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Development of a human lymphoblastoid cell line constitutively expressing human CYP1B1 cDNA: substrate specificity with model substrates and promutagens

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An AHH-1 TK$^{+/-}$ cell derivative was developed that stably expresses human cytochrome P4501B1 (CYP1B1) cDNA in an extrachromosomal vector which confers resistance to l-histidinol and co-expresses NADPH cytochrome P450 oxidoreductase (OR). The CYP1B1-expressing cell line was designated h1B1/OR. Microsomes prepared from CYP1B1 cDNA expressing cells exhibit elevated levels of 7-ethoxyresorufin deethylase (EROD), 7-ethoxy-4-trifluoromethylcoumarin deethylase (EFCD), benzo(*a*)pyrene hydroxylase (BPH), bufuralol 1'-hydroxylase, testosterone hydroxylase activities and spectrally quantifiable cytochrome P450. CYP1B1-containing microsomes did not contain detectable coumarin 7-hydroxylase, *p*-nitrophenol hydroxylase, lauric acid hydroxylase, (*S*)-mephenytoin 4'-hydroxylase or diclofenac 4'-hydroxylase activities. Kinetic parameters for selected substrates were compared among CYP1B1 and the two additional members of the CYP1 family, CYP1A1 and CYP1A2. For BPH and EFCD, the rank order of rates of substrate metabolism were CYP1A1>CYP1B1> CYP1A2. For EROD, the rank order of substrate metabolism was CYP1A1>CYP1A2>CYP1B1. For both EROD and EFCD the apparent K_m values for CYP1B1 were more similar to CYP1A1 than to CYP1A2. In order to begin to characterize the promutagen activating ability of CYP1B1, the mutagenicity of selected chemicals was examined in h1B1/OR cells; there was increased sensitivity (CYP1B1-expressing relative to control cells) to the mutagenicity of benzo(*a*)pyrene, cyclopenta(*c,d*)pyrene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and aflatoxin B_1 (AFB). CYP1B1, expressed in this system, appears to be particularly efficient at activating AFB.

Introduction

Cytochromes P450 (CYP) are a family of haemoproteins which are the principal enzymes involved in the activation of many promutagens and procarcinogens (Guengerich, 1988; Gonzalez, 1989; Gonzalez and Gelboin, 1994). These enzymes are expressed in many tissues *in vivo* with the highest levels of this class of enzymes found in the liver of mammals (Gonzalez and Gelboin, 1994). Individual cytochrome P450 forms have unique, yet often overlapping substrate specificity. For example, at least five different hepatic cytochrome P450 forms have been reported to activate aflatoxin B_1 (AFB) (Aoyama *et al.*, 1990) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Crespi *et al.*, 1991b; Smith *et al.*, 1992). Aromatic amines are usually activated by a single form (CYP1A2) (Butler *et al.*, 1989).

One of the more recently identified human cytochrome P450 forms, CYP1B1, was first recognized as being 2,3,7,8,-tetrachlorodibenxo-*p*-dioxin (TCDD)-inducible in human keratinocytes (Sutter *et al.*, 1994). The CYP1B1 protein is also expressed in MCF-7 cells upon treatment with TCDD (Spink *et al.*, 1994). Northern analysis of RNA isolated from different human tissues reveals that CYP1B1 is expressed in the kidney, prostate, uterus and mammary tissues, but at much lower levels in the liver (Sutter *et al.*, 1994). Mouse CYP1B1 (P450EF) is expressed in C3H 10T1/2 cells (Shen *et al.*, 1994; Savas *et al.*, 1994). Rat CYP1B1 is expressed at high levels in the adrenal gland of untreated rats (Otto *et al.*, 1991; Walker *et al.*, 1995) and in many additional tissues upon TCDD treatment. The significance of CYP1B1 in xenobiotic metabolism, in general, and procarcinogen metabolism, in particular, is not well understood.

In addition to CYP1B1, the CYP1 family contains two additional members, CYP1A1 and CYP1A2. CYP1A1 has been recognized as having a principal role in the metabolic activation of many polycyclic aromatic hydrocarbons (PAH) (Shimada *et al.*, 1992) and CYP1A2 has been recognized a principal role in the activation of many aromatic and heterocyclic amines (Butler *et al.*, 1989; Eaton *et al.*, 1995). CYP1A2 is primarily a hepatic enzyme, whereas, CYP1A1 and CYP1B1 appear to be principally extrahepatic and most readily detected following administration of inducers (Sesardic *et al.*, 1990; Sutter *et al.*, 1994).

Here we report the development of a human cell line stably expressing CYP1B1 cDNA. This cell line, designated h1B1/OR, metabolizes prototypic substrates for CYP1 and contains spectrophotometrically quantifiable and immunologically detectable CYP1B1 protein. The AHH-1 TK$^{+/-}$ human B-lymphoblastoid cell line, which was used in these studies, is a convenient vehicle to measure the induction of gene mutations at the hypoxanthine phosphoribosyl transferase (*hprt*) and thymidine kinase loci and also the induction of chromosomal aberrations, aneuploidy and micronuclei (Crespi *et al.*, 1987; Crespi *et al.*, 1991a; Crofton-Sleigh *et al.*, 1993). The CYP1B1 cDNA-expressing cell lines were characterized and used to study the P450 form-specific activation of mutagens at the *hprt* locus. Finally, we have compared properties of cDNA-expressed CYP1B1 with cDNA-expressed CYP1A1 and CYP1A2.

