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VALIDATION OF 4-NITROPHENOL AS AN *IN VITRO* SUBSTRATE PROBE FOR HUMAN LIVER CYP2E1 USING cDNA EXPRESSION AND MICROSOMAL KINETIC TECHNIQUES

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Abstract—The involvement of human cytochrome P450 (CYP) 2E1 in the hydroxylation of 4-nitrophenol (4NP) to 4-nitrocatechol (4NC) has been investigated using cDNA expression and liver microsomal kinetic and inhibitor techniques. 4NP hydroxylation by human liver microsomes and cDNA-expressed human CYP2E1 exhibited Michaelis–Menten kinetics; the respective apparent K_m values were 30 ± 7 and 21 μM. Mutual competitive inhibition was observed for 4NP and chlorzoxazone (CZ) (an alternative human CYP2E1 substrate) in liver microsomes, with close similarities between the calculated apparent K_m and K_i values for each individual compound. 4NP and CZ hydroxylase activities in microsomes from 18 liver donors varied to a similar extent (3.3- and 3.0-fold, respectively) and 4NP hydroxylase activity correlated significantly ($r_s \geq 0.75$, $P < 0.005$) with both CZ hydroxylation and immunoreactive CYP2E1 content. The prototypic CYP2E1 inhibitor, diethyldithiocarbamate, was a potent inhibitor of 4NC formation and decreased 4NP hydroxylation by cDNA-expressed CYP2E1 and human liver microsomes in parallel. Probes for other human CYP isoforms namely (α-naphthoflavone, coumarin, sulphaphenazole, quinidine, troleandomycin and mephenytoin) caused <15% inhibition of liver microsomal 4NP hydroxylation. These data confirm that, as in animal species, 4NP hydroxylation is catalysed largely by CYP2E1 in human liver and 4NP may therefore be used as an *in vitro* substrate probe for the human enzyme.

Cytochromes P450 (CYP‡) comprise a superfamily of enzymes (isoforms) responsible for the oxidative metabolism of a structurally diverse range of drugs, dietary chemicals, environmental pollutants, carcinogens and endogenous compounds. It is now well established that the various CYP isoforms exhibit distinct, but frequently overlapping, patterns of substrate specificities and tend to differ in terms of regulation [1]. Although CYP-mediated oxidation normally results in the formation of products with diminished biological activity, a number of CYP isoforms are known to have an important role in the bioactivation of numerous chemical carcinogens and toxins [2–3].

The ethanol-inducible isoform, CYP2E1, contributes to the metabolism of a range of aliphatic alcohols, ethers, halides and nitriles, and certain nitrosamines and aromatic compounds [3, 4]. Of particular interest is the ability of CYP2E1 to catalyse the biotransformation and DNA adduct formation of potential human carcinogens such as aniline, *N*-nitrosodimethylamine, urethane and vinyl chloride [3–5]. Additionally, CYP2E1 may play an important role in the metabolic activation of the hepatotoxins carbon tetrachloride, chloroform, enflurane, halothane and paracetamol [6–10].

Given the important role of CYP2E1 in xenobiotic metabolism and toxicity, there has been considerable interest in the development of model substrate and inhibitor probes for this enzyme. The availability of such probes is essential for the further investigation of CYP2E1 regulation and substrate specificity [11]. Among the compounds apparently metabolized by CYP2E1, 4-nitrophenol (4NP) has attracted attention as a CYP2E1 substrate probe. The microsomal hydroxylation of 4NP to form 4-nitrocatechol (4NC) is known to be highly inducible by ethanol in the rat [12], indicative of the involvement of CYP2E1 in 4NP metabolism in this species. A role for CYP2E1 in 4NP hydroxylation in the rabbit was further demonstrated using purified rabbit liver CYP2E1 and an antibody raised against this enzyme [13]. While the predominant contribution of CYP2E1 to 4NP hydroxylation in laboratory animals is now generally accepted [14–20], a role for the human isoform cannot be assumed automatically given the interspecies differences sometimes apparent in CYP2E1 activities [21].

