XENOBIOTICA , 1997, VOL. 27, NO. 10, 1025–1037

Identification of the cytochrome P450 isoforms involved in the *O*-demethylation of 4-nitroanisole in human liver microsomes

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Received 5 May 1997

1. 4-Nitroanisole is *O*-demethylated to 4-nitrophenol by human liver microsomes. Kinetic studies indicate that this metabolic route is mediated by two cytochrome P450 isoforms, one with a K_m = 2·1 μM and the other with a K_m = 220 μM.

2. Chemical inhibition and correlation studies in human liver microsomes indicate that the low K_m enzyme is CYP2A6 and the high K_m enzyme is CYP2E1 suggesting that 4-nitroanisole is not a general cytochrome P450 substrate.

3. Studies using expressed recombinant cytochrome P450s indicated that all the cytochrome P450s investigated metabolized 4-nitroanisole but CYP2A6 and CYP2E1 produced the highest rates. Kinetic studies with these two isoforms produced a K_m for CYP2A6 of 9 μM and 54 μM for CYP2E1.

4. The involvement of these two isoforms in the *O*-demethylation of 4-nitroanisole can be rationalized in terms of a hydrogen bond interaction with the nitro group and the active site of CYP2A6 and a hydrophobic interaction with the active site of CYP2E1.

Introduction

Cytochrome P450 isoenzymes play an important role in the oxidation of many drugs and xenobiotic compounds as well as many endogenous compounds (Gonzalez 1992) and consist of an enzyme superfamily comprised of a number of subfamilies (Nelson *et al*. 1993). The individual enzymes have subtly different but sometimes overlapping requirements in terms of their substrate specificity. In man the cytochrome P450 system has been shown to have the capacity to metabolize a range of molecules, from small molecules such as acetone (Koop 1992) to very large molecules such as cyclosporin (Smith and Jones 1992).

Many studies have been carried out to investigate the substrate specificity of these enzymes. It is recognized that within the human drug-metabolizing cytochrome P450s CYP2D6 relies on an ion-pair interaction, CYP2C9 on a hydrogen bond interaction and metabolism by CYP3A4 seems to occur as a result of chemical lability rather than a specific binding interaction (Smith and Jones 1992). Whilst a good deal of effort has been devoted to identifying specific probe substrates for each of the human drug-metabolizing cytochrome P450s, it is also useful to have compounds which are more non-specific and which measure the hepatic metabolic capacity of the cytochrome P450 system. Amongst these more non-specific substrates are compounds such as antipyrine and 4-nitroanisole (Souhaili-el Amri *et al*. 1986, Sharer and Wrighton 1996).

Antipyrine is used as a non-specific substrate both for *in vitro* and *in vivo* studies in man. Recently two studies investigated antipyrine with respect to identifying the cytochrome P450s involved in its metabolism in human liver microsomes (Engel *et al*. 1996, Sharer and Wrighton 1996). Both studies concluded that a number of

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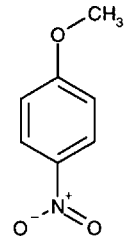

Figure 1. Structure of 4-nitroanisole.

cytochrome P450s including CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4 were involved to some extent in one or more of the routes of antipyrine metabolism. This involvement of a number of cytochrome P450s confirms that antipyrine is a promiscuous cytochrome P450 substrate.

4-Nitroanisole (figure 1) has also been widely used as a non-specific marker for cytochrome P450 activity. It has been used in a wide range of studies designed to examine the effect of xenobiotic compounds on the cytochrome P450 system, both in terms of induction (Kim and Kim 1996) and inhibition (Gelboin *et al*. 1995). 4-Nitroanisole has also been used as a substrate to investigate the effect of changes in physiological conditions, such as warm ischaemia and reperfusion of the liver, on the cytochrome P450 system (Izuishi *et al*. 1995). Its use as a non-specific cytochrome P450 marker spans a number of species including rat (Kim and Kim 1996), dog (Daling *et al*. 1994) and man (Gelboin *et al*. 1995). However, its use has not been confined to mammals since it has also been used to investigate the effect of compounds on the cytochrome P450 system in insects (Neal and Wu 1994).

One reason for the wide usage of 4-nitroanisole as a cytochrome P450 marker is the ease with which its major metabolite 4-nitrophenol can be assayed (Gibson and Skett 1986). This ease of assay, coupled with the suggestion that it is a general marker for cytochrome P450 activity, has lead to its use as a positive control for microsomal incubations within our laboratory. However whilst 4-nitroanisole is commonly used as a non-specific substrate, little is known about the cytochrome P450 isoforms which metabolize it. The purpose of this study was to elucidate which of the human cytochrome P450s metabolize 4-nitroanisole and hence confirm its choice as a general cytochrome P450 substrate.

Materials and methods

Chemicals and reagents

4-Nitroanisole, 4-nitrophenol and salicylamide were obtained from Aldrich Chemical Co. (Dorset, UK). Furafylline, 4′-hydroxy diclofenac, (*S*)-mephenytoin, 4-hydroxy mephenytoin, bufuralol, 1′-hydroxy bufuralol and sulphaphenazole were obtained from Salford Ultrafine Chemicals and Research (Manchester, UK). Specific CYP cDNA-transfected human B-lymphoblastoid-derived microsomes were obtained from Gentest Corp. (Woburn, MA, USA). All other reagents were obtained from Sigma Chemical Co (Dorset UK) and were of the purest grade available.