Material and methods

Cells, tissue culture, DNA introduction

AHH-1 TK$^{+/-}$ human B lymphoblastoid cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented to 9% v/v with horse serum. Plasmid vector was introduced into AHH-1 TK$^{+/-}$ cells by electroporation using a BTX model 600 Electro cell Manipulator (San Diego, CA, USA). Cells containing vectors were initially selected by resistance to hygromycin B, then shifted l-histidinol-selection to accomplish vector

amplification. Cells bearing recombinant plasmids were maintained in medium either with 100–400 μg/ml hygromycin B or without histidine and containing 2 mM l-histidinol (pEBVHistk-based vectors) (Crespi *et al.*, 1990a).

Enzymes and reagents

Restriction endonucleases, Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). 7-Ethoxy-4-trifluoromethylcoumarin and 7-hydroxy-4-trifluoromethylcoumarin were purchased from Molecular Probes, Eugene, OR, USA NNK and 3-hydroxybenzo(*a*)pyrene were obtained from the NCI Chemical Carcinogen Repository (Research Triangle Park, NC, USA). Radiolabelled lauric acid and (*S*)-mephenytoin were obtained from Amersham, Arlington Heights, IL, USA. All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Cytotoxicity and mutagenicity assays

Cytotoxicity was estimated by measuring cell growth after treatment. After cultures have resumed exponential growth, the cumulative growth of the mutagen-treated cultures was divided by the cumulative growth of the negative control cultures to yield relative survival. Induction of mutation at the *hprt* locus was measured by previously published protocols (Crespi *et al.*, 1987) with minor modifications Human lymphoblasts were exposed to the mutagen for 28 h. cDNA-derived NADPH cytochrome P450 oxidoreductase (OR) is expressed from the glucocorticoid-inducible mouse mammary tumour virus long terminal repeat. OR expression was increased by the addition of 1 μM dexamethasone. Each replicate culture contained 3×10^7 cells. After a 7 day phenotypic expression period, the mutant fraction was measured by plating 7.5×10^6 cells in three 96-well microtitre plates in the presence of 6-thioguanine (0.6 μg/ml) and 500 cells in two 96-well microtitre plates without selection. NNK was dissolved in water for delivery to the cell cultures all other chemicals were dissolved in dimethyl sulphoxide (DMSO)

DNA manipulations, vectors

Bacterial transformations, plasmid DNA isolations and construction of the expression vectors were performed according to standard protocols. For CYP1B1 expression, a derivative of the pRedHyHo vector (Crespi *et al.*, 1995), designated pBlueHyHo was used. pBlueHyHo was produced from pRedHyho by introduction of the *Lac*Z α complementation from pUC19 into the *Hin*dIII site between the *His*D expression unit and the OR expression unit. This construct was performed as follows: the polylinker in pUC19 was eliminated by digestion with *Eco*RI, treatment with the Klenow fragment of DNA polymerase, then digestion with *Hin*dIII and blunt end religation after treatment with mung bean nuclease. The *Lac*Z α complementation was isolated by digestion with *Eco*0109I and *Bsr*BI and made blunt ended by treatment with the Klenow fragment of DNA polymerase. This fragment was blunt-end ligated into gel-purified, single cut pRedHyHo which had been subjected to *Hin*dIII partial digestion and treatment with the Klenow fragment of DNA polymerase and calf intestine phosphatase.

The CYP1B1 cDNA was first introduced into the unique *Xba*I site of the pBlueHyHo vector as a 1.7 kb *Hin*dIII/Ssp I fragment of p128 (containing the entire, unmodified, native open reading frame (Sutter *et al.*, 1994)) modified by the addition of *Xba*I linkers. Vector containing the cDNA in the appropriate orientation was identified by restriction mapping and designated pBlueHyHo1B1 (Figure 1).

Preparation of microsomal samples

Microsomes were prepared according to (Penman and Crespi, 1987). For microsomes used in spectral studies, cells were incubated in the presence of 20 μg/ml 5-aminolevulinic acid and 0.2% DMSO and 1 μM dexamethasone for 2 days prior to microsome preparation. Microsomal protein concentrations were determined by the method of (Lowry *et al.*, 1951) using bovine serum albumin as the standard. Cytochrome P450 difference spectra were performed according to (Omura and Sato, 1964) using a Beckman Model 640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

