle differences in the amino acid composition of these 2C9/10 proteins can affect the functional specificities towards both tolbutamide and phenytoin.

INTRODUCTION

Cytochrome *P*-450 isoenzymes of the CYP2C subfamily are known to be of considerable importance in the metabolism of many drugs, including tolbutamide (Srivastava et al., 1991), phenytoin (Veronese et al., 1991), warfarin (Rettie et al., 1992) and mephenytoin (Srivastava et al., 1991), as well as some endogenous compounds (Leo et al., 1988). Recent investigations in this laboratory have demonstrated that a human CYP2C9 cDNA expressed in COS-7 cells is functionally similar to tolbutamide hydroxylase present in human liver (Veronese et al., 1991). Several other groups have also isolated cDNAs which appear to be allelic variants (i.e. $\geqslant 97\%$ similarity in protein sequence as defined by Nebert et al., 1991) of 2C9 differing by only a few residues in their amino acid composition (Kimura et al., 1987; Umbenhauer et al., 1987; Ged et al., 1988; Yasumori et al., 1987; Meehan et al., 1988; Romkes et al., 1991). It is not known whether these isolated variants represent different gene products as the appropriate CYP2C genes have not been isolated. At present it is also unclear which of the 2C9 variants represents the 'wild-type' until population studies are performed to determine which variant exists at a higher frequency. Many of these 2C9 variants and a closely related protein 2C8 (77% similar in amino acid sequence) have been shown in cDNA transfection experiments to catalyse the hydroxylation of tolbutamide (Brian et al., 1989; Relling et al., 1990; Veronese et al., 1991; Yasumori et al., 1991). However, comparison between the variants is complicated because of the use of different expression systems between laboratories and the lack of any kinetic characterization. Hence, the contribution of each of these individual microheterogeneous isoenzymes in the metabolism of tolbutamide remains unclear.

Evidence is accumulating from studies both *in vivo* and *in vitro* that tolbutamide methylhydroxylation and phenytoin 4-hydroxylation may be catalysed by the same human cytochrome *P*-450 isoenzyme(s). Briefly, these include: (i) a significant correlation between the two hydroxylations in human liver microsomes (Doecke et al., 1990a); (ii) inhibition of metabolism *in vivo* and *in vitro* by the specific inhibitor sulphaphenazole (Hansen et al., 1979; Doecke et al., 1990a; Veronese et al., 1990); (iii) occurrence of a low incidence of slow metabolizers identified for each drug (Inaba, 1990; Veronese et al., 1990), although no slow metabolizers of tolbutamide or phenytoin have been checked with the other drug; (iv) similar patterns of pharmacokinetic drug interactions between these two drugs (Skovsted et al., 1976; Andreassen et al., 1973; Pond et al., 1977); and more recently (v) significant correlation of unbound plasma clearances *in vivo* in man (Tassaneeyakul et al., 1992). However, although the metabolism of phenytoin and tolbutamide appear closely linked, the role of the above-mentioned CYP2C isoenzymes in phenytoin metabolism is also presently unclear.

In the present study we used site-directed mutagenesis and cDNA expression in COS cells to characterize in detail the kinetics of tolbutamide and phenytoin hydroxylations by various CYP2C proteins in order to define the effects of minor changes in amino acid sequences and the likely proteins responsible in the

Abbreviations used: HPPH, 5-(4-hydroxyphenyl)-5-phenylhydantoin; the cytochrome *P*-450 nomenclature used is that described by Nebert et al. (1991).

metabolism of these two drugs in man. The results suggest that 2C9/10 proteins are functionally similar to the tolbutamide and phenytoin hydroxylases in human liver and that small differences in the amino acid composition of these proteins can affect the functional specificity of these reactions.