Preparation of microsomes

Transplant-quality human liver tissue was obtained from the International Institute for the Advancement of Medicine (Exton, PA, USA). The donors ranged in age from 22 to 66 years and included eight males and four females. All the samples had no known drug history with the exception of HM-11 who had been treated with phenobarbitone, a known CYP3A4 inducer. None of the donors had a history of alcohol abuse. Hepatic microsomes were prepared from individual human livers by the process of differential centrifugation. Briefly, the liver tissue was homogenized in 50 mM Tris-HCl (pH 7·4) containing 250 mM sucrose and then centrifuged at 9000 *g* for 20 min to remove the cell debris and

nuclear fraction. The supernatant was removed and further centrifuged at 105 000 *g* for 60 min to pellet the microsomal fraction. This pellet was washed with 100 mM Tris-HCl (pH 7·4) and centrifuged at 105 000 *g* for 60 min to remove any contaminating haemoglobin. The final pellet was resuspended in 100 mM potassium phosphate (pH 7·4) and stored at −80 °C prior to use. A separate batch of microsomes was produced for the chemical inhibition experiments from a combination of equal amounts (~ 50 g from each sample) of four human livers (HM-11, HM-12, HM-13 and HM-14). Cytochrome P450 content was determined using the method of Omuro and Sato (1964) and protein concentration was determined using the method of Lowry *et al*. (1951), with bovine serum albumin as the protein standard.

Assay for 4-nitroanisole O-demethylation

The conversion of 4-nitroanisole to 4-nitrophenol by human liver microsomes or expressed recombinant cytochrome P450s was determined according to the following method. Each incubation (final volume 100 μl) was comprised of microsomal protein (0·5 mg/ml), 50 mM Tris-HCl (pH 7·4), 5 mM $MgCl_2$ and 5 μM $MnCl_2$. Reducing equivalents required for cytochrome P450 metabolism were provided by NADPH (1 mM), which was regenerated *in situ* by an isocitric acid (5 mM)/isocitric acid dehydrogenase (1 unit/ml) system. The incubation mixture was preincubated at 37 °C in the presence of substrate prior to addition of NADPH to initiate the reaction.

At the end of each incubation the reaction was terminated by the addition of 10 μl 600 mM perchloric acid to precipitate the microsomal protein. At this point the internal standard (salicylamide, 10 μl of 100 μg/ml solution) was added. The precipitated microsomal protein was pelleted by centrifugation at 3000 rpm for 5 min. The supernatant was removed and 80 μl subjected to hplc analysis.

Hplc analysis

Samples were chromatographed on a Inertsil-5-ODS2 column (15 cm × 4·6 mm i.d. Hichrom) which was eluted with 20 mM sodium perchlorate, pH 2·5/acetonitrile (55/45 v/v) at 1·0 ml min^{-1}. Quantitation was achieved using a UV-VIS detector (Shimadzu SPD-6A) operating at 310 nm. Calibration curves were constructed using authentic 4-nitrophenol over the range 5–500 ng.

Chemical inhibition

In the chemical inhibition experiments methanolic stock solutions of each inhibitor were added to the incubation prior to the initiation of the reaction. In all cases the methanol concentration did not exceed 0·1%. This resulted in a minimal effect (< 10%) on the rate of 4-nitroanisole *O*-demethylation. The concentration of 4-nitroanisole used in the chemical inhibition experiments was 10 μM. Each of the specific inhibitors was used at two concentrations, found to give 50 and 90% inhibition of the relevant isoforms activity determined using an appropriate substrate. Further detailed investigations into the inhibition of 4-nitroanisole *O*-demethylase activity by coumarin and chlorzoxazone were carried out at the same 4-nitroanisole concentration (10 μM), but with increasing inhibitor concentrations (0·01–100 μM for coumarin and 0·1–1000 μM for chlorzoxazone).

Correlation analysis

Correlations between the rate of 4-nitroanisole *O*-demethylation and cytochrome P450 isoform-specific activities were assessed in microsomes prepared from a panel of human livers (n = 12). These studies were carried out at a 4-nitroanisole concentration of 1000 μM. To ensure that all the data were normally distributed a log transformation of the data was carried out prior to the correlation analysis. This reduces the influence of very high or low activities on the correlation.

Metabolism by expressed recombinant cytochrome P450s

The incubations with microsomes derived from specific CYP cDNA-transfected human B-lymphoblastoid cells were conducted as described above, at two 4-nitroanisole concentrations (10 and 1000 μM) for 15 min at a protein concentration of 0·25 mg/ml. Kinetics in B-lymphoblastoid-derived microsomes were determined under the same conditions, but with 4-nitroanisole concentrations of 0·25–500 μM for CYP2A6 and 1–500 μM for CYP2E1.

