Biochemical Pharmacology, Vol. 53, pp. 171–178, 1997.

ELSEVIER

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00650-8

Variable Contribution of Cytochromes P450 2D6, 2C9 and 3A4 to the 4-Hydroxylation of Tamoxifen by Human Liver Microsomes

H. Kim Crewe, S. Wynne Ellis, Martin S. Lennard and Geoffrey T. Tucker*

University of Sheffield, Department of Medicine and Pharmacology, The Royal Hallamshire Hospital, Sheffield S10 2JF, United Kingdom

ABSTRACT. 4-Hydroxylation is an important pathway of tamoxifen metabolism because the product of this reaction is intrinsically 100 times more potent as an oestrogen receptor antagonist than is the parent drug. Although tamoxifen 4-hydroxylation is catalysed by human cytochrome P450 (CYP), data conflict on the specific isoforms responsible. The aim of this study was to define unequivocally the role of individual CYPs in the 4-hydroxylation of tamoxifen by human liver microsomes. Microsomes from each of 10 human livers catalysed the reaction [range = 0.6–2.9 pmol/mg protein/min (1 μM substrate concentration) and 6–25 pmol/mg protein/min (18 μM)]. Three of the livers with the lowest tamoxifen 4-hydroxylation activity were from genetically poor metabolisers with respect to CYP2D6. Inhibition of activity by quinidine (1 μM), sulphaphenazole (20 μM) and ketoconazole (2 μM), selective inhibitors of CYPs 2D6, 2C9 and 3A4, respectively, was 0–80%, 0–80% and 12–57%. The proportion of activity inhibited by quinidine correlated positively with total microsomal tamoxifen 4-hydroxylation activity ($r_s = 0.89$, $P < 0.01$), indicating a major involvement of CYP2D6 in this reaction. Recombinant human CYPs 2D6, 2C9 and 3A4 but not CYPs 1A1, 1A2, 2C19 and 2E1 displayed significant 4-hydroxylation activity. Similar inhibition and correlation experiments confirmed that tamoxifen N-demethylation is catalysed predominantly by CYP3A4. These findings indicate that the 4-hydroxylation of tamoxifen is catalysed almost exclusively by CYPs 2D6, 2C9 and 3A4 in human liver microsomes. However, the marked between-subject variation in the contribution of these isoforms underlines the need to study metabolic reactions in a sufficient number of livers that are characterised with respect to a range of cytochrome P450 activities. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:171–178, 1997.

KEY WORDS. tamoxifen 4-hydroxylation; human liver microsomes; cytochromes P450, CYP2D6, CYP2C9, CYP3A4

The oestrogen receptor antagonist tamoxifen[1] is used widely to treat breast cancer. However, it is only partially effective, with only about one-third of patients gaining any benefit from the drug [1]. Furthermore, most patients who do respond to treatment with tamoxifen eventually acquire resistance, leading to disease recurrence. Large trials are currently in progress to evaluate the efficacy of tamoxifen in the prevention of breast cancer in women who are at high risk for developing the disease [2]. However, controversial concern has been expressed over the potential of tamoxifen to cause hepatic and endometrial cancers [2], an important issue with regard to prophylactic drug therapy in healthy women.

The metabolism of tamoxifen shows considerable inter-individual variation, and this variation may be a determinant of the efficacy and toxicity of the drug [3]. Tamoxifen undergoes extensive hepatic metabolism and subsequent biliary excretion of metabolites [3]. The primary route of metabolism is N-demethylation, and minor routes include N-oxidation, and 4- and 4′-hydroxylation [3, 4]. There is strong evidence that human CYP† 3A4 is responsible for most of the N-demethylation [5–7], with a possible contribution from CYP1A [8].

Although plasma and tumour concentrations of 4-hydroxytamoxifen are only about 2% of those of the parent compound [9], this metabolite is about 100 times more potent as an oestrogen antagonist than tamoxifen is [10] and may contribute to the clinical response. Resistance to tamoxifen may be associated in part with isomerisation of *trans*-4-hydroxytamoxifen to its *cis*-isomer, which is a much less potent antioestrogen and has weak oestrogenic properties [11]. Furthermore, 4-hydroxytamoxifen may be important in the activation of species that form DNA adducts [12]. Thus, a knowledge of the CYP isoforms responsible for tamoxifen 4-hydroxylation should help to explain indi-

* Corresponding author: TEL: +44 114 2712578; FAX: +44 114 2720275.

† *Abbreviations:* CYP, cytochrome P450; HL, human liver; EM, extensive metaboliser; PM, poor metaboliser; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

[1] Unless otherwise stated, the names of tamoxifen and its metabolites refer to their *trans*-isomers.

Received: 19 June 1996; accepted 16 August 1996.

vidual variation in response to the drug with respect to efficacy, acquired resistance and carcinogenic risk.

A clear indication of the CYP isoform specificity of tamoxifen 4-hydroxylation has not been established. Previously, we concluded that CYP2D6, a human form of CYP exhibiting a marked genetic polymorphism, did not contribute significantly to 4-hydroxylation in HL [13]. This conclusion was based on a comparison of 4-hydroxytamoxifen formation by liver microsomes from two phenotypically EMs and a phenotypically PM with respect to CYP2D6. Phenotype was unrelated to 4-hydroxylation activity, and quinidine, a selective inhibitor of CYP2D6 activity, had no detectable effect on this reaction in liver microsomes from one of the EMs. However, we observed subsequently that recombinant human CYP2D6 expressed in yeast can catalyse the 4-hydroxylation of tamoxifen (unpublished data). Others have also noted 4-hydroxylation by recombinant CYP2D6 and CYP2E1 [14]. In addition, we and others have found evidence for the involvement of the CYP2C subfamily in this reaction [15, 16]. However, these data were obtained using immunoblotting and inhibitory antibodies raised against rat CYP2C, of questionable specificity with regard to the human forms of CYP2C. Thus, the aim of the present study was to identify unequivocally the isoforms of CYP responsible for the 4-hydroxylation of tamoxifen in microsomes from a range of HLs.