Enzyme assays

Benzo(*a*)pyrene hydroxylase (BPH) activity was measured according to (Nebert and Gelboin, 1968) and quantified using 3-hydroxybenzo(*a*)pyrene as standard. Benzo(*a*)pyrene (BP) was used at a final concentration of 25 μM. The other enzyme assays were conducted according to the following methods: 7-ethoxyresorufin *O*-deethylase (EROD, Burke *et al.*, 1977), 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylase (EFCD, DeLuca *et al.*, 1988), cytochrome *c* reductase (Phillips and Langdon, 1962), coumarin 7-hydroxylase (Greenlee and Poland, 1978), bufuralol 1′-hydroxylase (Kronbach *et al.*, 1987), diclofenac 4′-hydroxylase (Leeman *et al.*, 1993), *p*-nitrophenol hydroxylase (Reinke and Moyer, 1985), lauric acid hydroxylase (Clarke *et al.*, 1994) and (*S*)-mephenytoin 4′-hydroxylase (Wrighton *et al.*, 1993). NNK metabolism was analysed as previously described (Smith *et al.*, 1992). Apparent K_m and V_{max} values were calculated by non-linear kinetics using GraFit 3.0 (Erithacus Software, Middlesex, UK)

Antibodies and immunoblot detection

Amino terminal-hexa-histidine-tagged fusion polypeptide corresponding to human CYP1B1 amino acid residues 166-349 (Sutter *et al.*, 1994) was produced in *Escherichia coli* using the inducible expression vector, pTrcHis (Invitrogen Corp., San Diego, CA, USA). After cell lysis, the hexa-histidine-tagged proteins were purified by affinity chromatography (Hochuli, 1990), using a nickel–nitrilotriacetic acid agarose column according to the manufacturer's procedure (Qiagen Inc., Chatsworth, CA, USA). After elution from the column, these proteins were further purified by preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The denatured polypeptides were recovered by electrophoretic elution and dialysed against 0.5× phosphate-buffered saline (N.J.Walker, F.G.Crofts, and T.R.Sutter, unpublished results).

Male New Zealand White rabbits were immunized with the purified polypeptides according to the procedures of Spring Valley Laboratories (Sykesville, MD, USA). Serum was prepared from blood drawn 4 weeks after the second boost. Microsomal proteins were separated by SDS–PAGE (Laemmli, 1970), electroblotted (Towbin *et al.*, 1979), and incubated with anti-CYP serum at a dilution of 1:5000. Detection was by the enhanced chemiluminescence system (Amersham Life Sciences) after incubation with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (Ig)G secondary antibody, Promega, (Madison, WI, USA) at a dilution of 1:20 000.

Results and discussion

Cell line development/characterization

Human lymphoblastoid cell lines were developed to constitutively express transfected human CYP1B1. Vector-bearing, l-histidinol-resistant, clonal populations were isolated by plating in 96-well microtitre plates at limiting cell dilution. Seven clonal populations were assayed for sensitivity to BP cytotoxicity (BP concentrations ranged from 3 to 100 ng/ml, exposure time, 4 days). All populations had similar activities (range ~2-fold) and one population was chosen for further characterization and designated h1B1/OR cells.

The stability of cDNA-derived enzymatic activity in h1B1/OR cells was monitored as a function of time of growth in cell culture. Cells were propagated and monitored periodically for sensitivity to BP cytotoxicity. The cell line was stable for at least 2 months of continuous exponential growth. This level of stability is sufficient for most applications in toxicology and drug metabolism (Crespi *et al.*, 1991a).

Characterization of the enzyme

Microsomes prepared from h1B1/OR cells contained spectrophotometrically and immunologically detectable CYP1B1 protein. This protein was not detectable in control cells (without cDNA-bearing vector) (Figure 2). The level of expression (spectral P450 content) under culture conditions similar to those used in the mutagenicity assay was 14 pmol P450/mg microsomal protein. This content is slightly lower than that previously reported for CYP1A1 (h1A1v2 cells, 25 pmol P450/mg) and CYP1A2 (h1A2v2 cells, 38 pmol P450/mg). No spectrally active CYP was detectable in control microsomes CYP1A1 or CYP1A2 were not detectable by Western blot at loading up to 150 μg/lane (data not shown).

The level of OR activity, as measured by cytochrome c reductase, was elevated ~1.5-fold relative to microsomes prepared from control cells. We have found that when cytochrome P450 and OR cDNAs are co-expressed from a single vector, the levels of OR present can vary substantially for different P450 cDNAs. The results for CYP1B1 are at the low end of the range. For example, when CYP2C9/OR or CYP2E1/OR are co-expressed, the level of cytochrome c reductase activity is higher [250 or 80 nmol/(mg min) respect-