MATERIALS AND METHODS

Materials

NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and phenytoin sodium were obtained from Sigma (St. Louis, MO, U.S.A.). Tolbutamide and hydroxytolbutamide were kindly supplied by Hoechst Australia (Melbourne, Australia), sulphaphenazole by Ciba–Geigy (Sydney, Australia) and 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) by Warner–Lambert (Ann Arbor, MI, U.S.A.). [4-^{14}C]Phenytoin was purchased from du Pont (Wilmington, DE, U.S.A.). Restriction enzymes and other reagents used in molecular-biology techniques were obtained from New England Biolabs, Pharmacia LKB Biotechnology or IBI biochemicals. The Bluescript plasmid (II SK+) and XL-1 Blue cells were from Stratagene (La Jolla, CA, U.S.A.). The Erase-A-Base kit was from Promega (Madison, WI, U.S.A.). The COS vector p91023(B) (Wong et al., 1985) was generously given by the Genetics Institute (Boston, MA, U.S.A.). All other chemicals were of analytical reagent grade. The cDNA clone for human 2C8 has been characterized previously (Kimura et al. 1987) as have the 2C9 cDNA variants designated here as 2C9v1 and 2C9v2 by Veronese et al. (1991) and Kimura et al. (1987) respectively. The 2C9v2 and 2C8 clones were kindly provided by F. J. Gonzalez (NCI, NIH, Bethesda, MD, U.S.A.).

Site-directed mutagenesis

The introduction of specific base changes to the 2C9v2 cDNA were made with single-stranded cDNA templates in M13 vectors using the oligonucleotide-directed '*in vitro*' mutagenesis system (Amersham). The oligonucleotide primers synthesized to perform the amino acid changes Cys-144→Arg, Tyr-358→Cys and Gly-417→Asp were 5′ > CTTGAACACGGTCCTCAAT < 3′, 5′ > AGGTCAATGCATCTCTGGA < 3′ and 5′ > TTAAAATT-GTCACCTTCAT < 3′ respectively. All mutations were confirmed by sequencing using the dideoxy method with T7 DNA polymerase after ligation of the mutated cDNA fragments into the Bluescript plasmid and subsequent utilization of the Erase-A-Base kit as previously described (Veronese et al., 1991).

Expression and characterization in COS-7 cells

cDNA inserts from the *Eco*RI-digested Bluescript vectors were ligated into the unique *Eco*RI site of the p91023(B) COS expression vector. After appropriate subclones were characterized for the correct or reverse orientation of the cDNA with respect to the promoter element in p91023(B), plasmid preparations were prepared by the alkaline lysis method after two caesium chloride density-gradient centrifugations. Transfections were carried out as previously described (Mackenzie, 1986). Briefly, the plasmids containing the P4502C cDNAs were transfected into exponentially growing COS-7 cells [that had been previously cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Nuserum (Collaborative Research Incorporated, Bedford, MA, U.S.A.)], using the DEAE-dextran protocol and subsequent chloroquine treatment. The negative control consisted of cells transfected with the 2C9v1 cDNA inserted in the reverse orientation with respect to the promoter element of the COS vector. COS cells used for tolbutamide and phenytoin hydroxylase activities were harvested by scraping 50 h after transfection, washed twice with phosphate-buffered saline, pH 7.4, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, and immediately stored at −70 °C until required. Western blots were also performed on these cell lysates as previously described (McManus et al., 1987), using a purified polyclonal goat antibody directed against rabbit CYP2C3 (Doecke et al., 1990b). Quantification of immunoreactive bands was carried out using an LKB Ultroscan XL densitometer.

Tolbutamide and phenytoin hydroxylase assays

The enzymic hydroxylations of tolbutamide and phenytoin in both human liver microsomes and COS cell lysates were monitored using the incubation conditions and h.p.l.c. assays originally described by Miners et al. (1988) and Doecke et al. (1990b) with some minor modifications (Veronese et al., 1991).

Analysis of results

Initial estimates of the apparent K_m values for tolbutamide and phenytoin were determined by linear regression analysis of the Lineweaver–Burk plots. These values were then used as the first estimates for MKMODEL, an extended least-squares modelling program (Holford, 1985).