In the present study, cDNA expression and microsomal kinetic and inhibitor techniques have been utilized to demonstrate the specificity of 4NP as a human hepatic CYP2E1 substrate. Given the ready availability of 4NP and its hydroxylated metabolite (4NC) and the ease, specificity and

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‡ Abbreviations: CYP, cytochrome P450; CZ, chlorzoxazone; 4NC, 4-nitrocatechol; 4NP, 4-nitrophenol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate.

sensitivity by which 4NP hydroxylation may be measured in human liver microsomes [22], 4NP constitutes an ideal model *in vitro* substrate probe for human hepatic CYP2E1.

MATERIALS AND METHODS

Chemicals and reagents. 4NP, 4NC, salicylamide, chlorzoxazone (CZ), diethyldithiocarbamate, coumarin, troleandomycin, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and α-naphthoflavone from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 5-Fluoro-2(3*H*)-benzoxazolone and 6-hydroxychlorzoxazone were kindly provided by Dr R. Peter, University of Erlangen-Nurnburg (Erlangen, Germany). Other drugs were obtained from the following sources: mephenytoin from Sandoz Ltd (Basle, Switzerland), quinidine sulphate from Burroughs Wellcome (Sydney, Australia), sulphaphenazole from Ciba-Geigy (Sydney, Australia). The QIA *Escherichia coli* protein expression kit was obtained from QIAGEN Inc. (Chatsworth, CA, U.S.A.). Cell culture and molecular biology reagents were purchased mainly from Becton Dickinson Labware (Bedford, MA, U.S.A.), New England Biolabs Inc. (Beverley, MA, U.S.A.), Promega Corp. (Madison, WI, U.S.A.) and Difco Laboratories (Detroit, MI, U.S.A.). All other chemicals and solvents were of analytical reagent grade.

Preparation of microsomes. Microsomes were prepared from human liver obtained from renal transplant donors as described by Robson *et al.* [23]; approval of the Flinders Medical Centre Ethical Review Committee was obtained to use these livers for xenobiotic metabolism studies. Details of the donors of livers used (namely H5–H15 and H17–H23) have been described previously [24]. Microsomal pellets were suspended in 0.1 M phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, aliquoted and stored at −70° until used. Protein content was determined by the method of Lowry *et al.* [25] with bovine serum albumin as the standard. Since glycerol may inhibit CYP2E1-catalysed reactions at concentrations above 1.0% (v/v) [22], the final concentration of glycerol was kept to less than 1% (v/v) in all incubations.

PCR amplification of CYP2E1 *cDNA.* A full-length human *CYP2E1* cDNA was isolated from a human liver λgt11 cDNA library using the polymerase chain reaction (PCR) [26]. The forward and reverse oligonucleotide primers used were 5′-CAGAGATCTATGTCTGCCCTCGGAGTGAC-C-3′ and 5′-GTCAGATCTACACTCATGAGC-GGGGAATGA-3′, respectively. These primers, designed from the published nucleotide sequence of CYP2E1 [27], were complementary to the 5′ and 3′ flanking regions of the *CYP2E1* cDNA. Additionally, *Bgl* 11 sites were included at their 5′ termini to aid subcloning of the PCR product. PCR reactions contained DNA template (human liver cDNA library, 100 ng), 50 pmol of each primer, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, *Taq* DNA polymerase (1 U) and 1 × PCR reaction buffer (Bresatec, Adelaide, Australia) in a total volume of 100 μL. PCR reactions were performed using a Perkin–Elmer Cetus Thermocycler using an initial denaturation temperature of 94° for 4 min, followed by 94° × 45 sec; 60° × 60 sec; 72° × 90 sec for 10 cycles followed by 94° × 45 sec; 60° × 60 sec; and 72° × 60 sec for 25 cycles. A temperature of 72° was maintained for 20 min following completion of the 35 cycles. An aliquot (5 μL) from each of the PCR reactions was subjected to electrophoresis on a 1% agarose gel and an ≈1.5 kb fragment, which specified the full length *CYP2E1* cDNA, was visualized following ethidium bromide staining and UV transillumination. The ≈1.5 kb PCR product was purified from a 1% low melting point agarose gel, digested with *Bgl* II and ligated into the *Bam* HI site of the pBluescript II (SK+) plasmid (Stratagene, La Jolla, CA, U.S.A.) and transformed into competent XL-1 Blue *E. coli* cells (Stratgene). To determine the authenticity of the PCR product, complete sequence analysis was carried out by the dideoxy method using T7 DNA polymerase and the Erase-A-Base procedure according to the protocol of the suppliers (Promega, Madison, WI, U.S.A.). The nucleotide sequence of the PCR product was identical to that of the human CYP2E1 cDNA published by Song *et al.* [27].