MATERIALS AND METHODS

Drugs and chemicals

trans-Tamoxifen citrate and ketoconazole were purchased from Sigma–Aldrich Company Ltd. (Poole, U.K.). *trans*-4-Hydroxytamoxifen, *trans*-N-demethyltamoxifen and diethyldroloxifene were gifts from Klinge Pharma (Munich, Germany). Sulphaphenazole was provided by Ciga-Geigy (Basle, Switzerland). Furafylline was purchased from Salford Ultrafine Chemicals and Research Ltd. (Manchester, U.K.). Other chemicals and biochemicals were obtained commercially and were of the highest grade available.

HL samples

Samples of HL were obtained from 10 donors with the approval of the Hospital Ethics Committee and, where appropriate, with the knowledge of the local coroner. Clinical details of the donors are listed in Table 1. Phenotyping and genotyping analyses established that samples HL1, 3 and 8 were from PMs with respect to CYP2D6. HL1 and 3 were homozygous for the *CYP2D6B* allele and HL8 was heterozygous for the *CYP2D6B* and *CYP2D6T* alleles (unpublished data). The remaining livers were either homozygous or heterozygous for wild-type CYP2D6.

Preparation of liver microsomes

Liver tissue stored at −80° was thawed on ice. Tissue samples were homogenised in 10 volumes of 0.01 M potassium phosphate buffer (pH 7.25) containing 1.15% (w/v) KCl using an Omni Homogenizer System fitted with a rotor stator generator (Camlab, Cambridge, U.K.). Homogenisation was carried out at maximum rotor speed for 30 sec in a cooled (4°) stainless-steel vessel. Microsomes were prepared from the homogenate by ultracentrifugation, as described previously [17]. The microsomal pellets were resuspended in 0.25 M potassium phosphate buffer (pH 7.25) containing 30% glycerol (v/v). The microsomal protein concentration was measured by using the method of Lowry *et al.* [18], with bovine serum albumin (fraction V) as the standard.

Human recombinant CYPs

Microsomes from yeast cells expressing human wild-type CYPs 2D6, 1A1 and 1A2 were prepared as described previously [19–21]. Their CYP content was measured by the method of Omura and Sato [22]. Microsomes from lymphoblastoid cells expressing CYPs 2C9, 2C19, 2E1 and 3A4 were obtained commercially (Gentest Corporation, Woburn, U.S.A.). Values for their CYP content were supplied by the manufacturer.

Incubation conditions

The incubation mixture comprised ethanol/acetone (2 μl, 1:1 v/v) containing tamoxifen (1 μM or 18 μM), an NADPH-generating system consisting of 4 μmol G6P, 0.4 μmol NADP, 0.4 U G6PD and 2 μmol $MgCl_2$ dissolved in 0.2 M potassium phosphate buffer, pH 7.4 (0.4 mL), and the microsomal suspension (0.2 mL). The incubations contained 0.1 mg of HL microsomal protein, 10 pmol of CYP expressed in yeast or 5–45 pmol of CYP expressed in lymphoblastoid cells. The incubation volume was made up to 1 mL with 1.15% (w/v) KCl with or without inhibitor compounds. The reaction was started by the addition of microsomes except in experiments where furafylline and diethylthiocarbamate were added. These compounds were preincubated with microsomes and the NADPH-generating system for 10 min prior to addition of substrate. All incubations were carried out at 37° for 40 min in a shaking water bath. The reaction was stopped by the addition of 3.5 mL methyl *t*-butyl ether. Diethyldroloxifene (1.6 ng) was added as an internal standard.

Analysis of metabolites

4-Hydroxytamoxifen and N-demethyltamoxifen were assayed by using a modification of a previously described HPLC method [13]. Samples were mixed by using a vortex device (5 sec), and the methyl *t*-butyl ether layer was removed and evaporated to dryness under nitrogen at room temperature. Pending further analysis, the samples were stored in the dark at 4°C before reconstitution with a mixture of mobile phase and water (2:1 v/v, 250 μl). An aliquot (20–100 μL) was injected onto the chromatograph. HPLC

TABLE 1. Clinical details of the liver donors

Sample	Sex	Age	Circumstances of tissue donation/cause of death	Drug history	Smoking habits and alcohol consumption
HL1	Male	28	Renal transplant donor: cerebrovascular accident	Diazepam, mannitol, heparin, phenoxybenzamine, dopamine, pancuronium, phentolamine	Not known
HL3	Male	58	Renal transplant donor: posterior fossa bleed and pneumonia	Dexamethasone, dopamine, dobutamine, chlorpromazine, phentolamine, hydrallazine, dibenyline, frusemide, heparin, phenoxybenzamine	Not known
HL4	Male	46	Renal transplant donor: cerebrovascular accident	Cimetidine, indomethacin, prochlorperazine, bretylium, lignocaine, insulin, dexamethazone, phenoxybenzamine, chlorpromazine, frusemide, heparin, phentolamine	Not known
HL5	Male	Not known	Road traffic accident	Atracurium, morphine, propofol, dobutamine, dopamine, alfentanil, verapamil, frusemide, midazolam, metronidazole, ranitidine, cefuroxime	Nonsmoker, occasional drinker
HL6	Female	43	Primary hepatocellular carcinoma*	Ibuprofen, diclofenac, temazepam, paracetamol, morphine, spironolactone, fentanyl, propofol, atracurium, cefuroxime, metronidazole, isoflurane	Nonsmoker, nondrinker
HL7	Male	52	Secondary hepatocellular carcinoma*	Midazolam, temazepam, indomethacin, fentanyl, etomidate, atracurium, enflurane	Nonsmoker, occasional drinker
HL8	Male	26	Primary hepatocellular carcinoma*	Morphine, thiopentone, alfentanyl, atracurium, bupivacaine	Nonsmoker, occasional drinker
HL9	Male	64	Secondary hepatocellular carcinoma*	Fentanyl, propofol, atracurium, isoflurane, bupivacaine	Nonsmoker, occasional drinker
HL10	Male	60	Secondary hepatocellular carcinoma*	Bupivacaine, fentanyl, dopamine, alfentanyl, thiopentone, ampicillin, aprotinin, nitrofurantoin, glyceryl trinitrate	Nonsmoker, occasional drinker
HL11	Female	Not known	Secondary hepatocellular carcinoma*	Oxytetracycline, diazepam, chloral hydrate, fentanyl, thiopentone, atracurium, isoflurane	Nonsmoker, occasional drinker