RESULTS

Construction and expression of P4502C cDNAs

We have recently isolated a variant of 2C9 (designated here as 2C9v1) and shown in cDNA-transfection experiments that it is able to hydroxylate both tolbutamide and phenytoin (Veronese et al., 1991). This variant is one of seven other 2C9/10 'like' cDNA clones isolated by other workers to date and is identical in the coding region with the more recently isolated clone 25 of Romkes et al. (1991). Of the other published clones ours most closely resembles that of human form 2 of Yasumori et al. (1987) which differs by only one amino acid codon at position 359 (Leu versus Ile). A line-up of most of the currently isolated clones has been recently published (Romkes et al., 1991). We have studied another 2C9 variant, clone IIC1 of Kimura et al. (1987) (designated here as 2C9v2), and this has also been shown to hydroxylate tolbutamide (Relling et al., 1990). On resequencing this clone, 2C9v2 had codons for leucine and valine in positions 4 and 6 instead of isoleucine and serine in the published sequence. In comparison with the 2C9v1 cDNA characterized in this laboratory (Veronese et al., 1991) the 2C9v2 variant has cysteine and isoleucine at positions 144 and 349 compared with arginine and leucine. A CYP2C10 cDNA (mp8) originally isolated by Umbenhauer et al. (1987), when expressed also catalysed tolbutamide hydroxylation. This clone encodes a protein that differs by two amino acids compared with the human 2 protein of Yasumori et al. (1987) (Tyr-358→Cys and Gly-417→Asp). The 2C10 cDNA, mp8, may well be an allelic variant of 2C9, but has been classified at present as a separate gene product on the basis of differences in its 3′-non-coding region when compared with other 2C9 variants.

We were particularly interested in whether these small differences in amino acid sequence between the 2C9/10 proteins appreciably affect catalytic activity towards tolbutamide and phenytoin hydroxylation. Thus sequential site-directed mutagenesis of 2C9v2 was used to introduce amino acid changes to form the two clones: human form 2 and mp8. The 2C9v2 clone was mutagenized to change Cys-144→Arg so as to be equivalent to human form 2 clone (designated here as 2C9v2-A) and subsequently Tyr-358→Cys and Gly-417→Asp so as to be

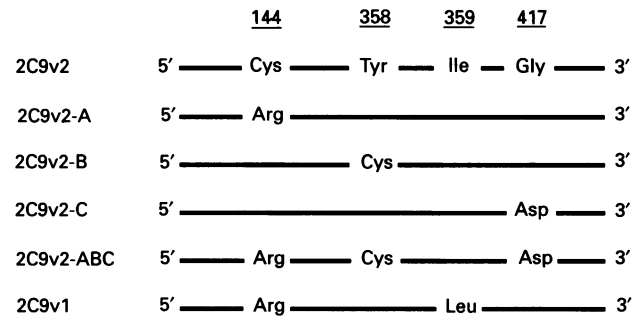

Figure 1 Diagram of the differences in the amino acid composition of the two 2C9 variants and the various mutants

2C9v1 and 2C9v2 are the cDNAs previously described by Veronese et al. (1991) and Kimura et al. (1987). Only the amino acid differences compared with the 2C9v2 sequence are noted. The mutants derived from 2C9v2 including the letters A, B, and C denote the presence of Arg-144, Cys-358 and Asp-417 respectively. 2C9v2-A and 2C9v2-ABC are equivalent to the human form 2 and mp-8 encoded proteins respectively, previously described by Yasumori et al. (1987) and Umbenhauer et al. (1987).