Other assays

Phenacetin *O*-deethylase activity was determined at 10 μM phenacetin and 0·5 mg/ml microsomal protein for 30 min. After 30 min the incubations were terminated by the addition of 7 ml tertiary butylmethyl ether followed by 100 μl 1 M HCl and 5 μl internal standard (3-hydroxy acetanilide, 5 μg/ml). After extraction the organic layer was removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 μl of hplc mobile phase and 80 μl was injected onto the hplc. Samples were chromatographed on a 25 cm Spherisorb-5-ODS2 column eluted at 1 ml min^{-1} with an isocratic mixture of 83% solvent A (water) and 17% solvent B (methanol) from 0 to 10 min, followed by a linear gradient to 70% B over 5 min, then isocratic at 70% B for 1 min and then a final linear gradient back to 17% B over 12 min. Detection was by UV absorbance (Shimazdzu SPD-10A) at 210 nm. Under these

conditions paracetamol had a retention time of approximately 9 min, 3-hydroxy acetanilide approximately 13 min and phenacetin approximately 19 min.

Diclofenac 4′-hydroxylase activity was determined at a substrate concentration of 500 μM with 0·25 mg/ml microsomal protein for 15 min. After 15 min the incubations were stopped by the addition of 7 ml t-butyl methyl ether followed by 10 μl internal standard (mefenamic acid, 10 μg/ml). Samples were extracted and the organic layer removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 μl of hplc mobile phase and 80 μl was injected onto the hplc. Samples were chromatographed on a 15-cm Spherisorb-5-ODS2 column with a mobile phase of 100 mM Na_2HPO_4/methanol/acetonitrile (60/22·5/17·5 v/v), adjusted to pH 7, at 1·3 ml min^{-1}. Detection was by UV absorbance (Shimadzu SPD-6A) at 280 nm. Under these conditions 4′-hydroxy diclofenac had a retention time of approximately 5 min, diclofenac approximately 16 min and mefenamic acid approximately 23 min.

The remaining probe substrate activities used were as described previously in the literature. Coumarin 7-hydroxylase activity was determined at a substrate concentration of 100 μM (Maurice *et al.* 1991), (*S*)-mephenytoin 4-hydroxylase activity was determined at a substrate concentration of 500 μM (Meier *et al.* 1985), bufuralol 1′-hydroxylase was determined at a substrate concentration of 10 μM (Kronbach *et al.* 1986), 4-nitrophenol 3-hydroxylase activity was determined at a substrate concentration of 1000 μM (Tassaneeyakul *et al.* 1993) and testosterone 6β-hydroxylase activity was determined at a substrate concentration of 250 μM (Funae and Imaoka 1987).

Analysis of results

All results are presented as mean ± SD. Determination of apparent K_m, V_{max} and IC_{50} were obtained using GraFit version 3 (Leatherbarrow 1992). Correlation and statistical analysis was carried out using Microsoft Excel.

Results

Enzyme kinetics

4-Nitroanisole is readily *O*-demethylated to 4-nitrophenol in human liver microsomes and the reaction rate was linear with time up to 20 min and with protein up to 2 mg/ml (data not shown). The apparent kinetic constants for this conversion were estimated using 4-nitroanisole concentrations up to 1000 μM. The conversion followed Michaelis–Menten kinetics in the four human livers investigated (figure 2). Examination of the Eadie–Hoftsee transformations (inset figure 2) revealed a marked biphasic nature suggesting at least two enzymes are involved in the reaction. The apparent Michaelis-Menten kinetic parameters were estimated with the assumption that two enzymes, by fitting the data to the following equation:

$$v = \frac{V_{max\ 1}.[S]}{K_{m1} + [S]} + \frac{V_{max\ 2}.[S]}{K_{m_2} + [S]},$$

where v is the velocity of formation of 4-nitrophenol, S is the concentration of 4-nitroanisole in the incubation mixture, K_{m_1} and K_{m_2} are the Michaelis constants for the two enzymatic components and $V_{max\ 1}$ and $V_{max\ 2}$ are the respective maximum velocities. The kinetic parameters obtained for the *O*-demethylation in the four human livers investigated are detailed in table 1. The mean apparent K_m for the two components were 2·1 and 220 μM.

Inhibitor studies

The effect of specific cytochrome P450 inhibitors on the rate of *O*-demethylation of 4-nitroanisole was investigated using furafylline (CYP1A2), sulphaphenazole (CYP2C9), quinidine (CYP2D6) and ketoconazole (CYP3A4) as the inhibitors. For isoforms for which there are no identified specific inhibitors, specific substrates were used as competitive inhibitors. These were coumarin (CYP2A6), (*S*)-mephenytoin (CYP2C19) and chlorzoxazone (CYP2E1). The inhibition studies were carried out

