Pergamon

Biochemical Pharmacology, Vol. 47, No. 8, pp. 1295–1307, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0116-O

AN EVALUATION OF CYTOCHROME P450 ISOFORM ACTIVITIES IN THE FEMALE DARK AGOUTI (DA) RAT: RELEVANCE TO ITS USE AS A MODEL OF THE CYP2D6 POOR METABOLISER PHENOTYPE

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(*Received* 3 *December* 1993; *accepted* 7 *December* 1993)

Abstract—The female dark agouti (DA) rat lacks CYP2D1, the equivalent enzyme in the rat to human CYP2D6 (debrisoquine hydroxylase), and shows impaired metabolism of a number of CYP2D6 substrates. However, from the data available in the literature it is not entirely clear whether the enzyme deficiency in the DA rat is restricted to CYP2D1, and whether factors such as age and substrate concentration are important determinants of interstrain differences in the activity of this enzyme. Given that the female DA rat is used as a model of the human CYP2D6 poor metaboliser phenotype, there is a need for a systematic evaluation of the P450 activities in the DA rat, and of its suitability as a model of the PM phenotype. In the present study metoprolol was used as a probe substrate to investigate CYP2D1 activity since both the α-hydroxylation and O-demethylation of this drug are catalysed by CYP2D6 in man. Formation of α-hydroxymetoprolol (AHM) and *O*-demethylmetoprolol (ODM) was 10- and 2.5-fold lower in liver microsomes from female DA rats compared with microsomes from age-matched female Wistar rats, the latter representing the extensive metaboliser strain. Kinetic analysis suggested that in both strains of rat both the α-hydroxylation and O-demethylation of metoprolol were catalysed by more than one enzyme. By using quinine as a specific inhibitor of the enzyme, CYP2D1 was identified as an intermediate affinity site in the Wistar strain and was shown to have impaired activity in the DA strain. The activities of lower and higher affinity sites were similar in the two strains. Thus, the only difference between the two strains with respect to both routes of metoprolol metabolism appeared to be in the activity of CYP2D1. Interstrain differences were found to be highly dependent on the choice of substrate concentration, being more marked at lower concentrations. We have also investigated the metabolism of a number of probe compounds for some of the other P450 isoforms commonly involved in drug metabolism to determine the selectivity of the deficiency in the DA strain. *p*-Nitrophenol hydroxylation and erythromycin N-demethylation were catalysed at higher rates by DA than by Wistar liver microsomes, indicating higher levels of activity of CYP2E1 and CYP3A in the former strain. Felodipine oxidation, tolbutamide hydroxylation and both the hydroxylation and N-demethylation of *S*-mephenytoin were catalysed at similar rates by microsomes from the two strains, indicating similar activities of enzymes in the CYP2C and CYP3A families. However, both the hydroxylation and N-demethylation of *R*-mephenytoin were impaired in the DA strain. This indicates that at least one other isoform of P450, thought also to be a member of the CYP2C or CYP3A families, in addition to CYP2D1 is deficient in the DA strain. Our findings indicate that whilst the female DA rat could be used as a preliminary screen to identify CYP2D6 substrates, because of interspecies differences in metabolism it could not be used to provide quantitative information regarding the contribution of CYP2D6 to an oxidation in man. In addition, a small number of false positives would be identified owing to other enzyme deficiencies; no false negatives would be expected. Comparisons between strains should be performed using female, age-matched animals and low substrate concentrations.

Key words: DA rat; CYP2D; P450; metoprolol metabolism

CYP2D6 (debrisoquine hydroxylase) is expressed polymorphically in man and is involved in the metabolism of a large number of clinically relevant drugs [1]. Substrates of CYP2D6 can be identified by a variety of methods which include studies using human liver microsomes in conjunction with antibodies [2] or the specific inhibitor quinidine [3], molecular modelling [4, 5] and heterologous expression systems [6, 7]. Animal tissues are used widely in drug metabolism studies. Candidates for animal models of the CYP2D6 polymorphism include the crabeater monkey (*Macaca fascicularis*) [8], the African green monkey (*Cercopithecus aethiops*) [9] and the female DA‡ rat [10]. These animal models are likely to be of use both as screens to identify CYP2D6 substrates, and for investigating the pharmacological implications of the polymorphism, especially with respect to the role of CYP2D6 in the brain.

The female of the DA inbred strain of rat was

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‡ Abbreviations: CYP, cytochrome P450; DA, dark agouti; PM, poor metaboliser; AHM, α-hydroxymetoprolol; ODM, o-demethylmetoprolol; IC_{50}, inhibitor concentration causing 50% inhibition of control activity; and EM, extensive metaboliser.

first proposed as a model of the PM phenotype of the human CYP2D6 polymorphism by Al-Dabbagh *et al.* in 1981 [10]. Thus, the mean urinary debrisoquine/4-hydroxydebrisoquine ratio was found to be 6-fold higher in this strain than in six other strains, indicative of an impaired ability to hydroxylate debrisoquine. These observations were extended by *in vitro* studies using liver microsomes [11] and purified enzymes [12]. The metabolic deficiency in the DA rat has been shown to arise from a lack of expression of the *CYP2D1* gene, the only gene of the *CYP2D* cluster in the rat which codes for an enzyme capable of catalysing the hydroxylation of bufuralol [13]. Genetic analysis has shown this to be the result of a single mutant allele which is thought to block gene transcription [13]. This is in contrast to human PMs in whom multiple mutant alleles of *CYP2D6* have been identified [14]. Nevertheless, the biochemical defect in the female DA rat is similar to that in human PMs, that is absence of debrisoquine hydroxylase, even though the underlying genetic cause is different.

The published data regarding drug metabolising enzyme activities in the female DA rat are summarised in Table 1. These data indicate that the enzyme deficiency in the female DA rat may be selective for CYP2D1 and that the activities of a number of other P450 isozymes are comparable to those observed in other strains. Thus, it would appear that the female DA rat may be a suitable model for identifying CYP2D substrates. However, some studies have suggested that the CYP2D1 deficiency in the female DA rat may be affected by factors such as age [15] and substrate concentration [11, 16]. Furthermore, kinetic analyses have indicated that deficiencies in the oxidations of debrisoquine [11] and thebaine [17] may not in fact be restricted to CYP2D1. Thus, there is clearly a need for a systematic evaluation of the activity of CYP2D1 and of other P450 isozymes in the female DA rat in order to assess its suitability as a screen for identifying CYP2D6 substrates.