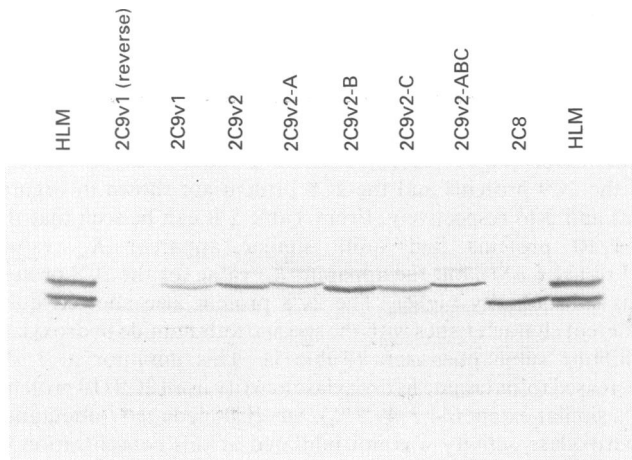

Figure 2 Western immunoblot of 2C8 and 2C9 related proteins

COS-cell homogenates (100 μg) transfected with 2C8 and the 2C9 variants and mutant cDNAs were subjected to SDS/10%-PAGE and Western immunoblotting with anti-(rabbit P4502C3) IgG. The control [P4502C9v1 (reverse)] consisted of COS cells transfected with the cDNA in the reverse orientation with respect to the promoter element of the COS vector. Human liver microsomes (HLM) were also loaded (15 μg).

equivalent to the mp8 clone (designated here as 2C9v2-ABC). Clones with the single mutations Tyr-358→Cys (2C9v2-B) or Gly-417→Asp (2C9v2-C) were also produced to study the effects of these mutations in isolation. The two 2C9 variants and mutants characterized here and their respective amino acid differences are shown in Figure 1.

Expression of each cDNA was monitored by immunoblotting with goat anti-rabbit CYP2C3 IgG (Figure 2). Proteins of molecular mass 55 kDa were detected in all lysates of COS cells transfected with 2C9/10 cDNAs, whereas the 2C8 transfected cells showed a protein with a molecular mass of 51 kDa. Proteins corresponding to these two molecular masses were also detected in human liver microsomes (Figure 2). Additional non-identified bands are also recognized by this antibody in human liver microsomes. The level of expression varied up to $\approx$ 3-fold between cDNAs as determined by densitometry of the immunoreactive bands, and activity determinations were adjusted for these differences (see the subsection below). The immunoreactivity of the variants of 2C9 would not be expected to be altered by mutagenization, since the antibody used was a polyclonal. Initial experiments with 2C9-transfected COS cells indicated that detection of immunoreactive protein by laser densitometry was linear up to at least 150 μg of cell protein lysate.