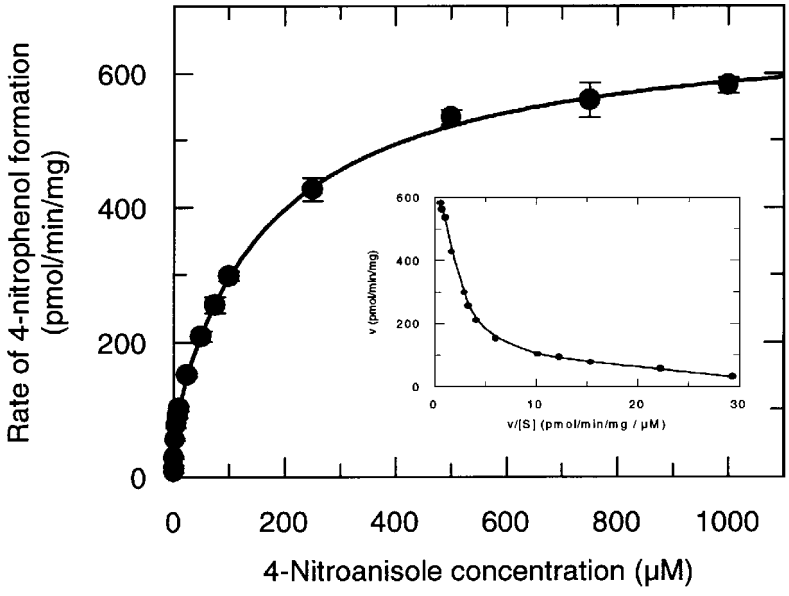

Figure 2. Representative velocity versus substrate concentration and Eadie–Hoftsee plots (inset) for the conversion of 4-nitroanisole to 4-nitrophenol in human liver microsomes. Data illustrated are from HM-14. Each data point represents the mean±SD of triplicate determinations.

Table 1. Michaelis–Menten kinetic parameters for the *O*-demethylation of 4-nitroanisole to 4-nitrophenol obtained using microsomes from four different human livers.

Liver	$V_{max\,1}$ (pmol/min/mg)	K_{m_1} (μM)	$V_{max\,2}$ (pmol/min/mg)	K_{m_2} (μM)
HM-11	359	2·1	776	214
HM-12	474	2·9	606	285
HM-13	138	1·2	397	200
HM-14	85	2·1	592	179
Mean (±SD)	264±159	2·1±0·6	593±134	220±40

using a batch of microsomes made from a combination of equal amounts (~ 50 g of each liver sample) of the same four livers used in the kinetic studies.

The results of this investigation are illustrated in figure 3. Compounds found to inhibit 4-nitroanisole *O*-demethylation by > 10% were coumarin, which inhibited the activity by 15 and 55% at 2·5 and 25 μM respectively, chlorzoxazone which inhibited the activity by 20 and 60% at 50 and 500 μM respectively, and ketoconazole which inhibited the activity by 20 and 70% at 2·5 and 25 μM respectively. Inhibition by coumarin and chlorzoxazone suggests CYP2A6 and CYP2E1 involvement. Further investigations of the effect of coumarin and chlorzoxazone on 4-nitroanisole metabolism at 10 μM produced an IC_{50} for coumarin of 19 and 254 μM for chlorzoxazone (figure 4).

The inhibition by ketoconazole is more difficult to interpret. Previous studies (Baldwin *et al*. 1995) have shown that low ketoconazole concentrations (1 μM) inhibit between 85 and 95% of CYP3A4 activity. However, this study also showed that at 1 μM ketoconazole inhibited CYP2E1, as measured by chlorzoxazone 6-hydroxylase activity, by approximately 30%. Hence it is not clear from this experiment alone

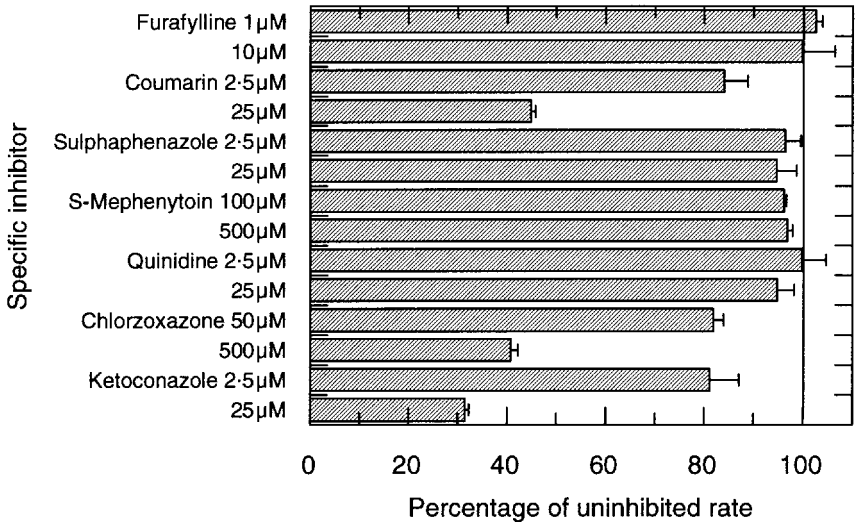

Figure 3. Effect of specific P450 inhibitors/substrates on the rate of *O*-demethylation of 4-nitroanisole (10 μM). Values are mean ± SD of triplicate determinations from microsomes made from a mixture of equal weights (approximately 50 g of each liver sample) of HM-11, HM-12, HM-13 and HM-14. 100% = 353 pmol/min/mg.