In the present study the nature of the CYP2D1 deficiency in the DA strain has been evaluated by comparing the kinetics of metoprolol oxidation by liver microsomes from female DA and Wistar rats. Previous studies have shown that the elimination of metoprolol by isolated perfused livers from DA rats is impaired significantly compared with that by livers from female Wistar age-matched controls [18]. The precise role of CYP2D6 in the oxidation of metoprolol by human liver microsomes has been elucidated using quinidine as a potent and selective inhibitor of the enzyme [3]. Thus, α-hydroxylation, a minor route of metoprolol metabolism in man, was shown to be catalysed entirely by CYP2D6 whereas the major pathway, O-demethylation, was catalysed partly by CYP2D6 and partly by another, as yet unidentified, P450 isoform. Quinine and quinidine have been shown to inhibit CYP2D catalysed oxidations in both human and rat liver microsomes but with a reversed order of potency in the former. Thus, quinine was a more potent inhibitor of debrisoquine hydroxylation than quinidine in the rat [19]. A similar rank order of potency has been reported for the inhibition of metoprolol metabolism *in vitro* [20]. In this study we have used quinine and quinidine as inhibitors to define the role of CYP2D1 in the metabolism of metoprolol by female DA and Wistar rat liver microsomes. By comparing our findings with those from human PM and EM liver microsomes [3] the accuracy with which the female DA rat represents the human PM with regard to CYP2D activity could be assessed.

In evaluating the DA rat as a model of the human PM, it is also important to consider the activities of other P450s in this strain in order to determine the selectivity of the deficiency. Thus, we also compared the metabolism of a number of substrates of other human P450 isozymes in microsomes from DA and Wistar rats, namely tolbutamide (CYP2C9 [21]), *p*-nitrophenol (CYP2E1 [22]), *S*-mephenytoin (CYP2Cmeph), erythromycin (CYP3A [23]) and felodipine (CYP3A [24]). These substrates were selected to include the P450 isoforms most commonly involved in xenobiotic metabolism. Mephenytoin was also included since its metabolism is polymorphic in man.

MATERIALS AND METHODS

Drugs and chemicals. Racemic metoprolol tartrate, α-hydroxymetoprolol *p*-hydroxybenzoate, *O*-demethylmetoprolol base and pamatolol sulphate were gifts from Hüssle (Mölndal, Sweden). Tolbutamide and chlorpropamide were gifts from The Upjohn Company (Kalamazoo, MI, U.S.A.), hydroxytolbutamide from Prof. J.O. Miners (Flinders Medical Centre, Adelaide, Australia). Nirvanol (*N*-demethylmephenytoin) was a gift from Dr P.J. Wedlund (University of KY, U.S.A.), 4-hydroxymephenytoin from Dr G.R. Wilkinson (Vanderbilt University, Nashville, TN, U.S.A.), *R*- and *S*-mephenytoin from Dr R. Chenery (Smith Kline Beecham, Welwyn Garden City, U.K.) and felodipine and UK-122,801 from Dr Barry Jones (Pfizer Central Research, Sandwich, U.K.). Quinidine sulphate, quinine hydrochloride, paranitrophenol and 4-nitrocatechol were purchased from the Sigma Chemical Co. (Poole, U.K.), sodium phenobarbitone from B.D.H. Ltd (Poole, U.K.) and glucose-6-phosphate dehydrogenase (grade II suspension), the disodium salts of D-glucose-6-phosphate and NADP from Boehringer Mannheim (Lewes, U.K.) All other chemicals were of analytical reagent grade.

Liver microsomes. Female DA and Wistar rats (15 weeks of age) were obtained from colonies bred by the University of Sheffield Field Laboratories. The rats were stunned and killed by cervical dislocation and their livers removed, rinsed in isotonic saline and frozen in liquid nitrogen. Livers were stored at $-80°$ prior to preparation of microsomes.

Microsomes from individual livers were prepared as described by Shaw *et al.* [25]. The microsomal pellets were resuspended in 0.25 M potassium-phosphate buffer (pH 7.25) containing 30% glycerol (v/v) and stored at $-80°$ prior to use. The microsomal protein concentration was measured by the method of Lowry *et al.* [26] using bovine serum albumin (fraction V) as the standard.

Table 1. Oxidations which are catalysed by CYP2D6 in man and which are impaired in the female DA rat, and oxidations which do not involve CYP2D6 in man and which are not impaired in the female DA rat

CYP2D6 reactions	Non-CYP2D6 reactions
Good evidence	
Debrisoquine 4-hydroxylation [11, 15]	Benzo[a]pyrene hydroxylase [12, 43, 56, 65]
Sparteine oxidation [37, 38, 56]	*p*-Nitroanisole *N*-dealkylase† [12, 40, 43, 45]
Bufuralol 1'-hydroxylation [16, 38, 57]	Arylhydrocarbon hydroxylase [43, 65]
Methoxyphenamine O-demethylation [58, 59]	*N*-Nitrosodimethylamine demethylase [12, 43]
Methoxyphenamine 5-hydroxylation [58, 59]	2-Acetyaminofluorene activation [43]
Dextromethorphan O-demethylation [60]	*N*-Nitrosomorphiline activation [43]
Perhexiline hydroxylation [61]	Aminopyrine N-demethylation†,‡ [37, 39, 43]
Codeine O-demethylation [62]	Benzyloxyresorufin *O*-dealkylase [66]
Propranolol 4-hydroxylation [12]	Epoxide hydrolase [41, 65]
Metoprolol α-hydroxylation*	Erythromycin *N*-demethylase*† [45]
Metoprolol O-demethylation*	Felodipine oxidase*
	Tolbutamide hydroxylase*
Putative CYP2D oxidations	Ethylmorphine *N*-demethylase† [45, 65]
Thebaine O-demethylation [17]	Benzphetamine *N*-demethylase† [37, 45]
TIQ hydroxylation [61]	Antipyrine hydroxylase [60, 67]
Bunitrolol hydroxylation [64]	7-Ethoxyresorufin *O*-dealkylase [42]
	7-Ethoxycoumarin *O*-dealkylase [42]
	7-Pentoxyresorufin *O*-dealkylase [42]
	7-Methoxycoumarin *O*-dealkylase [42]
	S-Mephenytoin 4'-hydroxylase*
	S-Mephenytoin *N*-demethylase*
	p-Nitrophenol hydroxylase*
Weak evidence	
Guanoxan oxidation [37]	Testosterone 6α-hydroxylase [43]
Phenformin oxidation [37]	Testosterone 6β-hydroxylase [43]
Encainide O-demethylation [12]	Testosterone 16α-hydroxylase [43]
	Aniline hydroxylase [12]
MDMA demethylenation [69]	Aldrin epoxidase [37]
MPTP oxidation [68]	Acetanilide *p*-hydroxylase [11]
	Cyclosporine A disposition [68]

Evidence was considered good if a detailed study had been performed, or if the finding had been confirmed by more than one study. Evidence was considered weak if an observation was reported by only one study with insufficient experimental details to evaluate its relevance. This latter group also includes comparisons between female DAs and males of other strains, and comparisons performed by *in vivo* studies.