Catalytic activities of cDNA-expressed cytochrome P-450 isoenzymes

Table 1 shows the results for tolbutamide and phenytoin hydroxylase activities determined in whole COS cell lysates for seven transfected P4502C cDNAs. All $V_{max.}$ data (i.e. data in columns 1 and 4) represented in this Table were from COS cells obtained in one large transfection experiment with all seven cDNAs so as to avoid batch-to-batch variability and were assayed for tolbutamide and phenytoin hydroxylase, in duplicate, concurrently on the same day. These $V_{max.}$ data were adjusted for variations in the expression level according to the amount of immunodetected protein (relative to 2C9v2) determined by Western blotting. It is possible that not all immunodetected protein may be functional. However, the low amounts of cytochrome *P*-450 synthesized in the COS system precludes quantification of functional protein by CO-difference spectrophotometry. In initial experiments with 2C9-transfected COS cells the methylhydroxylation of tolbutamide was shown to be linear with increasing cell protein and time up to at least 2 mg and 2 h respectively. The addition of purified human NADPH cytochrome *P*-450 reductase (McManus et al., 1987) as well as sonication had no effect on tolbutamide hydroxylase activity in CYP2C-transfected COS cell lysates (results not shown), indicating that reductase is not limiting in these cells. Phenytoin 4-hydroxylation was also shown to be linear with increasing protein and time up to at least 2 mg and 4 h respectively. There was a 3.3-fold range in tolbutamide hydroxylation activity among the 2C9 variants, with 2C9v2-A having the highest activity and 2C9v2 the lowest. 2C9v1, which has an arginine at amino acid position 144, similar to 2C9v2-A, also had high tolbutamide hydroxylase activity compared with 2C9v2, which has a cysteine residue at position 144. These results imply that the single amino acid change, namely Cys-144 → Arg, can increase tolbutamide hydroxylase activity. However, the triply mutated derivative 2C9v2-ABC [mp8 (2C10) equivalent], which also includes this amino acid change, only had marginally raised activity. Hence, the combined Tyr-358 → Cys and Gly-417 → Asp change appear to nullify the increased tolbutamide hydroxylase activity obtained with the Cys-144 → Arg change, although the individual mutants 2C9v2-B and 2C9v2-C, which have the single amino acid changes at position 358 and 417 respectively, do not have low tolbutamide hydroxylase activity. Recently, Yasumori et al (1991) have expressed the 2C9/10 forms, human 2 and mp8, equivalent to our 2C9v2-A and 2C9v2-ABC respectively, in a yeast system and, consistent with our results, they obtained higher activity (3.4-fold) for human 2 compared with mp8. In contrast with these results, Srivastava et al. (1991) recently showed a higher tolbutamide hydroxylase activity for the mp8 clone (1.9-fold higher than the human 2 equivalent, mp4). The tolbutamide hydroxylase activity of the 2C8 expressed cells was much lower (7–24-fold) than the 2C9 expressed cells, but was significantly higher (7-fold) than the negative control transfected cells, indicating significant, but low, activity for the 2C8 protein.

To characterize further the metabolic differences between the CYP2C proteins, the kinetics of tolbutamide metabolism were investigated using expressed 2C8 and 2C9 proteins, and the apparent K_m values are presented in Table 1. Over the substrate range studied, single-enzyme Michaelis–Menten kinetics best

Table 1 Characteristics of tolbutamide and phenytoin hydroxylations by cDNA-expressed cytochrome *P*-450 isoenzymes

Abbreviations used: TB, tolbutamide; PH, phenytoin; SPZ, sulphaphenazole. Rel. TB, Rel. PH, tolbutamide and phenytoin hydroxylation activities relative to 2C9v2 (original tolbutamide hydroxylase activity 188.5 pmol of hydroxytolbutamide/h per mg of protein; phenytoin hydroxylase activity 11.1 pmol of HPPH/h per mg of protein); activities were adjusted for expression level according to the amount of immunodetected protein (relative to 2C9v2) on Western blots; all data on the relative tolbutamide and phenytoin activities were performed on the same batch of COS cells obtained from a single transfection of all seven cDNAs. All results are the averages for duplicate incubations.

Protein	Rel. TB*	Apparent K_m (TB, μM)	Inhibition of TB by SPZ*‡ (%)	Rel. PH†
2C9v1 (reverse)	0.02	–	–	0.01
2C9v1	2.48	131.6	45.3	0.36
2C9v2	1.00	94.4	49.7	1.00
2C9v2-A	3.29	90.5	46.1	1.90
2C9v2-B	1.46	71.6	44.7	1.22
2C9v2-C	1.20	80.7	47.3	0.82
2C9v2-ABC	1.20	116.9	48.1	0.66
2C8	0.14	650.5	0.0	0.03

* Tolbutamide concentration 1 mM.
† Phenytoin concentration 150 μM.
‡ Tolbutamide hydroxylase inhibition by SPZ (μM).