* Tissue was obtained during surgery to remove a primary hepatocellular carcinoma or a single metastatic tumour arising from a primary carcinoma of the colon. Macroscopically normal tissue from close to the resection line up to 1 cm of the tumour was used.

was performed by using a stainless steel column (250 mm, 4.6 mm inner diameter) containing 5 μ Spherisorb ODS-1 reversed-phase packing material, fitted with a guard column packed with C_{18} reversed-phase material (40 μm). The analytical column was maintained at 40°C in a waterbath. The column eluent was irradiated with ultraviolet (UV) light while passing through 3.3 m of Teflon™ tubing (1.6 mm outer diameter, 0.5 mm inner diameter) crocheted into 5-mm loops and wound around a lattice fixed at 8 cm from a low pressure UV lamp (Osram HNS 10 W/U). The eluent was then passed through a fluorescence detector (Model 820FP, Jasko, Great Dunmow, U.K.) with excitation and emission wavelengths set at 260 and 375 nm, respectively. The mobile phase was 10 mM KH_2PO_4 (adjusted to pH 3.7 with orthophosphoric acid)/methanol/acetonitrile (2.5:3:4.5 v/v) except for the analysis of incubations containing quinidine when a ratio of 3:3:4 was used. Chromatography was performed isocratically at a flow rate of 1.3 mL/min. Intra-assay coefficients of variation (n = 6 replicates) were 6% for both metabolites at the lowest concentrations (1–3 pmol/mL) detected in HL microsomal incubations. The limit of assay of both metabolites was 0.1 pmol/mL.

Inhibition studies

Quinidine at a concentration of 1 μM was used as a selective inhibitor of CYP2D6 [23], furafylline at 10 μM was used as a selective inhibitor of CYP1A2 [24], sulphaphenazole at 20 μm was used as a selective inhibitor of CYP2C9 [25], ketoconazole at 2 μM was used as a selective inhibitor of CYP3A4 [25] and diethylthiocarbamate at 50 μM was used as a selective inhibitor of CYP2E1 [26].

Correlation studies

The rates of tamoxifen 4-hydroxylation and N-demethylation by microsomes from 10 HLs were correlated (Spearman rank test) with CYP1A2 activity measured by the O-demethylation of phenacetin (5 μM) [27], CYP2C9 activity measured by the methylhydroxylation of tolbutamide (100 μM) [28], CYP2C19 activity measured by the 4-hydroxylation of S-mephenytoin (200 μM) [29], CYP2D6 activity measured by the α-hydroxylation of metoprolol (40 μM) [23], CYP2E1 activity measured by the 6-hydroxylation of chlorozoxazone (30 μM) [30] and

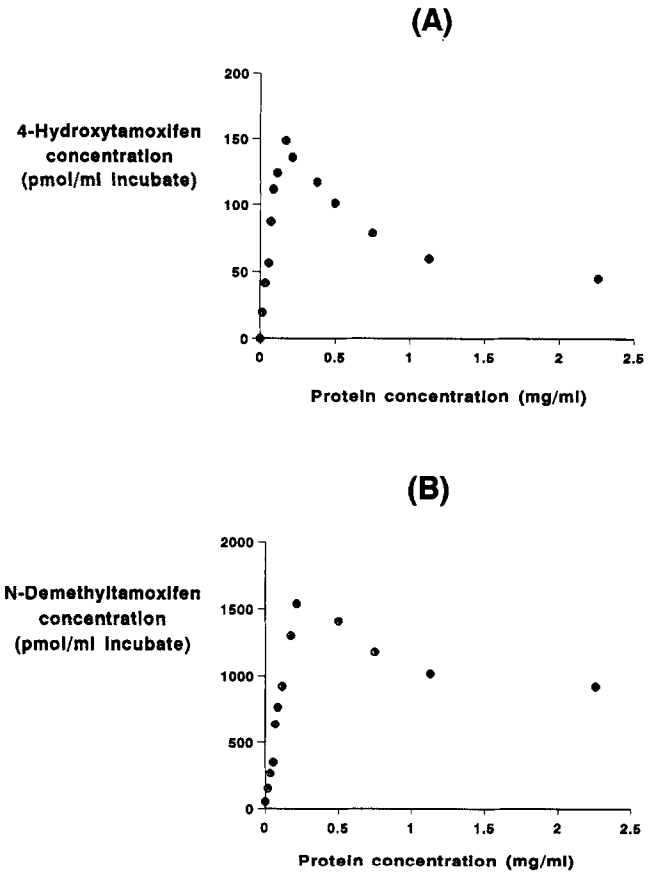

FIG. 1 **The effect of liver microsomal protein concentration on the (A) 4-hydroxylation and (B) N-demethylation of tamoxifen (1 μM) by HL microsomes. The data shown are the mean results of duplicate experiments with microsomes from HL10.**