* This study.

† Interstrain differences in activity [45].

‡ Age-dependent variation in activity [39].

Incubation conditions. The 1 mL incubation mixture consisted of 0.2 mL microsomal suspension [diluted to the required protein concentration with 1.15% (w/v) KCl], 0.2 mL substrate dissolved in 1.15% (w/v) KCl, 0.2 mL incubation buffer (0.2 M potassium phosphate buffer, pH 7.4), 0.2 mL 1.15% (w/v) KCl [with or without quinidine or quinine (see below)], and 0.2 mL of an NADPH-generating system. The NADPH-generating system consisted of 4 μmol glucose-6-phosphate, 0.4 μmol NADP, 0.4 U glucose-6-phosphate dehydrogenase and 2 μmol $MgCl_2$ dissolved in incubation buffer. All incubations were carried out at 37° in a shaking water bath. After preincubation for 2 min, the reaction was started by addition of the microsomal protein to the other reagents. Reactions were stopped by transferring 400 μL aliquots of the incubate to polypropylene vials containing 50 μL 6% (w/v) perchloric acid. The internal standard, pamatolol sulphate (80 ng), was added and the samples stored at −20° prior to analysis.

Metoprolol metabolites were assayed by HPLC using the method of Otton *et al.* [3], with methyl-tertiary-butyl ether as the extraction solvent. Published methods were used for the analysis of 4-hydroxy and *N*-demethylmephenytoin [27], hydroxytolbutamide [28], 4-nitrocatechol [29] and felodipine [30]. Erythromycin N-demethylation was measured as formaldehyde production [31]. The coefficients of variation for each assay were less than 5%. Limits of determination for the assays were: αHM/ODM, 0.02 nmol/mL; hydroxytolbutamide, 0.02 nmol/mL; 4'-hydroxymephenytoin, 0.01 nmol/mL; *N*-demethylmephenytoin, 0.03 nmol/mL and felodipine 0.001 nmol/mL.

For each substrate investigated, preliminary experiments were performed to determine the conditions under which metabolite formation was linear with respect to time and microsomal protein concentration, and to determine the approximate K_m of the reaction. The incubation conditions selected for subsequent experiments are listed in

Table 2. Incubation conditions for the comparison of oxidations by liver microsomes from female DA and Wistar rats

Substrate	Incubation time (min)	Protein concentration (mg/mL)	Substrate concentration (μM)
Metoprolol	5	1.0	20
Mephenytoin	12	0.8	100
Tolbutamide	40	1.2	20
p-Nitrophenol	12	1.2	50
Felodipine	15	0.5	1
Erythromycin	20	1.0	100

Table 2. For each substrate, microsomes from four individual rats of each strain were incubated in duplicate under the conditions listed in Table 2. To determine the potency of quinine and quinidine in inhibiting metoprolol metabolism, microsomes from two female rats of each strain were incubated in duplicate with 0.001 to 500 μM quinine or quinidine. For the kinetic analysis of metoprolol metabolite formation, microsomes from four individual rats of each strain were incubated with 0.2 μM to 10 mM metoprolol. Single incubations were performed at each concentration, with duplicate analyses. This kinetic analysis was repeated in the presence of 1 μM quinine using microsomes from two individual rats of each strain. This concentration of quinine was selected since it caused >95% inhibition of activity in the preliminary experiments.

Data analysis. The extent of inhibition of AHM and ODM appearance by quinine and quinidine was expressed as the concentration of inhibitor causing 50% inhibition of control activity, (IC_{50}). Analysis of the kinetics of metabolite formation in the presence and absence of 1 μM quinine was performed using an extended non-linear least-squares algorithm (ELSFIT [32]). The data were fitted by both 1- and 2-site enzyme models, and the goodness of fit of each model was judged by the significance of the -2 log likelihood value (Chi-squared distribution, $df = 2$).

RESULTS

α-Hydroxylation of metoprolol

The rate of appearance of AHM at a substrate concentration of 20 μM was 10-fold higher in female Wistar compared with female DA rat liver microsomes (Fig. 1). AHM formation was inhibited by quinine and quinidine in a concentration-dependent manner in microsomes from both strains of rat (Table 3). Quinine was 1 to 2 orders of magnitude more potent as an inhibitor than quinidine. Similar IC_{50} values were obtained in each strain of rat. However, α-hydroxylation could not be completely inhibited, even at higher inhibitor concentrations, leaving a similar level of residual activity in the two strains.

The kinetics of α-hydroxylation of metoprolol in the absence of quinine, were described better by a two site rather than a single site model for microsomes

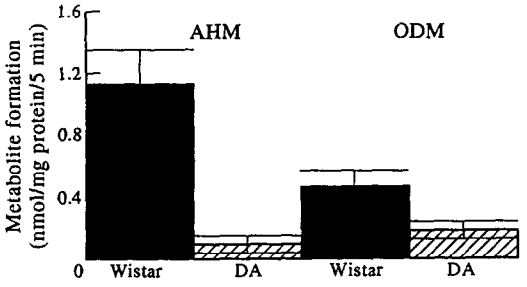

Fig. 1. Appearance of metabolites during incubation of metoprolol (20 μM) with female Wistar and DA liver microsomes. Values are means ± SD; N = 4 livers. Interstrain differences were significant ($P < 0.05$) for both routes.

Table 3. Parameters describing the inhibition of AHM formation by quinine and quinidine in DA and Wistar rat liver microsomes

	Quinine		Quinidine	
	IC_{50} (μM)	Maximum inhibition (%)	IC_{50} (μM)	Maximum inhibition (%)
Wistar	0.09	96	0.9	95
DA	0.03	75	3.0	62

Each value is the mean obtained from experiments performed using microsomes from two rats of each strain.

from both strains of rat. This indicates the presence of low-affinity, high-capacity and high-affinity, low capacity sites of oxidation (Fig. 2). Values for both the V_{max} and the K_m (Table 4) of the low affinity site were significantly lower in the DA than the Wistar strain but intrinsic clearance was similar. Values for the K_m of the high affinity site were similar in the two strains, whereas V_{max} values were 10-fold lower in the DA microsomes, resulting in an 8-fold lower value of intrinsic clearance at this site.