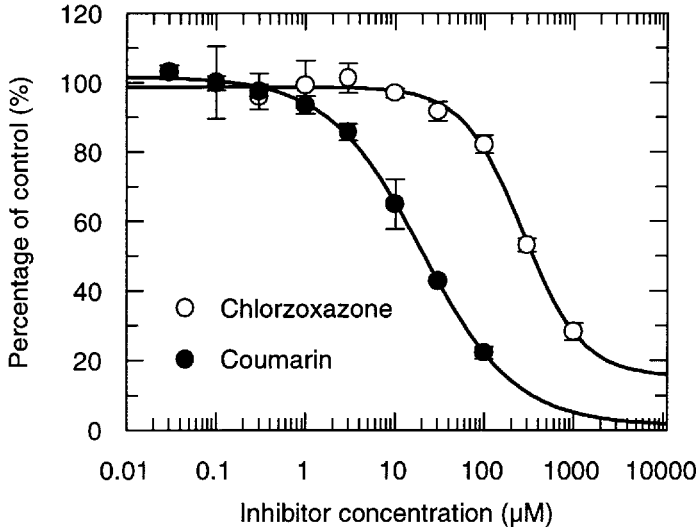

Figure 4. Effect of coumarin and chlorzoxazone on the *O*-demethylation of 4-nitroanisole (10 μM). Values are mean ± SD of triplicate determinations from microsomes made from a mixture of equal weights of HM-11, HM-12, HM-13 and HM-14. 100% = 245 pmol/min/mg.

whether ketoconazole is inhibiting a CYP3A4 or CYP2E1 component of 4-nitroanisole *O*-demethylation.

Correlation studies

The rate of 4-nitroanisole *O*-demethylation was determined in microsomes prepared from 12 individual human livers at a 4-nitroanisole concentration of 1000 μM. The rates of 4-nitrophenol formation observed exhibited a 7-fold range from 393 to 2721 pmol/min/mg. These rates were then correlated against the rates

Table 2. Correlation of the rate of *O*-demethylation of 4-nitroanisole with cytochrome P450 isoform-specific activities in a panel of microsomes produced from 12 individual human livers.

Cytochrome P450	Correlation coefficient (r)
1A2	0·71[a]
2A6	0·86[b]
2C9	0·38
2C19	0·59
2D6	0·20
2E1	0·85[b]
3A4	0·44

[a] $p < 0{\cdot}01$; [b] $p < 0{\cdot}001$. $n = 12$.

of probe substrates for the major human cytochrome P450s determined in these samples. The probe substrates assays used were phenacetin *O*-deethylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), diclofenac 4′-hydroxylase (CYP2C9), (*S*)-mephenytoin 4-hydroxylase (CYP2C19), bufuralol 1′-hydroxylase (CYP2D6), 4-nitrophenol 3-hydroxylase (CYP2E1) and testosterone 6β-hydroxylase (CYP3A4). To avoid liver samples with very low or very high activities giving undue weighting to the correlations all the activities where correlated as their logarithm. The correlation coefficients obtained are detailed in table 2.

Correlations ($p < 0{\cdot}001$) were obtained with CYP2A6 (coumarin 7-hydroxylase) and CYP2E1 (4-nitrophenol 3-hydroxylase activity) activities. A trend ($p < 0{\cdot}01$) was observed with CYP1A2, however there is a cross correlation between phenacetin *O*-deethylase activity and the 4-nitrophenol hydroxylase activity ($r = 0{\cdot}66$) within these liver samples which would explain the suggestion of a CYP1A2 involvement.

To clarify this correlation further, multivariate analysis was used to investigate the dependence of the 4-nitroanisole *O*-demethylase activity on the above three probe isoform activities. Each of the three activities (CYP1A2, CYP2A6 and CYP2E1) were combined in pairs to see if a combination of two activities gave an improvement in the correlation, i.e. an increase in the correlation coefficient when compared with the correlation with the individual activities. The inclusion of the CYP1A2 activity produced no improvement in the correlation coefficient (data not shown). Consequently this CYP1A2 activity was removed from the analysis. However, a combination of CYP2A6 and CYP2E1 produced a correlation which was an improvement on those with the individual activities ($r = 0{\cdot}98$ compared with 0·86 for CYP2A6 alone and 0·85 for CYP2E1 alone). This analysis produced the following equation relating both the CYP2A6 and CYP2E1 activities to the 4-nitroanisole *O*-demethylase activity:

$$\log \text{4-nitroanisole activity} = 0{\cdot}37\ (\pm 0{\cdot}05) \log \text{CYP2A6} + 0{\cdot}64\ (\pm 0{\cdot}11) \log \text{CYP2E1} + 2{\cdot}0\ (\pm 0{\cdot}20),$$

where $r = 0{\cdot}98$, standard error = 0·06 and $F = 85$.

This correlation is illustrated in figure 5 as the experimentally determined rate of 4-nitrophenol formation plotted against the rate calculated from this equation.

Metabolism by expressed recombinant cytochrome P450s

The *O*-demethylation of 4-nitroanisole to 4-nitrophenol was investigated in microsomes derived from human B-lymphoblastoid cells expressing recombinant CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (figure 6) at two 4-nitroanisole concentrations (10 and 1000 μM).