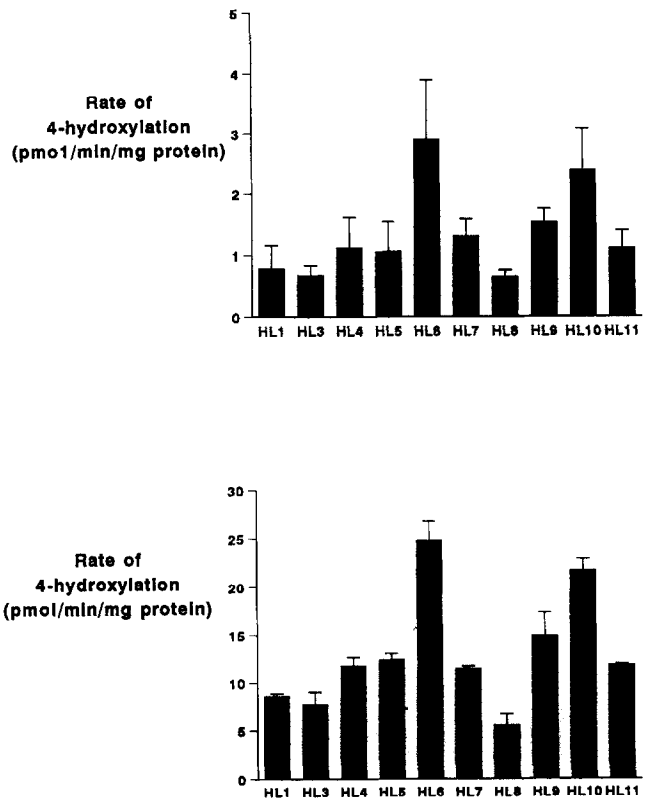

FIG. 2 **The rates of tamoxifen 4-hydroxylation by microsomes from 10 HLs at substrate concentrations of (A) 1 μM and (B) 18 μM. The data shown are the mean (±SD) results from 3–6 incubations.**

CYP3A4 activity measured by the 6β-hydroxylation of testosterone (20 μM) [31].

RESULTS

The rates of tamoxifen 4-hydroxylation and N-demethylation by HL microsomes at a substrate concentration of 1 μM were linear up to at least 40 min of incubation (data not shown). In contrast, the relationship between metabolite formation and microsomal protein content deviated markedly from linearity (Fig. 1). Thus, the reactions were linear only at protein concentrations below 0.2 mg/mL; above this level, there was a sharp decline in the rates of appearance of both 4-hydroxy- and N-demethyltamoxifen. All subsequent studies were carried out at a protein concentration of 0.1 mg/mL.

Tamoxifen 4-hydroxylation

Microsomes from all 10 HLs catalysed the 4-hydroxylation of tamoxifen. The rank order of activities within the liver bank was identical at substrate concentrations of 1 μM and 18 μM (Fig. 2). Activities ranged from 0.6 to 2.9 pmol/mg protein/min (1 μM) and from 6 to 25 pmol/mg protein/min (18 μM).

The HLs from the three PMs with respect to CYP2D6 had the lowest tamoxifen 4-hydroxylation activities. Quinidine (1 μM) inhibited 4-hydroxylation in six of the 10 HLs studied to 20–80% of control activity (Table 2; Fig. 3). 4-Hydroxylation activity in microsomes from the three CYP2D6 PM HLs was not decreased by quinidine. The apparent increase (42%) in 4-hydroxylase activity in microsomes from HL1, one of the PM livers, was a consistent finding. Paradoxically, quinidine had no effect on tamoxifen 4-hydroxylation activity in microsomes from HL5, the donor of which was genotyped as an EM and which had significant metoprolol α-hydroxylase activity. Mean inhibition of control activity was 0–80% for sulphaphenazole (20 μM) and 12–57% for ketoconazole (2 μM) (Table 2; Fig. 3). Furafylline (10 μM) and diethylthiocarbamate (50 μM) had negligible effects on tamoxifen 4-hydroxylation in the HLs (data not shown).

The proportions of 4-hydroxylation activity that were inhibited by quinidine and ketoconazole correlated highly with metoprolol α-hydroxylation (r_s = 0.88, $P < 0.01$), and testosterone β-hydroxylation (r_s = 0.89, $P < 0.01$) activities, respectively (Fig. 4). The correlation between sulphaphenazole-inhibitable tamoxifen 4-hydroxylation and tolbutamide hydroxylation activity was weaker (r_s = 0.54, $P >$

TABLE 2. Effect of quinidine (1 μM), ketoconazole (2 μM) and sulphaphenazole (20 μM) on the 4-hydroxylation of tamoxifen (1 μM) by microsomes from 10 HLs

	Velocity (pmol/mg prot/min)				
Liver	Control	Quinidine	Ketoconazole	Control	Sulphaphenazole
HL1	0.60 ± 0.06	0.85 ± 0.12	0.26 ± 0.08	0.51 ± 0.26	0.25 ± 0.13
HL3	0.47 ± 0.06	0.48 ± 0.05	0.22 ± 0.03	0.77 ± 0.03	0.14 ± 0.03
HL4	0.63 ± 0.10	0.29 ± 0.03	0.55 ± 0.05	0.99 ± 0.01	0.69 ± 0.24
HL5	0.69 ± 0.14	0.69 ± 0.10	0.36 ± 0.07	0.90 ± 0.34	0.34 ± 0.20
HL6	1.46 ± 0.03	0.29 ± 0.04	1.15 ± 0.15	2.99 ± 0.40	2.96 ± 0.27
HL7	1.84 ± 0.06	0.80 ± 0.05	1.00 ± 0.24	1.84 ± 0.06	2.03 ± 0.24
HL8	0.66 ± 0.08	0.72 ± 0.12	0.30 ± 0.06	0.59 ± 0.11	0.11 ± 0.06
HL9	1.55 ± 0.05	0.87 ± 0.07	1.33 ± 0.06	1.71 ± 0.30	0.75 ± 0.26
HL10	3.30 ± 0.75	1.66 ± 0.08	2.19 ± 0.26	3.30 ± 0.75	2.66 ± 0.36
HL11	1.14 ± 0.08	0.91 ± 0.14	0.72 ± 0.03	0.86 ± 0.42	0.65 ± 0.37

Data are the mean (±SD) results from triplicate incubations.