In the presence of quinine, the kinetics of α-

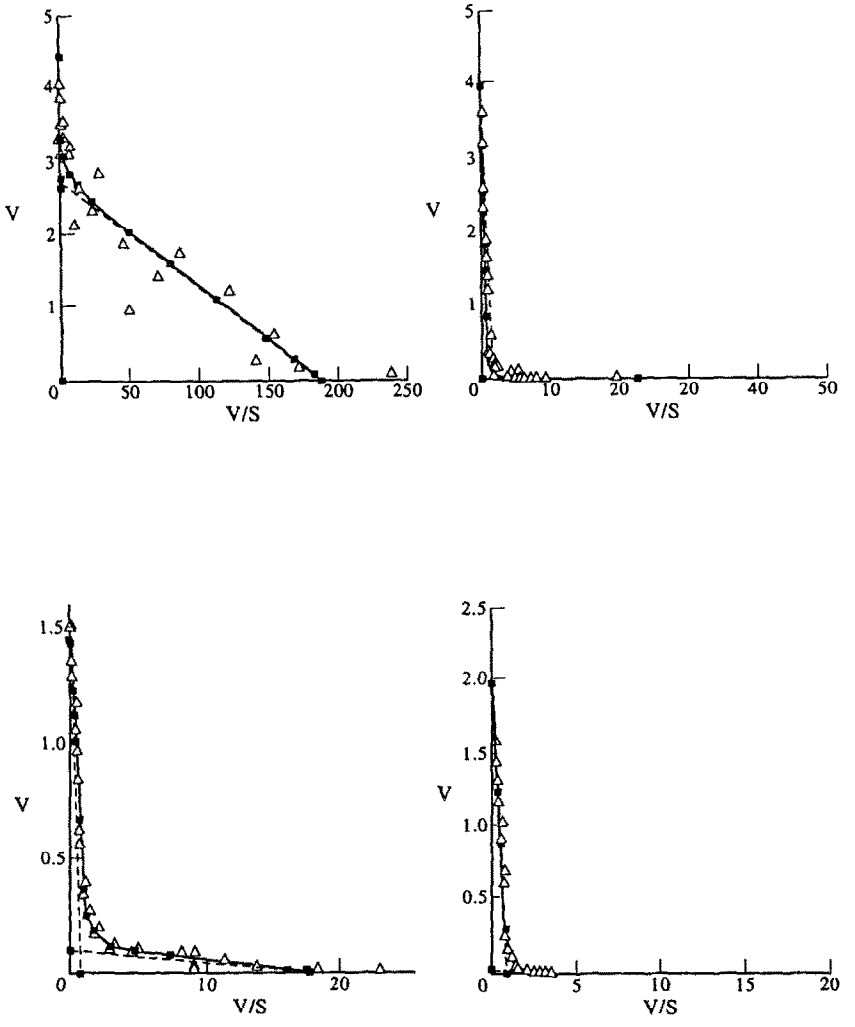

Fig. 2. Eadie–Hofstee plots for AHM formation by Wistar (top) and DA (bottom) rat liver microsomes in the absence (left) and presence (right) of quinine (1 μM). Each point represents the mean from duplicate analyses. Solid lines represent lines of best-fit for a two-site model, dotted lines represent the Eadie–Hofstee plots for the individual sites as calculated by ELSFIT. [V = metabolite formation velocity; nmol/mg protein/5 min; S = substrate concentration (mM)].

Table 4. Michaelis–Menten parameters (estimated using ELSFIT) describing the α-hydroxylation of metoprolol in the absence (N = 4 rats) and presence (each row of values is from experiments performed with microsomes from one rat of each strain) of quinine

	Site 1				Site 2			
	Wistar		DA		Wistar		DA	
	No inhibitor	+quinine (1 μM)	No inhibitor	+quinine (1 μM)	No inhibitor	+quinine (1 μM)	No inhibitor	+quinine (1 μM)
V_{max} (nmol/mg protein min)	0.55 ± 0.07	0.69 0.79	0.32 ± 0.04 *	0.39 0.31	0.40 ± 0.10	0.017 0.003	0.04 ± 0.02 *	0.006 0.001
K_m (mM)	5.04 ± 1.74	2.04 2.32	1.41 ± 0.40 *	2.57 2.18	0.013 ± 0.001	0.018 0.001	0.009 ± 0.004	0.015 0.005
CL_{int} (mL/min)	0.12 ± 0.04	0.34 0.34	0.24 ± 0.06	0.15 0.14	30.2 ± 5.1	0.94 4.57	4.23 ± 0.45	0.37 0.30

Site 1 is the low affinity, high capacity site.
* $P < 0.05$.