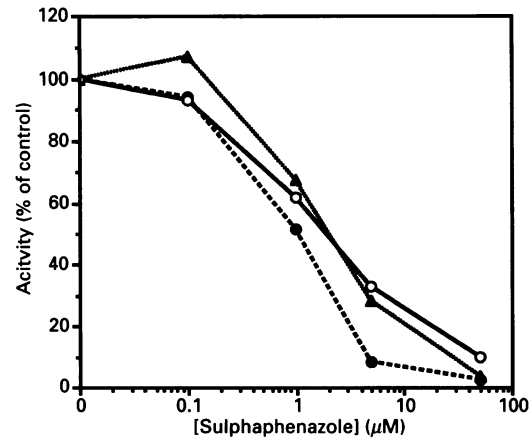

Figure 4 Sulphaphenazole inhibition of phenytoin hydroxylation in 2C9v2 (●) and 2C9v2-A (▲) transfected COS-7 cells and human liver microsomes (○)

Reaction mixtures contained either 1.5 mg of COS-cell protein or 0.5 mg of human liver microsomal protein and were incubated for 3 h. The phenytoin concentration was 150 μM. Uninhibited activities in the 2C9v2 and 2C9v2-A expressed cells and human liver microsomes were 8.9, 39.1 and 288.9 pmol of HPPH/h per mg of protein.

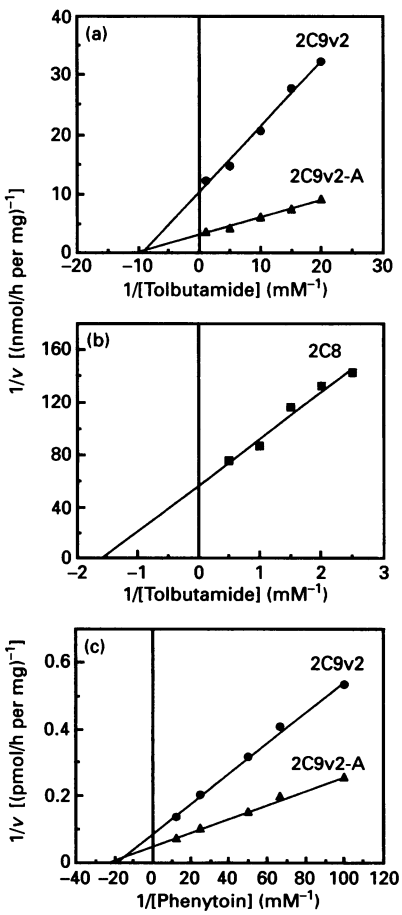

Figure 3 Lineweaver–Burk plots of the substrate-dependent formation of hydroxytolbutamide by (a) 2C9v2 and 2C9v2-A and (b) 2C8 proteins and (c) the formation of HPHH by 2C9v2 and 2C9v2-A proteins

described the data obtained from all expressed proteins. Typical Lineweaver–Burk representations of this data obtained with two of the 2C9 proteins and the 2C8 protein are shown in Figures 3(a) and 3(b) respectively. From Table 1 it can be seen that the 2C9/10 proteins had quite similar apparent K_m values (71.6–131.6 μM), but the apparent K_m value for the 2C8 protein was substantially higher. The 2C8 protein also showed quite different characteristics with the specific tolbutamide hydroxylase inhibitor sulphaphenazole (Table 1). This inhibitor at 1 μM decreased tolbutamide hydroxylase activity in all 2C9/10 proteins to a similar extent (44.7–49.7%), but 2C8-mediated tolbutamide hydroxylase activity was not inhibited at this concentration of sulphaphenazole. The extent of inhibition of the 2C9/10 proteins by sulphaphenazole is similar to that previously obtained with human liver microsomes (Doecke et al., 1990a).