CYP2E1 *expression in COS-7 cells.* The human *CYP2E1* cDNA was ligated into the pCMV4 mammalian expression vector [28] at the *Bgl* II site and then transfected in COS-7 cells [29]. Cells were harvested 48 hr post-transfection and stored at −70° until used. Cells transfected with pCMV4 carrying the *CYP2E1* cDNA in the reverse orientation (3′ → 5′ with respect to the promoter element), served as the negative controls for incubations of expressed CYP2E1.

Preparation of antibody against bacterially expressed CYP2E1. A *Bam* HI/*Hind* III fragment (nucleotides 449–1649 of the human *CYP2E1* cDNA [27]) was excised from the pBluescript/*CYP2E1* construct, ligated into the *Bam* HI/*Hind* III digested pQE11 *E. coli* expression vector, and transformed into *E. coli* strain SG13009 (Qiagen) containing the *lac* repressor-producing plasmid pREP4. Briefly, a 10 mL overnight culture of *E. coli* harbouring the CYP2E1 expression plasmid was added to 500 mL LB broth containing 100 μg/mL ampicillin and 25 μg/mL kanamycin at 37° and grown to a cell density of OD_{600} 0.7–0.9. Induction of protein expression was initiated by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside and the cells were shaken at 37° for a further 3 hr. The recombinant CYP2E1 protein was then purified from the pelleted cells, using denaturing conditions on a 2 mL Ni-nitrilotriacetate column according to the manufacturer's protocol (Qiagen). The CYP2E1 fragment was essentially pure, migrating as a single band (M_r ≈40,000) on SDS–PAGE (10% acrylamide). The purified protein was used as antigen to produce anti-recombinant human CYP2E1 antibody in a goat as described previously [30]. The purified IgG fraction recognised a single human liver microsomal protein band (M_r ≈54,000) as well as the recombinant human CYP2E1 protein fragment.

Assay for 4NP hydroxylation. The conversion of

4NP to 4NC by human liver microsomes or cDNA-expressed CYP2E1 was determined as described previously [22]. Briefly, standard 0.5 mL incubations contained human liver microsomes or COS cell lysate (0.2 mg protein) in phosphate buffer (0.1 M, pH 6.8), ascorbic acid (1 mM), NADPH generating system (consisting of 1 mM NADP, 10 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogerase and 5 mM $MgCl_2$), and 4NP (2.5–200 μM). Reactions were initiated by addition of the NADPH generating system and carried out in air at 37° for 30 min. Reactions were terminated by the addition of 0.25 mL of 0.6 M perchloric acid and the assay internal standard (salicylamide) was added. The mixture was saturated with ammonium sulphate (0.5 g) and extracted with diethyl ether (4 mL). 4NC in the dried extract was quantitated by high performance liquid chromatography (HPLC).