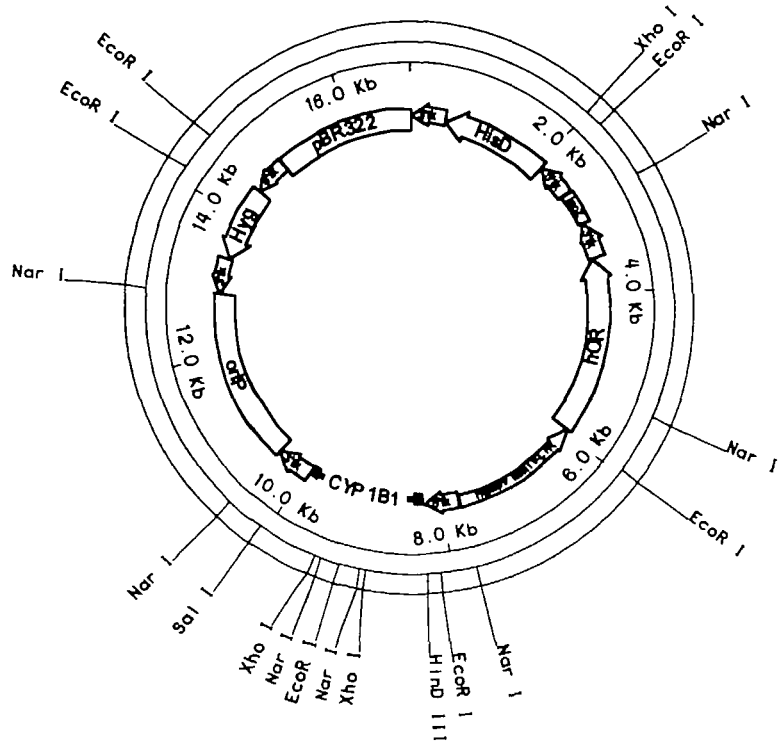

Fig. 1. Schematic map of the CYP1B1 cDNA expression vector pBlueHyHo1B1 cDNA expression units are transcribed in the direction of the arrow. The identity of the different DNA elements are as follows: 5′tk, herpes simplex virus thymidine kinase gene promoter; 3′tk, herpes simplex virus thymidine kinase gene polyadenylation signal; HaMSV MMTV-LTR, the Harvey murine sarcoma virus enhancer linked to the mouse mammary tumour virus long terminal repeat promoter; OriP, the origin of DNA replication from Epstein–Barr virus; hOR, the human NADPH:cytochrome P450 oxidoreductase; Hyg, the hygromycin B resistance gene; HisD, the *E.coli* histidinol dehydrogenase gene. The arrows indicate the direction of transcription. Selected restriction sites (*Eco*RI, *Nar*I, *Hin*dIII, *Xho*I and *Sal*I) are also shown.

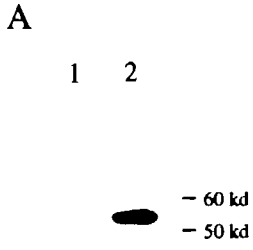

Fig. 2. Western immunoblot of microsomes prepared from human lymphoblastoid cells. Microsomal samples, 30 mg per lane, were analysed by SDS–PAGE and Western immunoblot. Immunoreactive proteins were detected by enhanced chemiluminescence procedure. Lane 1, control microsomes, P450 not detected, lane 2, h1B1/OR microsomes, 1 pmol P450. The positions of the 60 and 50 kDa protein markers are indicated at the right of the blot.

ively]. The native level of OR in AHH-1 $TK^{+/-}$ cells is not saturating, therefore, the level of OR can affect the rate of cytochrome P450 metabolism and hence, should be considered when interpreting data on rates of metabolism. For the comparisons made below, the other cytochrome P450 cDNAs were expressed in cells with native OR levels. Therefore, we would expect that the slightly higher OR levels with CYP1B1 will increase the rate of metabolism somewhat, but the magnitude of the effect should be small (<2-fold).

Analysis of metabolism of a panel of cytochrome P450 substrates which microsomes prepared from CYP1B1-expressing cells revealed elevated EROD, EFCD, BPH (Table I), bufuralol 1′-hydroxylase and testosterone hydroxylase (Table II). No detectable coumarin 7-hydroxylase, *p*-nitrophenol hydroxylase, lauric acid hydroxylase, (*S*)-mephenytoin 4′-hydroxylase or diclofenac 4′-hydroxylase activities were present.

The lack of coumarin 7-hydroxylase, a reaction catalysed by CYP2A6 (Yamano *et al.*, 1990), *p*-nitrophenol hydroxylase, a reaction catalysed by CYP2A6 and CYP2E1, lauric acid hydroxylase a reaction catalysed by CYP4A and CYP2E1 (Clarke *et al.*, 1994), (*S*)-mephenytoin 4′-hydroxylase, a reaction catalysed by CYP2C19 (Wrighton *et al.*, 1993) and diclofenac 4′-hydroxylase, a reaction catalysed by CYP2C9 (Leeman *et al.*, 1993) were consistent with the enzyme-selective nature of these substrates and supports the prediction of activities based on comparisons of CYP1B1 primary amino acid sequence to those of CYP1A1 and CYP1A2 (Sutter *et al.*, 1994).

The detection of bufuralol 1′-hydroxylase and testosterone hydroxylase activities were surprising. The rates of metabolism per unit enzyme were relatively low (~300-fold lower than CYP2D6/bufuralol or CYP3A4/testosterone) but the metabolite signal was substantially (10 fold) above machine background and/or incubations with control microsomes. CYP1B1-catalysed bufuralol 1′-hydroxylase activity was not inhibited by the addition of 1 μM quinidine, a CYP2D6-selective inhibitor (data not shown) indicating that the metabolism was not due to presence of CYP2D6. Concurrent incubations with cDNA-expressed CYP2D6 with and without 1 μM quinidine showed 95% inhibition. With CYP1B1, two hydroxylated testosterone metabolites were observed. These metabolites co-chromato-

Table I. Kinetic analyses of cDNA expressed CYP1B1, CYP1A1 and CYP1A2

Activity	CYP1B1 K_m (nM)	CYP1B1 Turnover No. (per min)	CYP1A1 K_m (nM)	CYP1A1 Turnover No. (per min)	CYP1A2 K_m (nM)	CYP1A2 Turnover No. (per min)
EROD	60	1 1	64	7.1	230	2.0
EFCD	580	0.74	1100	11	4.8	0.9
BPH	not done	0.27	not done	1.0	not done	0.007

Kinetic analyses were performed in CYP1B1, CYP1A1 and CYP1A2 microsomes. Incubations were performed in duplicate in at least two independent experiments with five to nine substrate concentrations. All activities (except EROD) were not detectable in control microsomes. Control microsomes had a EROD activity of 1 pmole/(mg×min).