0.1) (Fig. 4). The proportion of activity inhibited by quinidine correlated positively with total tamoxifen 4-hydroxylation activity (r_s = 0.89, $P < 0.01$) (Fig. 5). There was no correlation between sulphaphenazole-inhibitable activity (r_s = −0.09, $P > 0.1$) or ketoconazole-inhibitable activity (r_s = 0.04, $P > 0.1$) and total 4-hydroxylation activity. Tamoxifen 4-hydroxylation did not correlate with phenacetin O-demethylation (r_s = 0.15, $P > 0.1$), S-mephenytoin 4-hydroxylation (r_s = −0.08, $P > 0.1$) or chlorzoxazone 6-hydroxylation (r_s = −0.41, $P > 0.1$).

Significant tamoxifen 4-hydroxylation activity was displayed by recombinant human CYPs 2D6 (0.117 pmol/pmol P450/min, mean of duplicate incubations), 2C9 (0.048 pmol/pmol/ P450/min), and 3A4 (0.004 pmol/pmol P450/min) at a substrate concentration of 18 μM. Activity just above the limit of determination of the assay was observed for CYP2C19. No tamoxifen 4-hydroxylation by recombinant CYPs 1A1, 1A2 and 2E1 was detected.

Tamoxifen N-demethylation

Microsomes from all of the livers catalysed tamoxifen N-demethylation (range = 16–110 pmol/min/mg protein at a substrate concentration of 18 μM). N-demethylation correlated strongly with testosterone β-hydroxylation (r_s = 0.89, $P < 0.01$). In microsomes from nine HLs, ketoconazole inhibited most (84–94%) of the N-demethylase activity.

DISCUSSION

Difficulties were encountered in establishing optimal conditions for studying tamoxifen oxidation by HL micro-

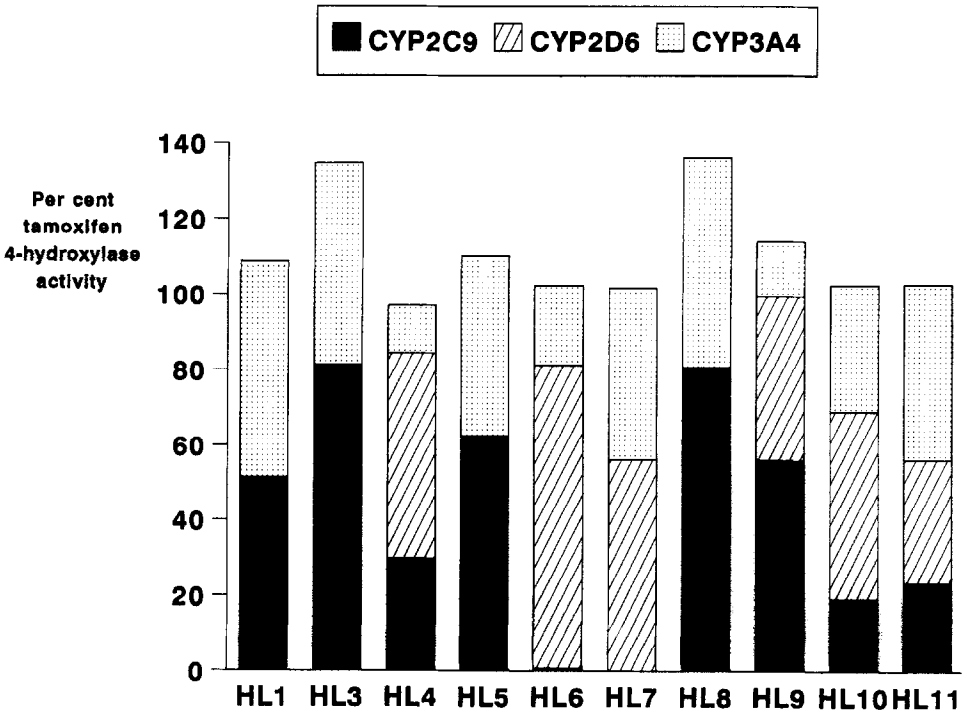

FIG. 3 The estimated contributions of CYPs 2D6, 2C9 and 3A4 to the 4-hydroxylation of tamoxifen (1 μM) by microsomes from 10 HLs based on inhibition by quinidine, sulphaphenazole and ketoconazole. HL1, 3 and 8 are from PMs with respect to CYP2D6.