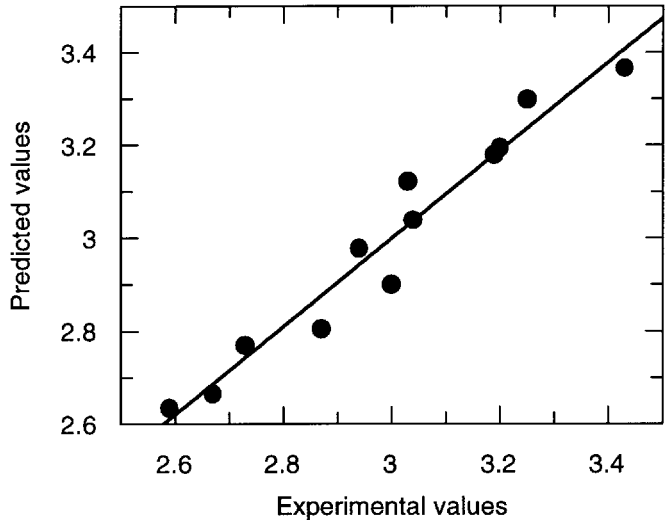

Figure 5. Plot of experimentally determined values for the rate of 4-nitroanisole *O*-demethylation against values obtained using multivariate analysis to produce an equation relating the logarithm of the rate of 4-nitroanisole *O*-demethylation to the logarithm of the enzymatic activities of CYP2A6 and 2E1 in a panel of 12 human livers. 4-Nitroanisole concentration was 1000 μM and each point represents the mean ± SD of triplicate determinations.

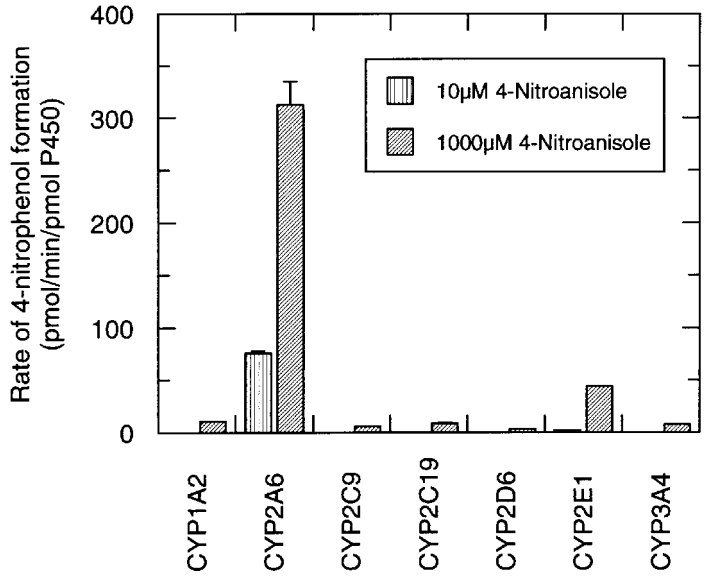

Figure 6. Rate of *O*-demethylation of 4-nitroanisole in a panel of microsomes derived from B-lymphoblastiod cells expressing either CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4 or no cytochrome P450 (control). Two 4-nitranisole concentrations were used, 10 and 100 μM and each assay was carried out in triplicate with the results expressed as mean ± SD.

At the low 4-nitroanisole concentration (10 μM) only CYP2A6 and CYP2E1 formed 4-nitrophenol. At the higher substrate concentration (1000 μM) all of the cytochrome P450s formed 4-nitrophenol to some degree including the control cell line which expresses low levels of CYP1A1. Again CYP2A6 and CYP2E1 had much