Phenytoin hydroxylase activity was also measured for all cDNA-expressed proteins, and these results are presented in Table 1. As Table 1 shows, generally the pattern of phenytoin hydroxylase activities of the various proteins is quite similar to that obtained for tolbutamide hydroxylase activity in these cells, except for one protein, namely that encoded by 2C9v1. This protein metabolized phenytoin at a lower relative rate (7-fold) than observed for tolbutamide hydroxylation. This result was confirmed by analysing the various proteins for tolbutamide and phenytoin hydroxylase activities concurrently in the same batches of COS cells on the same day. Interestingly, there is only one amino acid difference between this protein and the 2C9v2-A protein (which has relatively high tolbutamide and phenytoin hydroxylase activities) at position 359 where 2C9v2-A has a leucine and 2C9v1 an isoleucine respectively. One may speculate that the decreased phenytoin hydroxylase activity is the result of the Ile-359 → Leu change. The kinetics of phenytoin hydroxylation were also measured with two of the 2C9 proteins, and the Lineweaver–Burk representations are displayed in Figure 2(c). The 2C9v2 and 2C9v2-A encoded proteins had apparent K_m values of 52.3 μM and 44.5 μM respectively. Due to the low activity of expressed 2C8 and 2C9v1 proteins, their K_m values could not be determined. Inhibition of phenytoin metabolism by sulphaphenazole was also studied with the 2C9v2 and 2C9v2-A

proteins. As Figure 4 shows, sulphaphenazole was a potent inhibitor of phenytoin hydroxylation. This inhibitor at 1 μM decreased phenytoin hydroxylation in expressed 2C9v2 and 2C9v2-A proteins by 48.3 and 32.6%, respectively, this being similar to the extent of inhibition (37.9%) observed in human liver microsomes at this inhibitor concentration (Figure 4).

DISCUSSION

Evidence to date from cDNA transfection experiments performed by a number of groups have shown that the human P450s 2C8, 2C9 and 2C10 can all catalyse the hydroxylation of tolbutamide (Veronese et al., 1991; Srivastava et al., 1991; Relling et al., 1990; Yasumori et al., 1991) and that at least 2C9 can additionally metabolize phenytoin (Veronese et al., 1991). However, the contribution of the individual cytochrome *P*450 isoenzymes to tolbutamide and phenytoin hydroxylation in human liver remain unclear because of the lack of sufficient functional characterization of these cDNA-expressed proteins (i.e. kinetic as well as inhibition characteristics). This is further complicated by the isolation in different laboratories of variants of 2C9 differing by a few amino acids which may ultimately affect substrate specificity, as has been observed with other *P*-450 isoenzymes (Aoyama et al., 1989; Lindberg and Negishi, 1986; Matsunga et al., 1990). The present study is the first to characterize in detail the kinetic and inhibitor properties of seven cDNA-expressed CYP2C proteins (2C8 and 2C9/10 variants) towards both tolbutamide and phenytoin. We found the 2C9/10 proteins had apparent K_m values for tolbutamide hydroxylase (71.6–131.6 μM) which were within the range of apparent K_m values obtained previously in human liver microsomes (Doecke et al., 1990a; Miners et al., 1988). The inhibition of tolbutamide hydroxylase activity by sulphaphenazole in the 2C9/10 expressed cells was also similar to that observed in human liver microsomes (Doecke et al., 1990a). In contrast, the tolbutamide hydroxylase K_m for the 2C8 enzyme was substantially higher ($\approx$ 6-fold) than those obtained for the 2C9/10 proteins. Furthermore, the 2C8 enzyme had quite different inhibition characteristics with the specific tolbutamide hydroxylase inhibitor sulphaphenazole. Several workers have suggested a role for 2C8 in the metabolism of tolbutamide (Relling et al., 1990; Srivastava et al., 1991), but the qualitative differences in the functional characteristics of the expressed enzyme compared with those of human liver microsomal tolbutamide hydroxylase suggest that 2C8 plays only a minor role in tolbutamide hydroxylation in man. As the unbound plasma concentrations of tolbutamide during normal therapeutic use are generally no higher than 20 μM (Back and Orme, 1989), then the higher-affinity 2C9/10 proteins would be expected to play a greater role in the metabolism of tolbutamide *in vivo*.