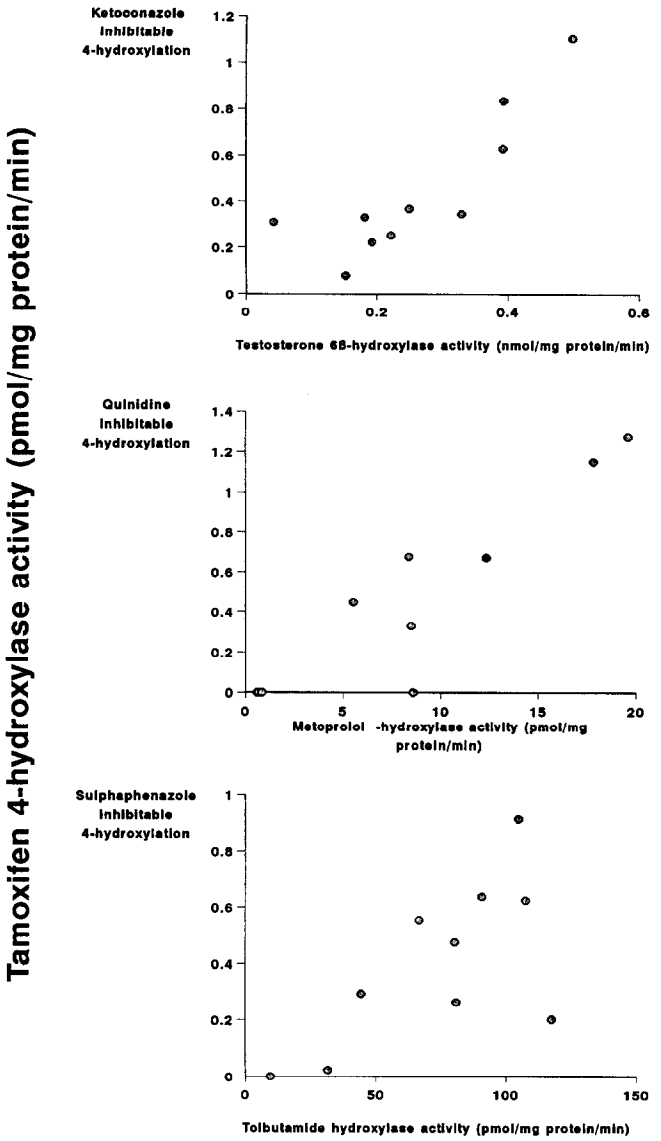

FIG. 4 Correlations between (A) quinidine-inhibitable tamoxifen 4-hydroxylase activity and metoprolol α-hydroxylase activity, (B) sulphaphenazole-inhibitable tamoxifen 4-hydroxylase activity and tolbutamide hydroxylase activity and (C) ketoconazole-inhibitable tamoxifen 4-hydroxylase activity and testosterone β-hydroxylase activity in microsomes from 10 HLs. Tamoxifen concentration = 1 μM. Data points are the mean values from triplicate incubations.

somes. The striking decrease in the rate of both 4-hydroxylation and N-demethylation at microsomal protein concentrations above 0.2 mg/mL was an unexpected finding. The basis of this nonlinearity was not studied further, but nonspecific binding of tamoxifen to or solubilisation in microsomal lipid leading to a decreased concentration of substrate available for metabolism at high protein concentrations may explain this observation. To determine whether more than one form of CYP might be responsible for the 4-hydroxylation of tamoxifen, attempts were made to characterise the kinetics of the reaction under conditions that were linear with respect to time and protein concentration. Unfortunately, Eadie-Hoftsee plots obtained over a wide concentration range were not interpretable and could not be fitted by simple Michaelis-Menten or Hill functions or by combinations of both. Studies were performed subsequently at two substrate concentrations. The high concentration (18 μM) is comparable to those for which published data are available (e.g. [16]), and the lowest concentration (1 μM) is within the range of those achieved in plasma at therapeutic doses of tamoxifen [3].

Our findings using microsomes from 10 HLs indicate the involvement of CYP2D6, CYP2C9 and CYP3A4 in the 4-hydroxylation of tamoxifen. Furthermore, all 4-hydroxylation activity could be accounted for by the sum of these activities (Fig. 4). In microsomes from two HLs (HL3 and HL8), net inhibition exceeded 100%, possibly reflecting some nonspecificity in the effects of one or more of the inhibitors. The strong positive correlation between quinidine-inhibitable 4-hydroxylation activity and net 4-hydroxylation activity indicates that metabolism by CYP2D6 is the most important determinant of the rate of 4-hydroxylation of tamoxifen in HL. When CYP2D6 activity is low or absent, CYP2C9 and CYP3A4 predominate, but the reaction proceeds at lower rate. A surprising and unexplained observation was that tamoxifen 4-hydroxylation by microsomes from HL5, a liver with significant metoprolol α-hydroxylation (CYP2D6) activity that was completely inhibited by quinidine (data not shown), was unaffected by quinidine. HL5 may contain a variant CYP2D6 enzyme with differing substrate specificity compared with the enzyme in the livers from the other EMs.

Our data for the N-demethylation of tamoxifen confirm those of others [5–7] and show that the reaction is catalysed predominantly by CYP3A4. A contribution of CYP1A2 to 4-hydroxylation, as reported by Simon *et al.* [8], could not be confirmed.

The present findings together with similar observations on the variable contribution of CYP2D6 to the N-dealkylation of MPTP [21] and propranolol [32] emphasise the importance of assessing the isoform profile of specific metabolic reactions with a sufficient range of liver samples characterised with respect to isoform activity. Data obtained by using microsomes from a small number of livers or microsomal mixtures pooled from several livers may be misleading because the relative importance of different enzymes contributing to the same reaction may show considerable variability between livers. This variability clearly led to our previous erroneous conclusion that CYP2D6 was not involved in the 4-hydroxylation of tamoxifen [13]. Thus, in microsomes from the three livers studied (from one PM and two EMs with respect to CYP2D6), we found that, at a substrate concentration of 36 μM, 4-hydroxylation activity in the PM liver (HL3 in the present study) was lower than that in EM2 (HL4) but higher than that in EM1 (HL5). Only one liver (HL5) was used in the experiments with quinidine, and unfortunately this proved to be the only EM liver we tested that possessed tamoxifen 4-hydroxylase activity not inhibitable by quinidine.