Table II. Catalytic activity for microsomes prepared from h1B1/OR cells

Assay	Activity
Cytochrome c reductase	42 nmol/(mg min)
Coumarin 7-hydroxylase	<0.06/min
Bufuralol 1′-hydroxylase	0.04/min
p-Nitrophenol hydroxylase	<0.06/min
Diclofenac 4′-hydroxylase	<0.06/min
Testosterone 6β-hydroxylase	0.08/min
Testosterone 16α-hydroxylase	0.05/min
Lauric acid hydroxylase	<0.1/min
(*S*)-Mephenytoin 4′-hydroxylase	<0.01/min

Turnover of selected substrates were analysed in microsomes prepared from h1B1/OR cells. Activities are expressed as either nmol product per mg min (cytochrome c reductase) or turnover number (pmol product per pmol P450) The level of cytochrome c reductase activity in control microsomes was 28 nmol/(mg min) None of the cytochrome P450-associated activities were detectable in control microsomes.

graphed with authentic 6β-hydroxytestosterone and 16α-hydroxytestosterone and were formed in approximately equimolar quantities. Unfortunately, the levels of metabolite production by CYP1B1 were too low to allow isolation of sufficient metabolite for mass spectral analysis. Therefore, the identity of these metabolites should be regarded as tentative. The testosterone metabolites produced by CYP1B1 did not co-elute with authentic 2α-, 6α-, 7α-, 11α-, 14α-, 15α-, 2β-, 11β- or 16β-hydroxytestosterone standards.

Testosterone 6β-hydroxylation is considered to be a prototypical CYP3A reaction. In order to test for any contribution of CYP3A to the observed 6β-hydroxylase activity, inhibition by troleandomycin was analysed (Chang *et al.*, 1994). CYP1B1-catalysed testosterone hydroxylase activity was not inhibited by 25 μM troleandomycin (95% activity of control) while cDNA-expressed CYP3A4 was inhibited by troleandomycin to 58% of the activity of control. Therefore, presence of CYP3A4 was not responsible for the observed 6β-hydroxylation of testosterone.

There is often considerable substrate overlap among the different enzymes comprising the CYP1 family. Indeed, relatively high levels of metabolite formation were observed with CYP1B1 for EROD, EFCD and BPH which are known CYP1A substrates. In order to further characterize these reactions, we examined kinetic constants for three model substrates for CYP1A1, CYP1A2 and CYP1B1 (Table I). The apparent K_m for CYP1B1-catalysed EROD was 60 nM, which is quite similar to the apparent K_m for CYP1A1 (64 nM) and lower than the K_m for CYP1A2 (230 nM). The turnover number for CYP1B1 (1.1/min) was lower than for either CYP1A1 (7.1/min) or CYP1A2 (2.0/min). The slightly higher OR levels present in CYP1B1 microsomes should not have affected this rank order. The apparent K_m for CYP1B1-catalyzed EFCD was 580 nM, which is similar to the apparent K_m for CYP1A1 (1100 nM) and much higher than the K_m for CYP1A2 (4.8 nM). The turnover number for CYP1B1 (0.74/min) was lower than for either CYP1A1 (11/min) or CYP1A2 (0.9/min). The slightly higher OR levels present in CYP1B1 microsomes again should not have affected this rank order. BPH activity was measured at a single substrate concentration (25 μM). The turnover number at this concentration for CYP1B1 (0.27/min) was intermediate relative to CYP1A1 (1.0/min) or CYP1A2 (0.007/min). The slightly higher OR levels present in CYP1B1 microsomes should not have affected this rank order.

Mutagenicity assays

The ability of cDNA-derived CYP1B1 to activate promutagens was examined using h1B1/OR cells. Four promutagens, benzo(*a*)pyrene (BP), cyclopenta(*c,d*)pyrene (CPP), AFB and NNK were examined for the ability to induce gene locus mutations at the *hprt* locus. We, and others, have previously demonstrated that AFB and NNK are activated by multiple CYP forms (Shimada and Guengerich, 1989; Forrester *et al.*, 1990; Aoyama *et al.*, 1990; Crespi *et al.*, 1991b,c; Smith *et al.*, 1992). The polycylic aromatic hyrdocarbons (PAH), BP and CPP are prototypical CYP1A1 substrates (Penman *et al.*, 1994).