Assay for CZ 6-hydroxylation. The assay conditions were primarily as described [31], with some modifications. Incubations (0.5 mL) contained human liver microsomes or COS cell protein (0.2 mg) in phosphate buffer (0.1 M, pH 7.4), NADPH generating system (see above) and CZ (10–800 μM). After 15 min reactions were terminated by the addition of 43% (w/v) phosphoric acid (50 μL) and the internal standard. 5-fluoro-2(3*H*)-benzoxazolone (2 μg/50 μL), was added. The mixture was then saturated with 0.5 g ammonium sulphate and extracted with 3 mL of dichloromethane/isopropanol (85:15, v/v) by vortex mixing for 1 min. The organic layer was separated by centrifugation (3000 *g* for 15 min) and evaporated to dryness under nitrogen. Residues were dissolved in 120 μL of a acetonitrile/water (4:6, v/v) and 20 μL aliquots were used for HPLC analysis. The chromatograph was fitted with a Spherisorb S5 Octyl column (15 cm × 4.6 mm i.d., ICI Instruments, Melbourne, Australia), which was eluted with acetonitrile–0.5% phosphoric acid (26:74, pH 3.0) at a flow rate of 1.8 mL/min. Quantitation was achieved using a UV-VIS detector operating at 287 nm. Calibration curves were constructed using authentic 6-hydroxychlorzoxazone.

Kinetic and inhibitor studies. 4NP hydroxylation and CZ 6-hydroxylation kinetics by human liver microsomes and cDNA-expressed CYP2E1 were determined over the concentration ranges 2.5–200 μM and 10–800 μM, respectively. Inhibitory effects of xenobiotics on human liver microsomal 4NP hydroxylation were determined at substrate (4NP) concentrations of 50 and 250 μM; the xenobiotics screened and their added concentrations in incubations are given in Figs 4 and 5. With the exception of α-naphthoflavone and troleandomycin, which were dissolved in 50% (v/v) methanol, xenobiotics were added to incubations as aqueous solutions; the presence of 0.5% (v/v) methanol in incubations had a minimal effect (≤15% inhibition) on 4NP hydroxylation. For the 4NP hydroxylation and CZ 6-hydroxylation correlation summarized in Fig. 3, the respective concentrations were 200 μM (4NP) and 400 μM (CZ).

Immunoblot analysis. Human liver microsomal proteins were separated by SDS–PAGE on gels containing 10% (w/v) acrylamide according to the method of Laemmli [32]. Following SDS–PAGE, proteins were electroblotted onto nitrocellulose [33]. After blocking non-specific binding sites, the nitrocellulose sheet was incubated for 1 hr at 37° with goat anti-recombinant human CYP2E1 IgG (1/5000). Blots were washed three times with phosphate-buffered saline containing Tween 20 (0.05%, v/v) and then incubated for 1 hr at room temperature in phosphate-buffered saline, 3% (w/v) bovine serum albumin and 5% (v/v) horse serum with 1/1250 donkey anti-sheep IgG conjugated to horseradish peroxidase. The antigenic components were visualised with 0.05% (w/v) diaminobenzidine in 20 mM imidazole buffer (pH 7.0) with 0.015% (w/v) hydrogen peroxide. Quantitation of immunoreactive CYP2E1 in immunoblots of microsomal proteins was accomplished by laser densitometry (LKB Ultrascan-XL, Bromma, Sweden).

Analysis of results. All results are presented as means ± SD. Initial estimates of apparent K_m and V_{max} values were obtained by linear regression of Eadie–Hofstee plots [34]. These values were then used as the first estimates for MKMODEL, an extended least squares modelling program [35].