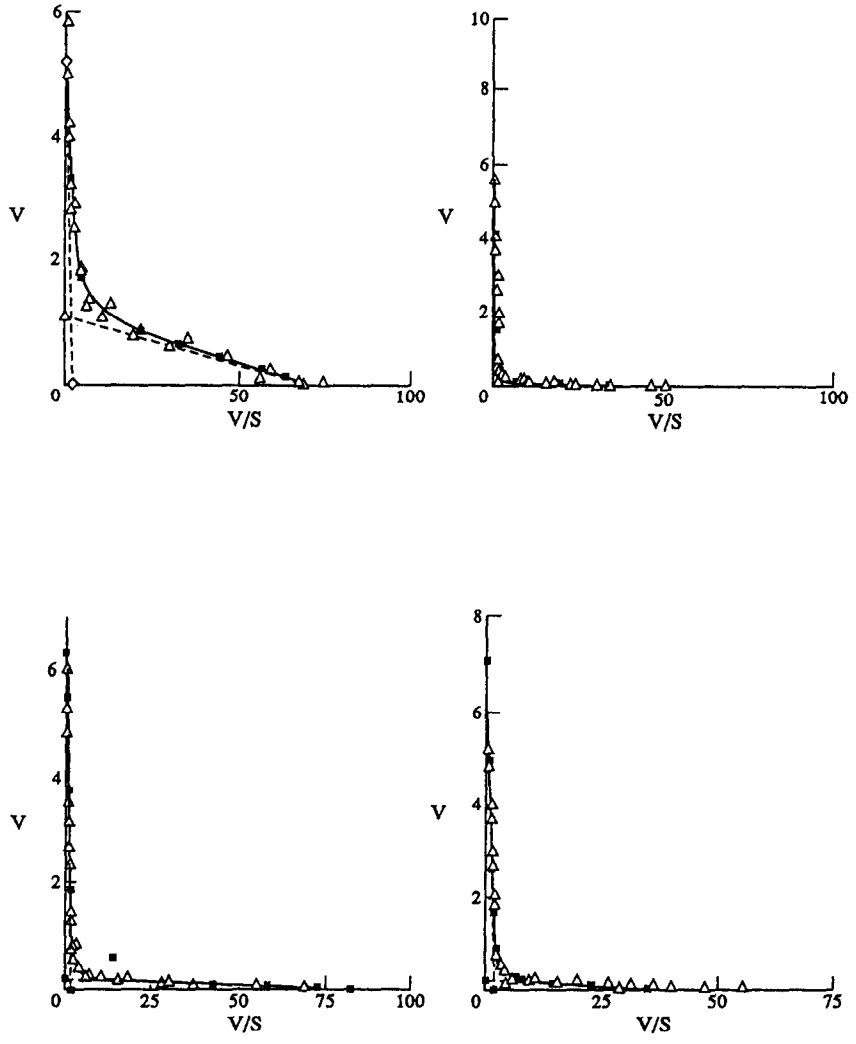

Fig. 3. Eadie–Hofstee plots for ODM formation by Wistar (top) and DA (bottom) rat liver microsomes in the absence (left) and presence (right) of quinine (1 μM). Each point represents the mean from duplicate analyses. Solid lines represent lines of best-fit for a two-site model, dotted lines represent the Eadie–Hofstee plots for the individual sites as calculated by ELSFIT. (V = metabolite formation velocity; nmol/mg protein/5 min; S= substrate concentration (mM)).

hydroxylation were also described better by a two rather than a single site model in both strains of rat (Fig. 2). However, from Eadie–Hofstee plots (Fig. 2) it appeared that the high affinity site was partially inhibited; values for the V_{max} and intrinsic clearance for this site were an order of magnitude lower in the presence than in the absence of quinine in both strains of rat. The residual uninhibited activity suggested that a third site of hydroxylation had been exposed. Values for the V_{max} and K_m (Table 4) of the low affinity site were similar in either the presence or absence of quinine, indicating that this site was not inhibited.

O-Demethylation of metoprolol

The rate of appearance of ODM at a substrate concentration of 20 μM was 2.5-fold higher in Wistar than in DA rat liver microsomes (Fig. 1). Hence, differences between the strains were less marked for this pathway than for the α-hydroxylation of metoprolol.

O-demethylation of metoprolol in Wistar microsomes was inhibited only partially by quinine and quinidine. The former was 35 times more potent than the latter and the IC_{50} values (quinine, 0.1 μM; quinidine, 3.5 μM.) were similar to those obtained for the inhibition of α-hydroxylation. 20–25% of the control *O*-demethylase activity in Wistar liver microsomes was insensitive to inhibition. In DA rat liver microsomes quinine and quinidine caused inhibition of metoprolol O-demethylation only at inhibitor concentrations in excess of 100 μM.

The kinetics of O-demethylation of metoprolol in Wistar microsomes in the absence of quinine were described better by a two-site than a one-site model (Fig. 3). From the Eadie–Hofstee plots it appeared

Table 5. Michaelis–Menten parameters (estimated using ELSFIT) describing the O-demethylation of metoprolol in the absence (N = 4 rats) and presence (each row of values is from experiments with microsomes from one rat of each strain) of quinine

	Site 1				Site 2			
	Wistar		DA		Wistar		DA	
	No inhibitor	+quinine (1 μM)	No inhibitor	+quinine (1 μM)	No inhibitor	+quinine (1 μM)	No inhibitor	+quinine (1 μM)
V_{max}		1.24		1.41		0.02		0.04
(nmol/mg prot/min)	1.29 ± 0.27	1.60	1.42 ± 0.29	1.39	0.19 ± 0.04	0.02	0.05 ± 0.01 *	0.03
K_m	4.34 ± 2.4	3.20	2.63 ± 0.82	3.86	0.016 ± 0.008	0.001	0.003 ± 0.001	0.006
(mM)		4.64		4.73		0.002		0.002
CL_{int}	0.33 ± 0.09	0.39	0.57 ± 0.15	0.37	13.0 ± 4.8	14.3	19.0 ± 3.7	7.0
(ml/min)		0.34		0.29		10.1		15.8

Site 1 is the low affinity, high capacity site
* P.

that addition of quinine inhibited the high affinity site. However, the data were still described better by a two-site than a one-site model. Values for the K_m and V_{max} (Table 5) of the low affinity site were similar in the presence and absence of quinine, indicating that this site was not inhibited. The V_{max} of the high affinity site appeared to be substantially lower in the presence of quinine whereas K_m and intrinsic clearance values were similar to those in its absence. Thus, although quinine had a marked effect on the shape of the Eadie–Hofstee plot, which suggested inhibition of the higher affinity site, the Michaelis–Menten parameters do not appear to reflect this. These findings suggest that a third site involved in O-demethylation may have been revealed.

In DA liver microsomes the kinetics of O-demethylation in the absence of quinine were also described by a two-site model (Fig. 3). Addition of quinine did not alter the kinetics of O-demethylation. Michaelis–Menten parameters were similar in the presence and absence of inhibitor and Eadie–Hofstee plots were superimposable. Michaelis–Menten parameters (Table 5) for the O-demethylation of metoprolol were not significantly different between the two rat strains, with the exception of the V_{max} of the high affinity site. Eadie–Hofstee plots for this oxidation by Wistar microsomes in the presence of quinine resembled those for the O-demethylation of metoprolol in DA microsomes in the presence or absence of quinine, and Michaelis–Menten parameters for the three sets of data were similar.

Metabolism of substrates of other P450 isoforms

No significant differences between DA and Wistar microsomes were observed for tolbutamide hydroxylation, *S*-mephenytoin hydroxylation and N-demethylation, and felodipine oxidation (Table 6). The rates of *p*-nitrophenol hydroxylation and erythromycin N-demethylation were significantly higher in the DA strain than the Wistar strain.

The 4′-hydroxylation of *R*-mephenytoin proceeded rapidly in Wistar microsomes (at a 15 times greater rate than hydroxylation of *S*-mephenytoin) but was barely detectable in DA microsomes. The N-demethylation of *S*-mephenytoin was significantly lower in DA than in Wistar microsomes. N-demethylation of the *R*-enantiomer was also lower in the DA strain, although this difference was not significant.