BP was clearly mutagenic to h1B1/OR cells (Figure 3). BP induced a monotonic increase in mutant fraction in the concentration range 10–1000 ng/ml. Over this same concentration range BP is non-mutagenic to control cells (Penman *et al.*, 1994). BP was relatively toxic to h1B1/OR cells. Comparison of this dose–response data with that previously published for CYP1A1 (Penman *et al.*, 1994) and taking into consideration that the h1A1 and h1B1/OR cell lines have roughly comparable cytochrome P450 contents, indicates that CYP1B1 and CYP1A1 have roughly comparable abilities to activate BP to a gene mutagen. The slightly higher OR levels present in h1B1/OR cells should not affect this conclusion. The activation of BP by CYP1B1-expressing human lymphoblasts stands in contrast to the lack of a response induced by BP with CYP1B1 present in the *Salmonella typhimurium umu* gene expression system (Shimada *et al.*, 1996). The bacterial system does indicate that both CYP1A1 and CYP1A2 are capable of activating BP, therefore, the difference cannot be completely attributed to a lack of sensitivity of the system to this compound. The activation of BP by CYP1B1 in human lymphoblasts is consistent with the detection of BP metabolites by fluorometric assay (Table I), although the phenols detected by this assay are not expected to be directly mutagenic. Metabolism of BP by CYP1A1 and CYP1A2 expressed in human lymphoblasts was also detectable (Table I). The discordance between the systems cannot be attributed to the presence of microsomal epoxide hydrolase in human lymphoblasts (and

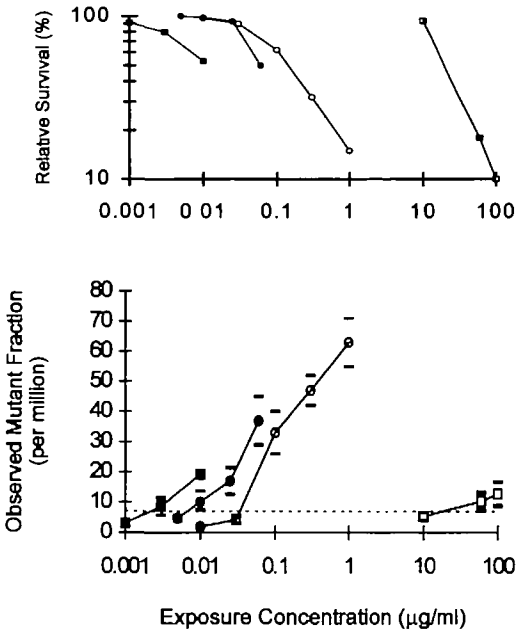

Fig. 3. Mutagenicity (lower panel) and cytotoxicity (upper panel) of AFB (closed squares), CPP (closed circles), BP (open circles) and NNK (open squares) to h1B1/OR cells. Mutagenicity data are plotted as the mean and SEM. Relative survival data are plotted as the mean only. The dotted line is the 99% upper confidence limit from the historical negative control data base at the *hprt* locus (a measure of statistical significance) The mean and SEM for the negative controls were 1.8×10^{-6} and 0.6×10^{-6} respectively (n = 12).

its absence in the bacterial system) since the lymphoblastoid cell line does not have detectable microsomal epoxide hydrolase activity (Crespi *et al.*, 1985). In contrast to BP, several BP diols produced a detectable response with CYP1B1 in the *S.typhimurium umu* gene expression system (Shimada *et al.*, 1996). Therefore, different responses of BP in the two systems could be due to differences in sensitivity of the different host cells to the range of electrophiles produced by BP metabolism. Additional experiments are required to clarify this point.

CPP was also clearly mutagenic to h1B1/OR cells (Figure 3). A monotonic increase in mutant fraction was observed over the concentration range 5–60 ng/ml. CPP was non-mutagenic to control cells over the concentration range tested (Penman *et al.*, 1994). Like BP, CPP is relatively toxic to h1B1/OR cells. Comparison of this dose–response data with that previously published for CYP1A1 (Penman *et al.*, 1994) and taking into consideration that the h1A1 and h1B1/OR cell lines have similar cytochrome P450 contents, indicates that as with BP, CYP1B1 and CYP1A1 have roughly comparable abilities to activate CPP to a gene mutagen. The slightly higher OR levels present in h1B1/OR cells should not affect this conclusion. Further analysis, with metabolite analyses and epoxide hydrolase co-expression may reveal more pronounced differences between CYP1B1 and CYP1A1 for this substrate.