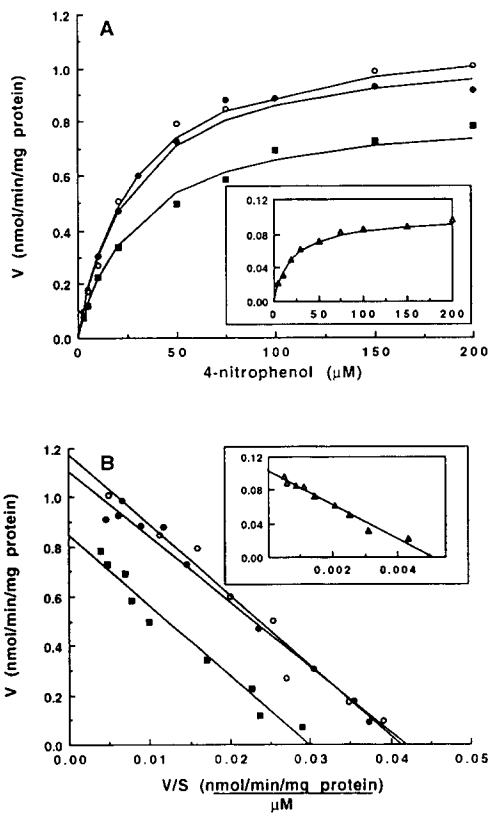

Fig. 1. Velocity versus substrate–concentration plots (panel A) and Eadie–Hofstee plots (panel B) for the conversion of 4NP to 4NC. Data are presented for microsomes from livers H7 (■), H10 (●), H15 (○) and for cDNA-expressed CYP2E1 (inset, △). Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

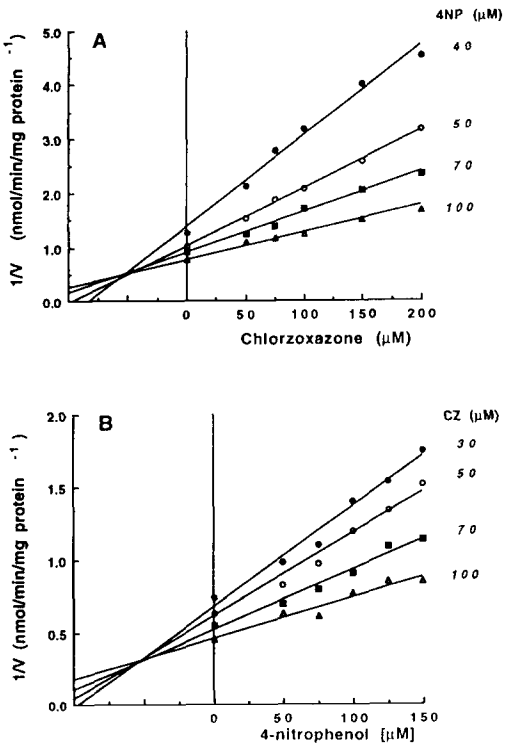

Fig. 2. Representative Dixon plot for the inhibition of 4NP hydroxylase activity by CZ (panel A) and for the inhibition of CZ 6-hydroxylase activity by 4NP (panel B). Microsomes were from the liver H8. Substrate concentrations used are shown on the right of the plots.