DISCUSSION

The CYP2D1 deficiency in the DA rat has been suggested to be dependent on age, based on the measurement of the debrisoquine metabolic ratio [15]. However, studies using the isolated perfused rat liver to investigate hepatic function in the absence of other influences such as renal function, showed that this was not the case. Thus, the elimination of metoprolol, the oxidation of which is mediated by CYP2D6 in man [3, 33] was 50% lower in female DA rat livers compared with female Wistar rat livers from animals aged between 10 and 32 weeks [34]. Furthermore, mRNA for CYP2D1 was found to be absent in 1 and 5 month old DA rats [13]. Thus, it would appear that CYP2D1 activity does not vary with age in the DA strain. Therefore, the apparent age-dependence of the metabolic ratio for debrisoquine described by Vincent-Viry *et al.* [15] may arise from variation in renal rather than hepatic function. The female DA rat is inherently small compared with other strains of rat. Thus, the selection of rats from different strains which are of similar body weight will yield groups which are of significantly different ages. Since the activities of other P450s vary with age in the rat [35, 36], we matched groups of rats for comparison by age rather than by weight.

The aim of this part of the present study was to evaluate CYP2D1 activity in the female DA rat using metoprolol as a probe substrate. From the results it is clear that the metabolism of metoprolol is more complicated in the rat than in man, both in terms of regioselectivity of metabolism and in the

Table 6. Formation velocities (pmol/mg protein/min mean ± SD; N = 4 livers) for the metabolism of probe compounds by female DA and Wistar rat liver microsomes

Substrate	Oxidation	Wistar	DA
Tolbutamide	Hydroxylation	18.7 ± 2.0	15.0 ± 3.0
R-Mephenytoin	4'-Hydroxylation	92.0 ± 11.0	3.3 ± 0.05*
	N-Demethylation	36.9 ± 7.6	18.2 ± 4.7*
S-Mephenytoin	4'-Hydroxylation	6.7 ± 1.0	5.7 ± 0.8
	N-Demethylation	32.7 ± 5.7	22.2 ± 4.0
p-nitrophenol	Hydroxylation	355.0 ± 86.0	484.0 ± 77.0*
Felodipine	Oxidation	77.0 ± 8.0	73.5 ± 7.0
Erythromycin	N-Demethylation	230.5 ± 11.6	389.3 ± 53.0*

* Significant ($P \leq 0.05$) interstrain differences.

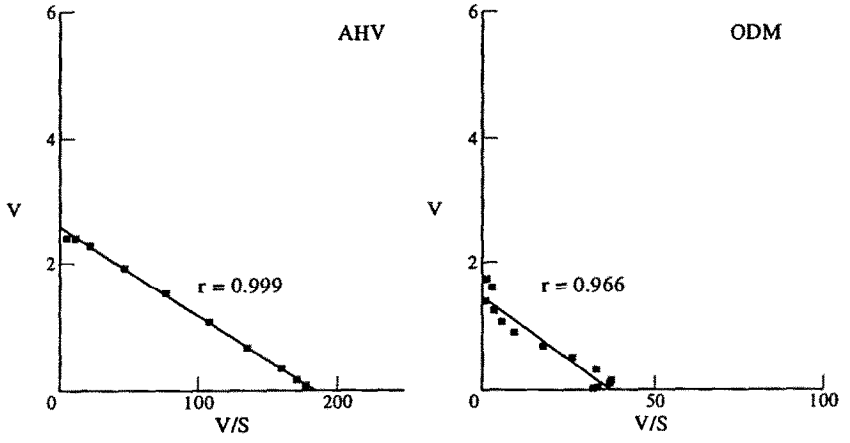

Fig. 4. Eadie–Hofstee plots illustrating the contribution of CYP2D1 to the formation of AHM (left) and ODM (right) by Wistar liver microsomes, calculated from the difference between Eadie–Hofstee plots obtained in the presence and absence of quinine. K_m values were 15 and 40 μM for AHM and ODM formation, respectively. V_{max} values were 0.44 and 0.3 nmol/min/mg protein for AHM and ODM formation, respectively.

number of enzymes catalysing each route of oxidation.

Metoprolol α-hydroxylation

In human EM liver microsomes the α-hydroxylation of metoprolol is catalysed solely by CYP2D6 [3]. However, in female Wistar liver microsomes this route of metabolism clearly involves more than one enzyme. The low affinity site was not inhibited by quinine and is, therefore, unlikely to be a CYP2D enzyme. The activity of the high affinity site was inhibited significantly, but not completely, by quinine. Furthermore, two enzymes were clearly involved in the α-hydroxylation of metoprolol even in the presence of quinine. Therefore, this suggests that the α-hydroxylation of metoprolol normally involves at least three enzymes. Subtracting values for the velocity of metabolite formation in the presence of quinine from those in its absence and transforming the data to an Eadie–Hofstee plot (Fig. 4) enables the contribution of CYP2D1 to be visualised, showing that CYP2D1 is an intermediate affinity site.

Human PM liver microsomes are unable to catalyse the α-hydroxylation of metoprolol [3] whereas this reaction was catalysed by DA rat liver microsomes and appeared to involve two or more enzymes. The activity of the low affinity enzyme was similar to that observed in Wistar liver microsomes and was not inhibited by quinine. The activity of the high affinity component was significantly lower in DA than in Wistar liver microsomes. However, the activity of this site could be decreased by addition of quinine in both strains, leaving a similar level of residual activity in each case. This quinine-sensitive activity observed in the DA strain is unlikely to reflect a low level of expression of CYP2D1 since the mRNA for CYP2D1 is absent in female DA rats at 1 and 5 months of age [13]. The *CYP2D* gene locus in the rat consists of five genes. Expression of the cDNAs in COS cells produced immunodetectable proteins, of which only CYP2D1 was able to catalyse bufuralol

hydroxylation [13]. However, little is known about the catalytic activity of the other enzymes, and it is possible that they differ in substrate specificity. Therefore, the intermediate-affinity site of metoprolol α-hydroxylation in DA liver microsomes may be either CYP2D1 or another member of the CYP2D family which is sensitive to inhibition by quinine.

These findings indicate that although the hydroxylation of metoprolol involved more enzymes in the rat than in man, the only difference between the two strains of rat with respect to this pathway of metoprolol metabolism appears to be in the activity of CYP2D1. Similarly, the only difference between the human EM and PM is in the activity of CYP2D6.