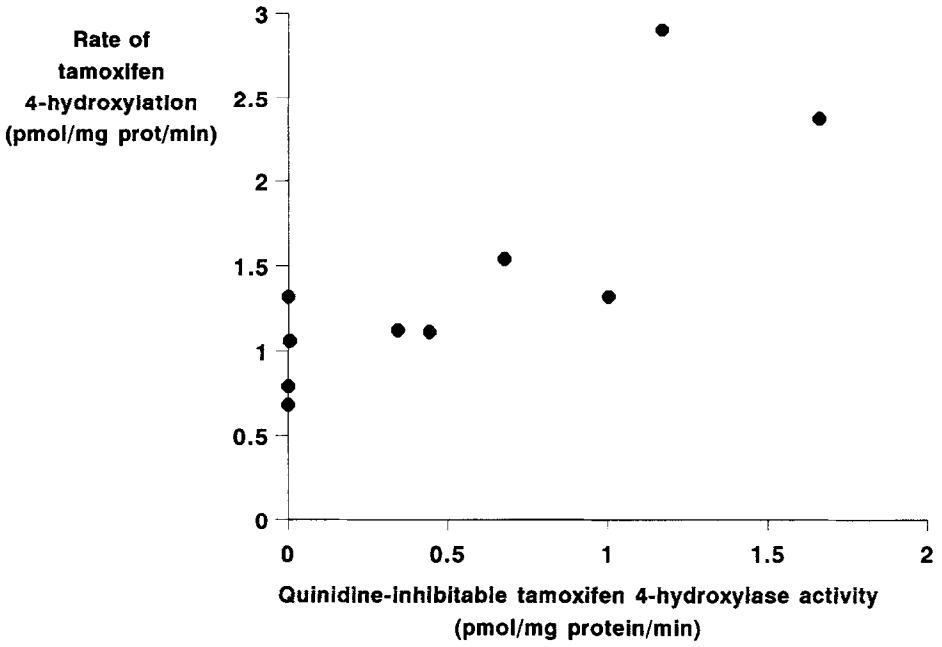

FIG. 5 Correlation between quinidine-inhibitable tamoxifen 4-hydroxylase activity and net 4-hydroxylase activity in microsomes from 10 HLs. Tamoxifen concentration = 1 μM. Data points are the mean values from triplicate incubations.

Another pitfall in using a limited number of livers is the possibility that prediction of specific drug interactions may be compromised. Because tamoxifen inhibits CYP [33], patients may be at particular risk for drug interactions mediated through the specific isoforms of CYP that metabolise tamoxifen, namely CYP2D6, CYP2C9 and CYP3A4. For example, this mechanism may explain the life-threatening interaction between tamoxifen and warfarin [34, 35], which is metabolised predominantly by CYP2C9 [36].

The other clinical implication of our findings for tamoxifen is that patients who are CYP2D6 PMs would be expected to have lower plasma and tissue concentrations of the pharmacologically active 4-hydroxy metabolite than would EMs, which may lead to differences in response.

We thank Dr. Ulrich Zanger (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart) for genotyping HL8 and Dr. Nigel Bird (University of Sheffield Department of Surgical Sciences) for his help in the provision of liver samples. This work was partly supported by the Commission of the European Communities (contract BMH1-CT 94–1622).

References

1. Catherino WH and Jordan VC, A risk-benefit assessment of tamoxifen therapy. *Drug Safety* **8:** 381–397, 1993.
2. Jordan VC, Tamoxifen: toxicities and drug resistance during the treatment and prevention of breast cancer. *Annu Rev Pharmacol Toxicol* **35:** 195–211, 1995.
3. Buckley MT and Goa KL, Tamoxifen: a reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic use. *Clin Pharmacokin* **37:**451–490, 1989.
4. Lim CK, Yuan Z-X, Lamb JH, White INH, De Matteis F and Smith LL, A comparative study of tamoxifen metabolism in female rat, mouse and human liver microsomes. *Carcinogenesis* **15:** 589–593, 1994.
5. Jacolot F, Simon I, Dreano I, Beaune P, Riche C and Berthou F, Identification of the cytochrome P450 IIA family as the enzymes involved in the N-demethylation of tamoxifen in human liver microsomes. *Biochem Pharmacol* **41:** 1911–1919, 1991.
6. Mani C, Gelboin HV, Park SS, Pearce R, Parkinson A and Kupfer D, Metabolism of the antimammary cancer antiestrogenic agent tamoxifen. I. Cytochrome P450-catalysed N-demethylation and 4-hydroxylation. *Drug Metab Dispos* **21:** 645–656, 1993.
7. Mani C, Pearce R, Parkinson A and Kupfer D, Involvement of cytochrome P4503A in catalysis of tamoxifen activation and covalent binding to rat and human liver microsomes. *Carcinogenesis* **15:** 2715–2720, 1994.
8. Simon I, Berthou F, Riche C, Beaune P and Ratanasavanh D, Both cytochromes P4501A1 and 3A4 are involved in the N-demethylation of tamoxifen. *Proceedings of the Fifth European ISSX Meeting*, Tours, France, Abstract No. 44, 1993.
9. Daniel PC, Gaskell J, Bishop H, Campbell C and Nicholson R, Determination of tamoxifen and biologically active metabolites in human breast tumour and plasma. *Eur J Cancer Clin Oncol* **17:** 1183–1189, 1981.
10. Jordan VC, Collins MM, Rowsby L and Prestwich G, A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* **75:** 305–316, 1977.
11. Williams ML, Lennard MS, Martin IJ and Tucker GT, Inter-individual variation in the isomerisation of 4-hydroxytamoxifen by human liver microsomes: involvement of cytochromes P450. *Carcinogenesis* **15:** 2733–2738, 1994.
12. Moorthy B, Sriram P, Pathak DN, Bodell WJ and Randerath K, Tamoxifen metabolic activation: comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed *in vivo*. *Cancer Res* **56:** 53–57, 1996.
13. Blankson EA, Ellis SW, Lennard MS, Tucker GT and Rogers K, The metabolism of tamoxifen by human liver microsomes is not mediated by cytochrome P450IID6. *Biochem Pharmacol* **42:** S209–S212, 1991.
14. Styles JA, Davies A, Lim CK, De Matteis F, Stanley LA, White INH, Yuan Z-X and Smith LL, Genotoxicity of tamoxifen, tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochromes P450. *Carcinogenesis* **15:** 5–9, 1994.