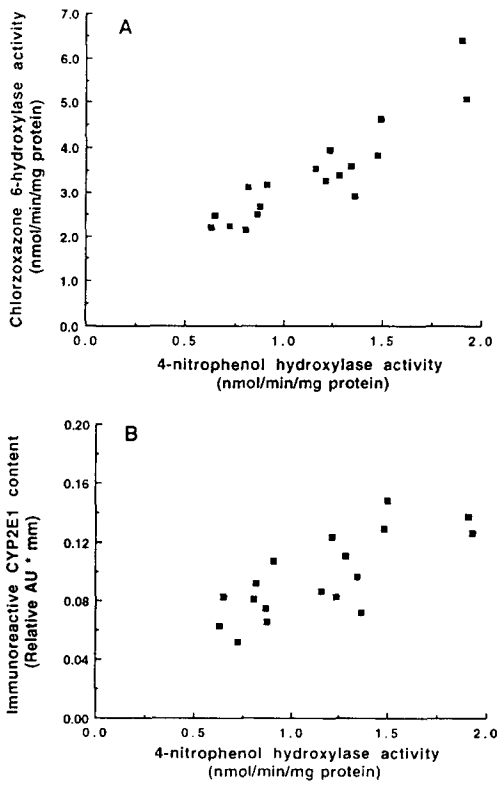

Fig. 3. Correlation of 4NP hydroxylase and CZ 6-hydroxylase activities (panel A) and 4NP hydroxylase and immunoreactive CYP2E1 content (panel B) in microsomes from 18 human livers. 4NP and CZ concentrations were 200 and 400 μM, respectively.

Apparent K_i values were calculated according to Dixon [36]. Correlations between human liver microsomal 4NP hydroxylation and CZ 6-hydroxylation or immunoreactive CYP2E1 content were determined using Spearman's rank method.

RESULTS

The conversion of 4NP to 4NC by both human liver microsomes and cDNA-expressed CYP2E1 followed Michaelis–Menten kinetics (Fig. 1). Apparent K_m values for human liver microsomes and CYP2E1-catalyzed 4NP hydroxylation were 30 ± 7 μM (N = 3 livers) and 21 μM, respectively. The mean V_{max} for human liver microsomal 4NP hydroxylation was 0.99 ± 0.22 nmol/min/mg.

Mutual competitive inhibition was observed with 4NP and CZ, an alternate CYP2E1 substrate (Fig. 2). The mean apparent K_i value for 4NP inhibition of CZ 6-hydroxylation was 42 ± 19 μM, which is similar to the mean apparent K_m for 4NP hydroxylation in the three livers studied (see above). The mean apparent K_i value for CZ inhibition of 4NP hydroxylation was 47 ± 10 μM. Again, this value is close to the mean apparent K_m (namely 56 ± 6 μM) determined for CZ 6-hydroxylation in the same livers studied, and the apparent K_m for CZ 6-hydroxylation by cDNA-expressed CYP2E1 (namely 69 μM) (data not shown).

4NP hydroxylase and CZ 6-hydroxylase activities were compared using liver microsomes from 18 donors. The 4NP and CZ hydroxylase activities of the 18 livers varied to a similar extent (3.3-fold and 3.0-fold, respectively) and were significantly correlated ($r_s = 0.88$, $P < 0.001$; Fig. 3A). The immunoreactive CYP2E1 content, determined in the same set of microsomes, correlated significantly with both the 4NP hydroxylase ($r_s = 0.75$, $P < 0.005$; Fig. 3B) and CZ 6-hydroxylase ($r_s = 0.80$, $P < 0.001$; data not shown) activities.

Microsomal 4NP hydroxylase and CZ 6-hydroxylase activities were decreased in parallel by increasing concentrations (0.01–1000 μM) of the protypic CYP2E1 inhibitor diethyldithiocarbamate (Fig. 4A). The profile of diethyldithiocarbamate inhibition of the two reactions catalysed by expressed CYP2E1 was essentially identical to that observed in human liver microsomes (Fig. 4B). IC_{50} values for diethyldithiocarbamate inhibition of the 4NP and CZ hydroxylations were within the range 2–3 μM for both enzyme sources.

With the exception of diethyldithiocarbamate, a range of xenobiotics characterized previously as human CYP isoform-specified substrate and/or inhibitor probes were shown to have little inhibitory effect (<15%) on human liver microsomal 4NP