Metoprolol O-demethylation

O-Demethylation was shown to be the major route of metoprolol metabolism in both human EM and PM liver microsomes [3] whereas in the rat α-hydroxylation is the major route. In human EM liver microsomes the O-demethylation of metoprolol is catalysed by two enzymes of which CYP2D6 is the high-affinity and low-capacity site. In the presence of quinine, and in PM liver microsomes, O-demethylation is catalysed by a low-affinity, high-capacity site. The present study using rat liver microsomes revealed that the O-demethylation of metoprolol in DA rats may be catalysed by two enzymes, in contrast to the human PM. Addition of quinine did not alter the Eadie–Hofstee plots or the derived Michaelis–Menten parameters. These findings indicate that although CYP2D1, or another quinine-sensitive enzyme, make a minor contribution to the α-hydroxylation of metoprolol in the female DA rat, this is not the case for the demethylation route. The O-demethylation of metoprolol in Wistar microsomes appears to involve multiple enzymes of which CYP2D1 is an intermediate affinity site. This is supported by the fact that addition of quinine caused a change in the Eadie–Hofstee plot to one which can be superimposed with plots obtained using DA rat liver microsomes. This also indicates that the only difference between the two strains with respect to the O-demethylation of metoprolol is in the activity of CYP2D1.

From these experiments it is clear that both routes of metoprolol metabolism involve multiple enzyme sites in the rat. The contribution of CYP2D1 has been identified using quinine to inhibit its activity. The metabolism of several CYP2D6 substrates appears to be more complex in the rat than in man. Thus, the oxidations of bufuralol [16] and debrisoquine [11] were shown to involve enzymes in addition to CYP2D1 in the rat. In these studies indirect methods such as enzyme inhibition and competitive inhibition using other CYP2D6 substrates were used in an attempt to define the role of CYP2D1. For both substrates, the DA rat appeared to have deficiencies in more than one enzyme, although the evidence was largely indirect. This is in contrast to the present study in which the two strains of rat were found to differ in their ability to metabolise metoprolol only with respect to the activity of CYP2D1. The activities of the other enzymes were similar in each strain.

Interstrain differences in the metabolism of debrisoquine and bufuralol were also shown to depend on the choice of substrate concentration [11, 16]. In the present study interstrain differences in both routes of metoprolol metabolism were more marked at lower substrate concentrations. Thus, although the α-hydroxylation of metoprolol in the DA rat was impaired compared with the Wistar rat at all substrate concentrations, a deficiency in the O-demethylation of metoprolol was apparent only at lower substrate concentrations. This implies catalysis by more than one enzyme. For both routes of metoprolol oxidation CYP2D1 was identified as an intermediate affinity site in the Wistar strain and was deficient in the DA strain, whereas the activities of the higher and lower affinity sites were similar in the two strains. Thus, interstrain differences in metabolism were more marked at lower substrate concentrations, at which CYP2D1 makes a major contribution to metabolism. As saturation of this enzyme occurs at higher substrate concentrations, the reactions become catalysed predominantly by the higher capacity site which was found to have similar activity in the two strains. Thus, interstrain differences are less marked at higher substrate concentrations.

The activity of other P450 enzymes in the DA strain

As well as investigating the activity of CYP2D1 in the female DA rat it is also important to determine the activities of other P450 isoforms in order to assess the specificity of the defect and thus the suitability of the DA rat as a model of the human PM phenotype. Total concentrations of P450 have been shown to be similar in the DA and other strains of rat [12, 37, 38]. Moreover, immunoquantitation studies have shown that the female DA rat has similar levels of CYP1A1 ($P450_{\beta\text{-NF}'}$), CYP3A2 ($P450_{PCN\text{-}E}$) and CYP2A1 ($P450_{UT\text{-}F}$) as male SD rats, and higher levels of CYP2B1/2 ($P450_{PB\text{-}B/D}$), CYP2C6 ($P450_{PB\text{-}C}$) and CYP1A2 ($P450_{ISF\text{-}G}$) [12], although this does not provide any information regarding the catalytic activities of these enzymes. Concentrations of NADPH-cytochrome P450 reductase [39, 40] and of cytochrome b_5 [39] are also similar in the DA and other strains. Thus, the DA rat clearly does not have a generalised deficiency in drug metabolism. In this study other P450 isoforms were examined in the DA rat to determine whether its deficiency is restricted to CYP2D1. The rate of 4-nitrocatechol production from *p*-nitrophenol was significantly higher in DA than in Wistar liver microsomes, showing that there is no deficiency in the activity of CYP2E1 [22] in the DA strain. The O-demethylation of ethoxycoumarin, which is also catalysed by CYP2E1 [41], has also been shown to be similar in female DA and SD liver microsomes [42] and in female DA and Lewis rat liver microsomes [43].

The oxidation of felodipine was catalysed at similar rates by female DA and Wistar liver microsomes, whereas the N-demethylation of erythromycin was catalysed at significantly higher rates by DA liver microsomes. The latter oxidation has been shown to be catalysed by CYP3A1 and/or CYP3A2 in the rat [44, 45] whereas the oxidation of felodipine is catalysed by members of the CYP3A and CYP2C

families [46]. The hydroxylation of tolbutamide, catalysed by CYP2C9 in man [21], was catalysed at a faster rate by DA than by Wistar liver microsomes. It is not known whether an orthologous enzyme catalyses this oxidation in the rat.

The metabolism of mephenytoin is highly stereoselective in man, the *S*-enantiomer being preferentially 4′-hydroxylated and the *R*-enantiomer undergoing N-demethylation [47]. The elimination of mephenytoin proceeds with a reversed stereochemistry in the rat compared to that in man [48]. In the present study the two enantiomers of mephenytoin were N-demethylated at similar rates by microsomes from Wistar rats. In contrast, 4′-hydroxylation was markedly stereoselective, the *R*-enantiomer being 4′-hydroxylated more than 10-fold faster than the *S*-enantiomer. This may provide an explanation for the faster elimination of *R*-mephenytoin observed *in vivo* [48].