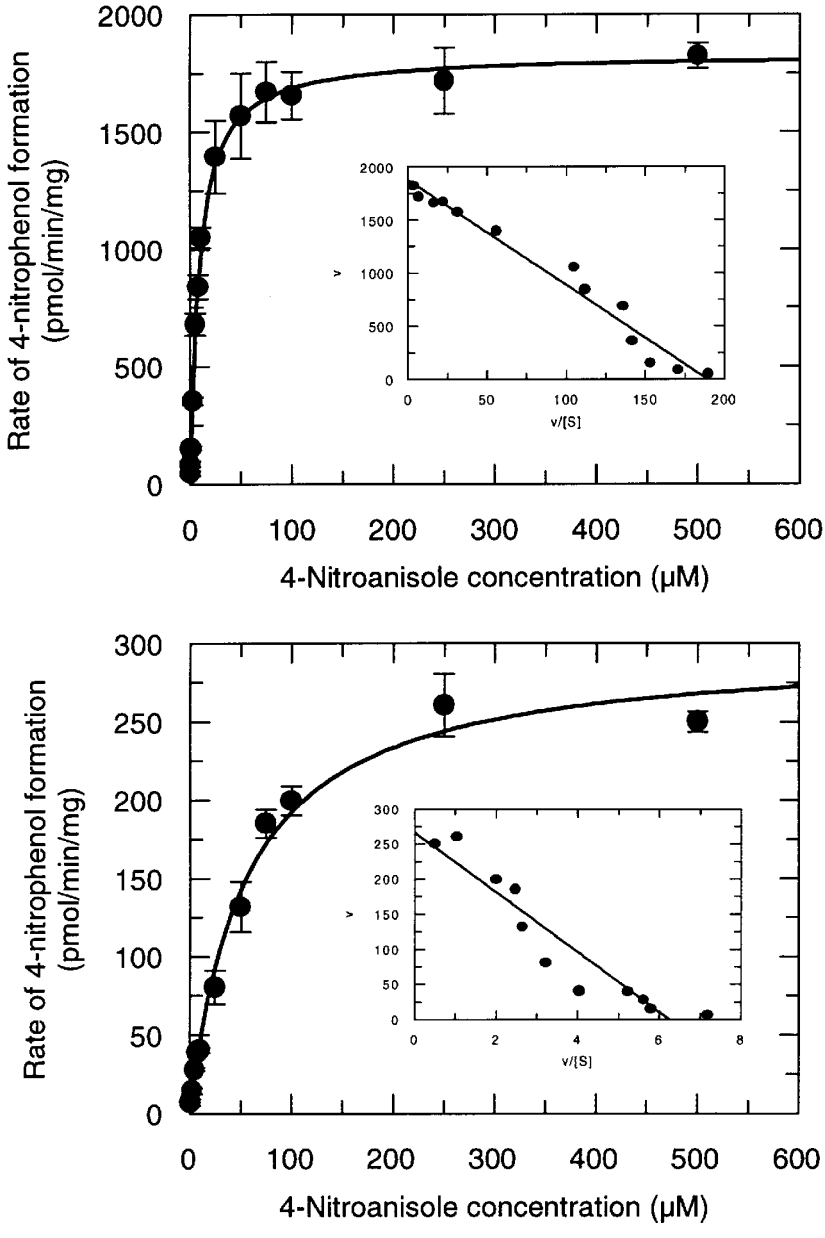

Figure 7. (a) Velocity versus substrate concentration and Eadie–Hoftsee plots (inset) for the conversion of 4-nitroanisole to 4-nitrophenol in microsomes derived from B-lymphoblastiod cells expressing CYP2A6. Each data point represents the mean ± SD of triplicate determinations. (b) Velocity versus substrate concentration and Eadie–Hoftsee plots (inset) for the conversion of 4-nitroanisole to 4-nitrophenol in microsomes derived from B-lymphoblastiod cells expressing CYP2E1. Each data point represents the mean ± SD of triplicate determinations.

higher rates of formation. The rate of formation by CYP2A6 was approximately 30-fold higher than the other cell lines and CYP2E1 was 4-fold higher.

The combination of experiments performed above indicates CYP2A6 and CYP2E1 as the dominant isoforms in 4-nitroanisole metabolism. Consequently the formation kinetics of 4-nitrophenol from 4-nitroanisole were determined for both

these isoforms. Under conditions of time and protein linearity both isoforms produced Michaelis–Menten saturation curves with increasing substrate concentration. These plots are shown in figure 7a for CYP2A6 and figure 7b for CYP2E1. Transformation into Eadie–Hoftsee plots suggested that a single isoform was mediating this metabolism in each case. The apparent K_m for CYP2A6 was 9 μM and for CYP2E1 it was 54 μM. This is in keeping with the low K_m isoform mediating the *O*-demethylation of 4-nitroanisole in human liver being CYP2A6 and the high K_m isoform being CYP2E1.

Discussion

4-Nitroanisole is *O*-demethylated to 4-nitrophenol by human liver microsomes and is mediated by at least two cytochrome P450 isoforms. The mean kinetic parameters determined for this reaction in four human livers are $K_{m_1} = 2{\cdot}1 \pm 0{\cdot}6$, $K_{m_2} = 220 \pm 40$ μM, $V_{max_1} = 264 \pm 159$ and $V_{max_2} = 593 \pm 134$ pmol 4-nitrophenol formed/min/mg. Further studies using a combination of chemical inhibition, correlation analysis and metabolism by expressed recombinant cytochrome P450s identified the low K_m isoform as CYP2A6 and the high K_m isoform as CYP2E1. Hence the conclusion from this study is that 4-nitroanisole is not a general substrate in human liver microsomes, but is metabolized mainly by CYP2A6 and CYP2E1. These two isoforms are present in human liver in approximately equal amounts (Shimada *et al*. 1994). It is possible to rationalize 4-nitroanisole as a substrate for both CYP2A6 and CYP2E1 using available protein models of these isoforms.

There are now a number of substrates identified as being metabolized by CYP2A6. The most widely investigated is coumarin which is specifically oxidized at the 7 position by this isoform and is often used as a probe. The majority of the substrates identified for CYP2A6 are small molecules such as methyl tert-butyl ether (Jun *et al*. 1997), nicotine (Nakajima *et al*. 1996) and quinoline (Reigh *et al*. 1996). Studies with purified CYP2A6 have shown it to be involved in the metabolism of relatively large molecules such as aflatoxin B1 although it only plays a minor role in this case (Yun *et al*. 1991). 4-Nitroanisole fits into the small molecule substrate class.

Very little has been described with respect to the substrate specificity of CYP2A6. However, protein models of the CYP2A family have been produced using the sequence of CYP102 ($P450_{bm3}$) as a template (Lewis and Lake 1995). Using the CYP2A6 model two key binding interactions were identified for coumarin. These interactions are a hydrogen bond between the carbonyl group of coumarin and the threonine at residue 184, and a π–π interaction with the phenylalanine at residue 181. The K_m for the CYP2A6-mediated 7-hydroxylation of coumarin is approximately 1 μM (Pearce *et al*. 1992). This is very similar to the K_m for CYP2A6-mediated *O*-demethylation of 4-nitroanisole. If the affinity is representative of the binding interactions of the molecule it is reasonable to speculate that both substrates undergo similar interactions with the enzyme. Under these conditions it is possible to envisage that π–π interaction with the phenylalanine residue would be similar for both molecules, but that 4-nitroanisole would form the hydrogen bond via the nitro group. This would allow the orientation of the methoxy group towards the haem which would facilitate oxidation of the methyl carbon atom and lead directly to *O*-demethylation (figure 8).