15. Daniels L, Blankson EA, Henderson CJ, Harris AR, Wolf CR, Lennard MS and Tucker GT, Delineation of human cytochromes P450 involved in the metabolism of tamoxifen. *Br J Clin Pharmacol* **34:** 153P–154P, 1992.
16. White INH, De Matteis F, Gibbs AH, Lim CK, Wolf CR, Henderson C and Smith LL, Species differences in the covalent binding of [^{14}C]tamoxifen to liver microsomes and the forms of cytochrome P450 involved. *Biochem Pharmacol* **49:** 1035–1042, 1995.
17. Shaw L, Lennard MS, Tucker GT, Bax NDS and Woods HF, Irreversible binding and metabolism of propranolol by human liver microsomes—relationship to polymorphic oxidation. *Biochem Pharmacol* **36:** 2283–2288, 1987.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
19. Ellis SW, Hayhurst GP, Smith G, Lightfoot T, Wong MMS, Simula A, Ackland MJ, Sternberg MJE, Lennard MS, Tucker GT and Wolf CR, Evidence that aspartic acid 301 is a critical substrate-contact residue in the active site of cytochrome P450 2D6. *J Biol Chem* **270:** 29055–29058, 1996.
20. Ching MS, Lennard MS, Tucker GT, Woods HF, Kelly DE and Kelly SL, The expression of human cytochrome P450IA1 in the yeast *Saccharomyces cerevisiae*. *Biochem Pharmacol* **42:** 753–758, 1991.
21. Coleman T, Ellis SW, Martin IJ, Lennard MS and Tucker GT, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is N-demethylated by CYPs 2D6, 1A2 and 3A4—implications for Parkinson's disease. *J Pharmacol Exp Ther* **277:** 685–690, 1996.
22. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239:** 2370–2378, 1964.
23. Otton SV, Crewe HK, Lennard MS, Tucker GT and Woods HF, Use of quinidine inhibition to define the role of the sparteine/debrisoquin cytochrome P450 in metoprolol oxidation by human liver microsomes. *J Pharmacol Exp Ther* **247:** 242–247, 1988.
24. Clarke SE, Ayrton AD and Chenery RJ, Characterisation of the inhibition of P4501A2 by furafylline. *Xenobiotica* **24:** 517–526, 1994.
25. Baldwin SJ, Bloomer JC, Smith GJ, Ayrton AD, Clarke SE and Chenery RJ, Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 2C9. *Xenobiotica* **25:** 261–270, 1995.
26. Guengerich FP, Kim D-H and Iwasaki M, Role of human cytochrome P-450IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4:** 168–179, 1991.
27. Sesardic D, Edwards RJ, Davies DS, Thomas PE, Levin W and Boobis AR, High affinity phenacetin O-deethylase activity is catalysed specifically by cytochrome P450d (P4501A2) the liver of rat. *Biochem Pharmacol* **39:** 489–498.
28. Miners JO, Smith KJ, Robson RA, McManus ME, Veronese ME and Birkett DJ, Tolbutamide hydroxylation by human liver microsomes. *Biochem Pharmacol* **37:** 1137–1144, 1988.
29. Meier UT, Dayer P, Male P-J, Kronbach T and Meyer UA, Mephenytoin hydroxylation polymorphism: characterisation of the enzyme deficiency in liver microsomes of poor metabolisers phenotyped *in vivo*. *Clin Pharmacol Ther* **38:** 488–494, 1985.
30. Peter R, Bocker R, Beaune PH, Iwaski M, Guengerich FP and Yang CS, Hydroxylation of chlorzoxazone as a specific probe cytochrome P-450IIE1. *Chem Res Toxicol* **3:** 566–573, 1990.
31. Waxman DJ, Attisano C, Guengerich JR and Lapenson DP, Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P450 enzyme. *Arch Biochem Biophys* **263:** 424–436, 1988.
32. Rowland K, Ellis SW, Lennard MS and Tucker GT, Variable contribution of CYP2D6 to the N-dealkylation of S-(-)-propranolol by human liver microsomes. *Br J Clin Pharmacol* **42:** 394–397, 1996.
33. Ruenitz PC and Toledo MM, Inhibition of rabbit liver microsomal oxidative metabolism and substrate binding by tamoxifen and the geometric isomers of clomiphene. *Biochem Pharmacol* **29:** 1583–1587, 1980.
34. Lodwick R, McConkey B, Brown AM and Beeley L, Life threatening interaction between tamoxifen and warfarin. *Br Med J* **295:** 1141, 1987.
35. Tenni P, Lalaich DL and Byrne MJ, Life threatening interaction between tamoxifen and warfarin. *Br Med J* **298:** 93, 1989.
36. Rettie AE, Korzekwa KR, Kunze KL, Laurence RF, Eddy AC, Ayoma T, Gelboin HV, Gonzalez FJ and Trager WF, Hydroxylation of warfarin by human cDNA expressed cytochrome P450: a role for P4502C9 in the etiology of (S)-warfarin drug interactions. *Chem Res Toxicol* **5:** 54–59, 1992.