Phenytoin hydroxylation also appears to be chiefly mediated by 2C9/10 proteins. The apparent K_m values obtained for phenytoin hydroxylation with the two 2C9 proteins studied (44.5 and 52.3 μM) were close to the range of apparent K_m values previously obtained in human liver microsomes (29–30 μM) (Doecke et al., 1990a) and *in vivo* (Inaba, 1990). The two 2C9 proteins also displayed similar sulphaphenazole inhibition profiles to that observed in human liver microsomes and paralleled the inhibition profiles for tolbutamide hydroxylation (Veronese et al., 1991; Doecke et al., 1990a). In the present study the two expressed 2C9 proteins showed identical inhibition by 1 μM sulphaphenazole with both phenytoin and tolbutamide hydroxylations (cf. Table 1 and Figure 4). The phenytoin hydroxylase activity of the 2C8 protein was very low, being at least 10-fold less than that of any of the 2C9/10 proteins. This suggests only a minor role for 2C8 in the metabolism of phenytoin. The similarities in the functional characteristics of the expressed 2C9/10 enzymes to both the tolbutamide and phenytoin hydroxylases in human liver microsomes suggest that the 2C9/10 enzymes are chiefly responsible for the metabolism of these two drugs *in vivo*.

Another interesting aspect of this study was the observation that small differences in amino acid composition of the various 2C9/10 proteins could alter the catalytic activities towards tolbutamide and phenytoin by more than 5-fold. The single amino acid change Cys-144 → Arg could increase tolbutamide and phenytoin hydroxylase activities up to 3-fold. However, the higher-activity protein (2C9v2-A) also appears to require one or both of the amino acids, Tyr-358 and Gly-417 to maintain this higher activity. Also, the single amino acid change (Leu-359 → Ile) increased phenytoin hydroxylation over 5-fold, but only increased tolbutamide hydroxylation 1.3-fold. The observation that these 2C9/10 variants have various activities and that more than one of these variants can be expressed in a single human liver (Ged et al., 1988; Romkes et al., 1991) may in part explain the large inter-individual differences observed in the disposition of tolbutamide and phenytoin in man. The later phenomenon of a single amino acid change causing a greater effect on one substrate than another has also been previously reported for the rat CYP2D1 enzyme (Matsunga et al., 1990). Here it was shown that a single amino acid change (Ile-380 → Phe) specifically decreased the $V_{max.}$ of CYP2D1 for bufuralol, but not debrisoquine hydroxylation. In either case the changes involve the relatively conservative substitution of one non-polar amino acid with another. How this would have major effects on the conformation of the enzyme or would play important catalytic roles at the active site presently remains unclear. It is noteworthy that position 359 of 2C9, like position 380 of 2D1, lies in close proximity to one of several substrate-contacting domains predicted for the human CYP17 enzyme (positions 369–371) by computer modelling based on the crystal structure of the bacterial enzyme CYP101 (Laughton et al., 1990). It has also been shown for mouse CYP2A4 that an amino acid change near this region (position 365) plays an important role in the enzymic function of this cytochrome (Lindberg and Negishi, 1989).

In conclusion, the successful expression of seven cDNAs encoding 2C8 and variants of 2C9/10 enzymes, has allowed us to elucidate further the role of these individual *P*-450 forms in tolbutamide and phenytoin metabolism. Enzyme kinetic studies and sulphaphenazole-inhibition characteristics indicate that the 2C9/10 proteins are functionally similar to the isozyme(s) responsible for tolbutamide and phenytoin hydroxylations in human liver microsomes. However, the 2C8 enzyme is functionally quite distinct, indicating a minor role in tolbutamide and phenytoin metabolism. In addition, subtle differences in the amino acid composition of the 2C9/10 proteins could produce marked differences in substrate specificities and may in part explain the wide inter-individual differences in the metabolic handling of both tolbutamide and phenytoin. However, these data obtained using the COS expression system will need to be confirmed in other expression systems (such as bacteria, or yeast) which are better suited to obtain the quantity of *P*-450 required for spectral analysis. These studies are currently under investigation in our laboratory.

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