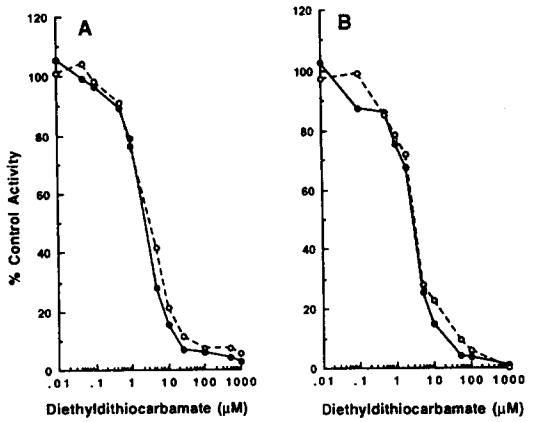

Fig. 4. Inhibitory effect of diethyldithiocarbamate on 4NP hydroxylation (●) and CZ 6-hydroxylation (○) by human liver microsomes (liver H10) (panel A) and cDNA-expressed CYP2E1 (panel B). The concentration of 4NP and CZ was 50 μM, the approximate apparent K_m for both substrates.

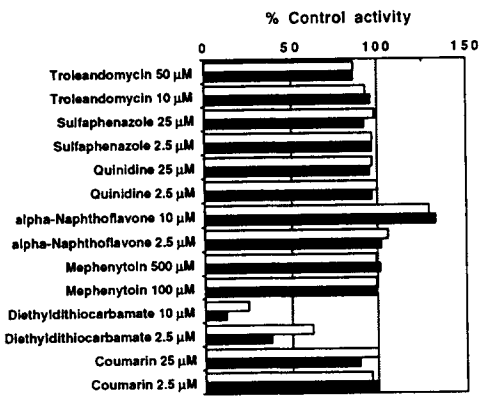

Fig. 5. Effects of various xenobiotics on human microsomal 4NP hydroxylase activity. 4NP concentrations were 50 μM (□) and 250 μM (■); the control activities were 0.92 and 1.29 nmol/min/mg protein, respectively. Concentrations of inhibitors were as shown.

hydroxylase activity. Compounds shown to inhibit 4NP hydroxylation <15% were: α-naphthoflavone (CYP1A), coumarin (CYP2A6), sulphaphenazole (CYP2C9/10), quinidine (CYP2D6), troleandomycin (CYP3A) and mephenytoin (*S*-mephenytoin hydroxylase) (Fig. 5). Consistent with this inhibitory profile, 4NP hydroxylase activities in microsomes from 18 livers (see above) were found not to correlate significantly with the high affinity caffeine 3-demethylase (CYP1A2), tolbutamide hydroxylase (CYP2C9/10) and benzo[*a*]pyrene hydroxylase (CYP3A) activities determined previously for these livers [37–39]. At an added concentration of 10 μM, α-naphthoflavone caused slight activation (*ca.* 25%) of 4NP hydroxylation (Fig. 5). Maximal activation of microsomal 4NP hydroxylation, about 60%, was further shown to occur at an α-naphthoflavone concentration of 25 μM, but at higher α-naphthoflavone concentrations 4NC formation declined back towards baseline activity (data not shown). α-Naphthoflavone concentrations ≥15 μM were further demonstrated to activate microsomal CZ 6-hydroxylation by approximately 30% but, in contrast to 4NP hydroxylation, this effect plateaued rather than declined with increasing concentration.

DISCUSSION

The data presented here strongly suggest that the human liver micosomal hydroxylation of 4NP (to form 4NC) is catalysed predominantly by CYP2E1. Thus, it has been demonstrated that: (i) apparent K_m values for 4NP hydroxylation by human liver microsomes and cDNA-expressed human CYP2E1 were similar; (ii) mutual competitive inhibition occurred for 4NP and CZ, an alternative CYP2E1 substrate (see below), with close similarities between the apparent K_m and K_i values for the individual compounds; (iii) 4NP hydroxylase activities in liver microsomes from 18 donors correlated significantly with CZ 6-hydroxylation and immunoreactive CYP2E1 content; (iv) 4NP hydroxylations by human liver microsomes and cDNA-expressed CYP2E1 were inhibited in parallel by low concentrations of the prototypic CYP2E1 inhibitor diethyldithiocarbamate; and (v) microsomal 4NP hydroxylation was largely unaffected by a range of inhibitors and/or substrates for isoforms other than CYP2E1.