AFB induced a monotonic increase in mutant fraction over the concentration range 1–10 ng/ml (Figure 3). AFB was toxic to h1B1/OR cells. The response of control cells to AFB has been previously reported four times (Crespi and Thilly, 1984; Crespi *et al.*, 1990a,b, 1991c). In these reports AFB was non-mutagenic to control cells at exposure concentrations up to 1000 ng/ml. Comparison of this dose–response data with that previously published for CYP1A1 (Penman *et al.*, 1994), CYP1A2 (Crespi *et al.*, 1991c), CYP2A6 (Crespi *et al.*, 1990a), CYP3A4 (Crespi *et al.*, 1991c) indicate that CYP1B1 is among the most efficient enzymes for activating AFB to a gene mutagen. Relevant parameters are summarized in Table III. We have listed the minimum effective concentration of AFB (A in Table III) which was calculated as the point where the concentration-response curve crosses the 99% upper confidence limit from the historical negative control data base (a mutant fraction 7×10^{-6}, all cell lines appear to have similar negative control mutant frequencies). Comparison of these values indicates the relative sensitivities of the different cell lines to AFB mutagenicity. However, the cell lines have different cytochrome P450 contents (B in Table III) and the effect of increasing cytochrome P450 content is to shift the cellular concentration–response curve to lower concentrations (Crespi *et al.*, 1990a; Penman *et al.*, 1994). Therefore, a comparison of the abilities of the different enzymes to activate AFB should consider the differences in P450 contents. The parameter of the minimum effective concentration multiplied by the cytochrome P450 content (A×B in Table III) should be proportional to the intrinsic ability of the enzyme to activate AFB. With this analysis, the abilities of CYP1A2 and CYP1B1 are similar (about two-fold). CYP1A1, CYP2B6 and CYP2A6 are ~10–20-fold less active than CYP1B1 and CYP3A4 is about five-fold less active than CYP1B1. The slightly higher OR levels present in h1B1/OR cells should not affect this conclusion.

The efficient activation of AFB by CYP1B1-expressing human lymphoblasts stands in sharp contrast to the complete lack of a response induced in the *S.typhimurium umu* gene expression system (Shimada *et al.*, 1996). This bacterial system does give positive responses for AFB when either CYP1A1 and CYP1A2 are present. However, the difference in response between CYP1A1 and CYP1A2 is less pronounced in the *S.typhimurium* system relative to the human lymphoblast system (three-fold versus 40-fold; Penman *et al.*, 1994) and substantially higher AFB concentrations were needed to generate a relatively weak response in *S.typhimurium*. It is possible that CYP1B1 is a relatively low capacity enzyme for AFB activation and insufficient electrophillic metabolite is formed to generate a detectable response in *S.typhimurium*. Further experiments are needed to clarify this point.

AFB is a known human (and rat) hepatocarcinogen and it has been established that among the mixture of enzymes present in human liver, CYP1A2 appears to be the most important enzyme at low AFB concentrations (Eaton *et al.*, 1995), although CYP3A4 may be involved at high AFB exposures. Given the extrahepatic nature of CYP1B1, one would expect little role of this enzyme in hepatic AFB activation. However, it is possible that CYP1B1 may play a role in extrahepatic AFB activation, particularly for individuals exposed to inducers.

NNK induced a monotonic increase in mutant fraction over the concentration range 10–100 μg/ml (Figure 3). NNK was also toxic to h1B1/OR cells. NNK is non-toxic and non-mutagenic to control cells at exposure concentrations up to 300 μg/ml (Crespi *et al.*, 1991b). We had previously demonstrated that multiple human P450 forms can activate NNK (Crespi *et al.*, 1991b; Penman *et al.*, 1994). After allowing for differences in cellular P450 content (using the same approach used for AFB above), the ability to metabolically activate NNK is approximately CYP1A1 = CYP1A2 = CYP1B1 = CYP2A6 > CYP2E1 = CYP2D6. It is interesting to note that, per unit induced mutant fraction, NNK is much more toxic

Table III. Comparison of the abilities cDNA expressed cytochromes P450 to activate aflatoxin B_1 to a gene mutagen. The minimum detectable concentration was calculated as the AFB concentration where the dose–response curve crossed the 99% upper confidence limit for the historical negative control data base (7×10^{-6}) (Penman and Crespi, 1987)

Enzyme	Cell line	**A.** Minimum detectable concentration (ng/ml)	**B.** P450 content (pmol/mg microsomes)	Product of **A** × **B**	Reference
CYP1A1	h1A1	62	12	740	Penman *et al.*, 1994
CYP1A2	1A2/Hyg	10.5	1.4	15	Crespi *et al.*, 1991c
CYP1B1	h1B1/OR	2.4	14	34	this report
CYP2A6	2A3/Hol	7	55	385	Crespi *et al.*, 1990a
CYP3A4	3A4/Hol	88	1.7	150	Crespi *et al.*, 1991c

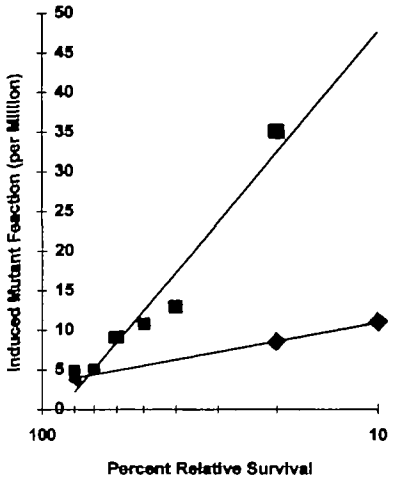

Fig. 4. Plot of relative survival versus induced mutant fraction for NNK activated by CYP1B1 (diamonds) or all other cytochrome P450s (squares) The data for all other cytochrome P450s is taken from (Crespi *et al.*, 1990a; Penman *et al.*, 1994) Survival data were grouped in increments of 10% decreases in survival and the induced mutant fractions for the individual cell lines were averaged.