DA and Wistar rat liver microsomes catalysed both routes of oxidation of *S*-mephenytoin at similar rates. However, marked interstrain differences were observed in the metabolism of *R*-mephenytoin. Whereas 4′-hydroxylation accounted for two-thirds of the total metabolism of the *R*-enantiomer in the Wistar strain this route was virtually absent in the DA strain. The N-demethylation of the *R*-enantiomer was also impaired significantly in the DA strain. This finding indicates that at least one other P450 isoform is deficient in the DA strain in addition to CYP2D1. However, its is not clear which particular isoform is affected. The observed deficiency in *R*-mephenytoin metabolism is unlikely to be related to CYP2D1 since mephenytoin is a weak acid, and all known substrates of CYP2D are basic [5]. Furthermore, human CYP2D6 expressed in yeast did not catalyse the oxidation of mephenytoin [6]. Hydroxylation of *S*-mephenytoin in man is catalysed by CYP2Cmeph, a member of the CYP2C family which is distinct from tolbutamide hydroxylase [21]. Hydroxylation of the *R*-enantiomer is thought to be catalysed by either tolbutamide hydroxylase [49] or a member of the CYP3A family [50]. However, the results of the present study indicate that the hydroxylations of *R*-mephenytoin and tolbutamide are catalysed by different enzymes in the rat since only the former was deficient in the DA strain. Members of the CYP2C and CYP3A subfamilies show marked differences in their substrate specificities in man and rat [46]. Thus, the hydroxylation of *S*-mephenytoin is thought to be catalysed by a member of the CYP3A family in the rat but by a CYP2C isoform in man [44, 46]. The N-demethylation of both enantiomers is thought to be catalysed by members of the CYP3A family in man [46, 50]. A recent report has indicated that the hydroxylation of *R*-mephenytoin in the rat is catalysed by a member of the CYP2C or CYP3A family [51]. Thus, although we have shown that the DA rat is deficient in another enzyme in addition to CYP2D1, it is unclear exactly which isoform this is. It is likely to be a member of the CYP2C or CYP3A families, distinct from the isoforms involved in tolbutamide hydroxylation, erythromycin N-demethylation and felodipine oxidation, since these latter oxidations were not impaired in the DA strain.

Use of the female DA rat as a screen to identify CYP2D6 substrates

Table 1 lists the oxidations of CYP2D6 substrates which have been found to be impaired in the DA strain. To date, every CYP2D6 substrate which has been investigated in the DA strain has been found to have impaired metabolism. Thus, it is anticipated that the female DA rat would not fail to identify a substrate of the human CYP2D6 enzyme. From the present study it is clear that whilst the DA rat can be used to predict the involvement of CYP2D6 in an oxidation, it cannot be used to provide quantitative information regarding the contribution of the human enzyme to an oxidation. This is a reflection of interspecies differences in both the regioselectivity of metabolism and in the multiplicity of enzymes involved in the oxidations.

A large number of oxidations which do not involve CYP2D6 in man have also been investigated in the female DA rat (Table 1). These oxidations were found to be catalysed at similar rates in the DA and other strains of rat. The oxidations listed in Table 1 involve a variety of different P450 isoforms. Thus, it is clear that, in general, the female DA rat has similar enzyme activities to other strains of rat. This is an important consideration in the use of the female DA rat as a preliminary screen to identify CYP2D6 substrates with regard to the identification of "false-positives", that is the false prediction of a drug as a CYP2D6 substrate. We have identified three possible sources of false prediction. Firstly, as described above, the DA rat has a deficiency in at least one other P450 isoform in addition to CYP2D1, which is probably a member of the CYP2C or CYP3A families. Secondly, the female DA rat has an impaired ability to catalyse the 3-hydroxylation of lignocaine [52, 53]. This indicates that this route of metabolism is catalysed by CYP2D1 in the rat and thus may be predicted to be catalysed by CYP2D6 in man. However, 3-hydroxylation is only a minor route of lignocaine metabolism in man [54, 55] and probably does not involve CYP2D6 since this oxidation was not catalysed by CYP2D6 expressed in yeast [6]. Such differences in activity between human CYP2D6 and rat CYP2D1 indicate that the two enzymes possess different active sites [19, 46]. It has been predicted [46] that the aromatic hydroxylation of a number of arylamines such as benzphetamine and amphetamine may be catalysed by rat CYP2D1 but not human CYP2D6, owing to differences in the active site of the enzyme. Thus, these routes of metabolism would be expected to be deficient in the female DA rat but not in the human PM. However, the false prediction of a role of CYP2D6 in these oxidations in man would be avoided provided that there is an understanding of interspecies differences in the regioselective metabolism of such compounds.

Finally, the activities of CYP3A enzymes have been shown to vary considerably between different strains of rat [45] such that the DA rat may appear to have other enzyme deficiencies when compared with some strains of rat but not others. For example, the erythromycin *N*-demethylase and *p*-nitroanisole *O*-demethylase activities of female DA rat liver

microsomes were similar to those in female Lewis and Wistar microsomes but were 50% lower than observed in female SD or Fischer rat liver microsomes. Ethylmorphine *N*-demethylase activity in DA liver microsomes was lower than that of female Fischer microsomes but similar to that of female SD, Lewis or Wistar microsomes. Thus, it is important to evaluate the activity of other P450 isoforms in the strain of rat against which the DA is compared to avoid making false identifications which are due to interstrain differences in the activities of enzymes other than CYP2D1. The number of false-positives identified is likely to be relatively small and in many cases may be rationalised on the basis of interspecies or interstrain differences in metabolism.

Conclusions

The female DA rat is deficient in both the α-hydroxylation and O-demethylation of metoprolol. Both routes of metoprolol oxidation involve additional enzymes in the rat compared to man. However, the only difference between the two strains was in the activity of CYP2D1, which was identified as an intermediate affinity site. Similarly, the only difference between human EMs and PMs with respect to the metabolism of metoprolol is in the activity of CYP2D6. Thus, the DA rat would have predicted the involvement of CYP2D6 in both routes of metabolism. However, regioselective differences in metoprolol metabolism between man and rat, and the involvement of additional enzymes in these oxidations in the rat, indicate that the DA rat cannot be used to provide quantitative predictions regarding the involvement of CYP2D6 in these oxidations in man. Interstrain differences in CYP2D activity were most marked at low substrate concentrations, at which CYP2D is the principal enzyme involved in the metabolism of CYP2D6 substrates. Interstrain differences were less marked at higher substrate concentrations owing to saturation of CYP2D1 and the involvement of other enzymes which show no difference in activity between the strains.

By measuring the ability of rat liver microsomes to catalyse the oxidation of probe compounds for a range of different P450 isoforms, the DA rat was shown to have normal activities with respect to members of the CYP2C, CYP2E and CYP3A subfamilies. However, the 4′-hydroxylation of *R*-mephenytoin, catalysed by a member of CYP2C or CYP3A subfamilies, was also found to be deficient in the DA rat compared with the Wistar rat. Thus, the DA rat clearly has a deficiency in another enzyme in addition to CYP2D1. Thus, if the DA rat is used as a screen to identify CYP2D6 substrates, a small number of false-positives would be identified.

In conclusion, we suggest that the DA rat can be used as a preliminary screen to identify substrates of CYP2D6. Interstrain comparisons should be made using microsomes from female, age-matched rats and low substrate concentrations.

Acknowledgements—This work was supported by the Habershon Rhodes PhD Scholarship awarded to HMB by the University of Sheffield and by The Hallamshire Therapeutics Research Trust.

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