CYP2E1 was also identified as being involved in the *O*-demethylation of 4-nitroanisole. As with CYP2A6, CYP2E1 is associated with the metabolism of

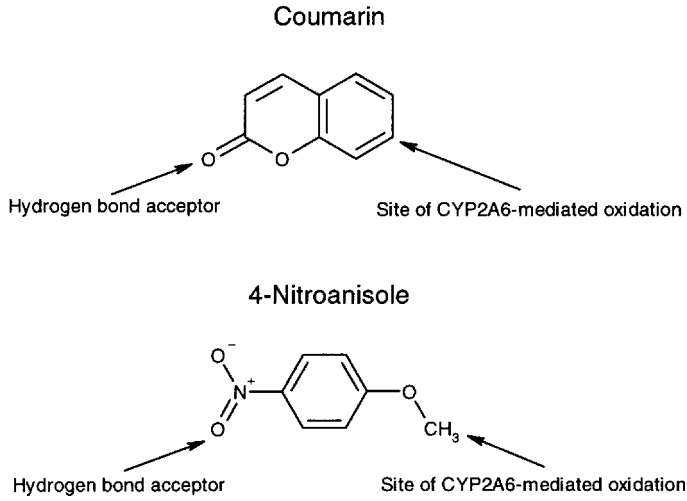

Figure 8. Suggested site of hydrogen bond interaction between coumarin and CYP2A6 which would yield the 7-hydroxy metabolite and how 4-nitroanisole could adopt a similar orientation to produce 4-nitrophenol.

small molecules such as acetone, hexane and benzene (Koop 1992). Unlike CYP2A6, CYP2E1 is also responsible for the metabolism of drug substrates including acetaminophen, zoxazolamine and gaseous anaesthetics (halothane and enflurane). CYP2E1 has also been shown to metabolize endogenous molecules such as fatty acids (Amet *et al*. 1995). This latter metabolic activity is similar to the endogenous activity of CYP102 and hence has been used to support the assumption that the active sites of these enzymes are similar. The crystal structure of CYP102 has been used as the template for a model of CYP2E1 (Tan *et al*. 1997). The model produced suggests that the active site of CYP2E1 is small and populated with hydrophobic residues. Hence interactions with substrates are via relatively non-specific hydrophobic interactions with the primary determinant of the substrate–structure activity relationship being molecular size. In the absence of any specific binding interactions such as ion-pairs or hydrogen bonds, therefore, CYP2E1 binds its substrates largely via hydrophobic interactions with these residues. This is supported by inhibition studies with CYP2E1 and a series heterocytes capable of hydrogen bond, ion pair and hydrophobic interactions (Hargreaves *et al*. 1994). Only molecules such as pyridine, which have aromatic nitrogen atoms capable of forming a ligand interaction directly with the haem, produced high affinity ($< 25\ \mu M$) inhibition of CYP2E1 activity. The hydrophobicity of 4-nitroanisole and suitability as a substrate is indicated by its log $P_{octanol}$ = 2·1. This is similar to the 1·85 for the CYP2E1 probe substrate 4-nitrophenol.

Comparison of the V_{max} for 4-nitroanisole *O*-demethylation in rat microsomes versus human microsomes shows a 7-fold increase in V_{max} in human microsomes (0·18 nmol/min/mg in rat versus 1·31 in human microsomes; Souhaili-el Amri *et al*. 1986). This difference can be explained by consideration of the properties of the different members of the CYP2A family in these species. Whilst rats do metabolize coumarin, 7-hydroxylation represents only 0·3% of the total metabolism compared with 84% in human liver microsomes (Pearce *et al*. 1992). The rat orthologues of CYP2A6: CYP2A1 and CYP2A2 catalyse the 7α and 15α-hydroxylation of

testosterone but have no coumarin 7-hydroxylase activity (Pearce *et al*. 1992). Assuming that the same active site interactions, suggested above, are required for 4-nitroanisole as for coumarin then it is unlikely that either CYP2A1 or CYP2A2 will *O*-demethylate 4-nitroanisole. Hence the difference in the rate of 4-nitroanisole *O*-demethylation between rat and human microsomes may arise because of a difference in substrate specificity between different members of the CYP2A family. Consequently, 4-nitroanisole, as a probe substrate, should be used with caution when comparing metabolism across species since in the rat, the *O*-demethylation will be mediated by largely CYP2E1 whilst in man CYP2A6 is the major enzyme involved.

In conclusion, the *O*-demethylation of 4-nitroanisole is mediated by both CYP2A6 and CYP2E1 in human liver microsomes. The involvement of both isoforms in the *O*-demethylation can be rationalized in terms of their substrate–structure activity relationships. Caution should therefore be placed on interpreting data on the rate of metabolism of 4-nitroanisole as related to the whole cytochrome P450 system since it is likely to relate to specific or selective changes in the amounts of these two isoforms.

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