Based on the use of hepatic microsomes from animals treated with known CYP2E1 inducers (e.g. acetone, ethanol, imidazole, pyrazole), reconstituted CYP2E1 protein or anti-CYP2E1 antibodies, a number of previous studies have a demonstrated a role for CYP2E1 in 4NP hydroxylation in rabbit [13], rat [12], hamster [14] and chicken [15]. 4NP has recently been used as a substrate for human CYP2E1 [40, 41], but until now this extrapolation has not been validated. The apparent specificity of 4NP for CYP2E1 in all species investigated contrasts with the interspecies variability reported for some CYP2E1-catalysed reductions [21].

Apart from 4NP, a number of compounds have been investigated as potential CYP2E1 substrate probes. These include acetone [42, 43], alcohols (e.g. butanol, ethanol) [44], aniline [45], benzene [46–48], *N*-nitrosodimethylamine [4, 49, 50], paracetamol [10, 51] and CZ [30]. Acetone, alcohols and benzene are volatile and this creates technical difficulties when they are used as substrates in microsomal incubations [52]. The oxidation of both aniline and paracetamol is also carried out by isoforms other than CYP2E1 [51, 53]. *N*-Nitrosodimethylamine is a known carcinogen and because only the high affinity component of *N*-nitrosodimethylamine demethylase is mediated by CYP2E1, the assay for this activity necessitates the use of a radiolabelled substrate [54]. Like 4NP hydroxylation, the 6-hydroxylation of CZ has been reported to be mediated only by CYP2E1 in human liver [30]. Further evidence presented here supports

the specificity of CZ as a CYP2E1 substrate. HPLC assays available for both the 4NP and CZ hydroxylase activities are straightforward and provide good sensitivity, precision and specificity [22, 30]. The specificity of 4NP and CZ for CYP2E1 and the availability of convenient, validated assays makes these two compounds ideal *in vitro* substrate probes for human hepatic CYP2E1. The product of 4NP hydroxylation (4NC) is, however, more readily available than the 6-hydroxylated metabolite of CZ.

Previous studies have demonstrated that diethyldithiocarbamate is a potent mechanism-based inhibitor of CYP2E1 [5]. In the present work the 4NP and CZ hydroxylations were inhibited in parallel by diethyldithiocarbamate. Greater than 80% inhibition of the two hydroxylations was observed at a diethyldithiocarbamate concentration of 10 μM. The prototypic CYP isoform substrate and/or inhibitor probes α-naphthoflavone (CYP1A), coumarin (CYP2A6), sulphaphenazole (CYP2C9/10), quinidine (CYP2D6), trioleandomycin (CYP3A) and mephenytoin (*S*-mephenytoin hydroxylase) caused <15% inhibition of 4NP hydroxylation, indicating none of these CYP isoforms contribute significantly to 4NP hydroxylation. Interestingly, α-naphthoflavone concentrations of 10 and 25 μM caused modest activation ($\approx$25–60%) of human liver microsomal 4NP hydroxylation. This observation may indicate that CYP2E1 can be activated by α-naphthoflavone or that CYP3A isoforms contribute to 4NP and CZ hydroxylation to a minor extent. Although there was a small inhibitory effect of troleandomycin on 4NP hydroxylation, activation of enzyme activity did not increase with increasing α-naphthoflavone concentrations above 50 μM suggesting that any involvement of CYP3A in this reaction is minor.

In summary, it has been demonstrated using cDNA-expressed and human liver microsomal kinetic, inhibition and correlation approaches that the conversion of 4NP to 4NC is catalysed predominantly (at least 85%) by CYP2E1. 4NP therefore constitutes a convenient substrate probe for the measurement of human hepatic CYP2E1 activity *in vitro*.

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