to CYP1B1-expressing cells than to cells expressing other cytochrome P450 enzymes (Figure 4). NNK is an asymmetric nitrosamine and this observation may be indicative of a different pathway of metabolism for CYP1B1 than for CYP1A1, CYP1A2, CYP2A6, CYP2D6 or CYP2E1. In order to test this hypothesis, NNK metabolism was analysed in microsomes prepared from h1B1/OR cells. Unfortunately, when sodium bisulphite (5 mM) was added to the incubation mixture to trap the keto aldehyde, thereby allowing quantification of the two α-hydroxylation pathways (Peterson *et al.*, 1991), the metabolism of NNK was inhibited by 50%. Therefore, sodium bisulphite was not added to the assay mixtures, and the metabolite peak was quantified as a mixture of keto aldehyde and keto alcohol. The apparent K_m and V_{max} values were 1.2 mM and 1.8/min respectively. The apparent K_m value for CYP1B1 was 3–10-fold higher than that reported for CYP2D6 (Penman *et al.*, 1993), CYP1A2 (Smith *et al.*, 1992) or CYP2A6 (Patten *et al.*, 1996). Since humans are exposed to low levels of NNK, and CYP1B1 exhibited a high K_m, other cytochrome P450s, such as CYP2A6, may play a more important role in the activation of NNK *in vivo*. With regard to activation of other nitrosamines, *N*-nitrosodimethylamine was negative for CYP1B1 activation using the *S.typhimurium umu* gene expression system (Shimada *et al.*, 1996). Therefore, it is unclear how broadly CYP1B1 activates nitrosamines.

The development of the h1B1/OR cell line complements the other isogenic AHH-1 $TK^{+/-}$ human B-lymphoblastoid cell lines expressing human cytochromes P450 (Crespi *et al.*, 1990a,b, 1991c; Penman *et al.*, 1993, 1994). For all of these expressed human P450s, spectrophotometric determination of P450 content is possible, allowing comparisons of metabolism and protoxin activation on both a per cell and per pmole P450 basis. This enables a quantitative analysis of the relative efficiency of different P450 enzymes to carry out specific biotransformations in an intact cellular system.

The present study, in combination with the study by (Shimada *et al.*, 1996) indicate that CYP1B1 activates a broad range of structurally diverse procarcinogens including, polycyclic aromatic hydrocarbon (and dihydrodiol derivatives), aromatic amines and amides, heterocyclic amines, nitro polycyclic aromatic hydrocarbons, AFB and NNK. This broad substrate specificity, coupled with the expression of CYP1B1 in multiple human tissues (Sutter *et al.*, 1994; Shimada *et al.*, 1996), suggests that CYP1B1 could have a significant role in cancer induction in humans. The striking differences in response for the activation of BP and AFB in the *S.typhimurium umu* gene expression system and human lymphoblasts serves to underscore the need to test procarcinogens in multiple systems.

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References

Aoyama,T., Yamano,S., Guzelian,P.S., Gelboin,H.V. and Gonzalez,F.J. (1990) Five of twelve forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B_1. *Proc. Natl. Acad. Sci. USA*, **87**, 4790–4793.

Burke,M.D., Prough,R.A. and Mayer,R.T. (1977) Characteristics of a microsomal cytochrome P-448-mediated reaction ethoxyresorufin *O*-deethylation. *Drug Metab. Disp.*, **5**, 1–8.

Butler, M.A., Iwasaki, M., Guengerich,F.P. and Kadlubar,F.F. (1989) Human cytochrome P450PA (P4501A2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. USA*, **86**, 7696–7700.

Chang,T.K.H., Gonzalez,F.J. and Waxman,D.J. (1994) Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethyldithiocarbamate as selective probes for inhibition of human cytochromes P450. *Arch. Biochem. Biophys.*, **311**, 437–442.

Clarke,S.E., Baldwin,S.J., Bloomer,J.C., Aryton,A.D., Sozio,R S. and Chenery,R.J. (1994) Lauric acid as a model substreate for the simultaneous determination of cytochrome P450 2E1 and 4A in hepatic microsomes. *Chem. Res. Toxicol.*, **7**, 836–842.

Crespi,C.L. and Thilly,W.G. (1984) Assay for gene mutation in a human lymphoblastoid line, AHH-1, competent for xenobiotic metabolism. *Mutat. Res.*, **128**, 221–230.

Crespi,C.L., Altman,J.D. and Marletta,M.A. (1985) Xenobiotic metabolism in a human lymphoblastoid cell line. *Chem. Biol. Interact.*, **53**, 257–272.

Crespi,C.L., Seixas,G.M. and Penman,B.W. (1987) The induction of aneuploidy by nitrogen mustard in a human lymphoblastoid cell line. *Mutat. Res.*, **190,** 69–76.

Crespi,C.L., Penman,B.W., Leakey,J.A.E., Arlotto, M.P., Stark,A., Turner,T., Steimel,D., Rudo,K., Davies,R.L. and Langenbach,R.L. (1990a) Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. *Carcinogenesis*, **8,** 1293–1300.

Crespi,C.L., Steimel,D.T., Aoyama,T., Gelboin,H.V. and Gonzalez,F.J. (1990b) Stable expression of human cytochrome P4501A2 cDNA in a human lymphoblastoid cell line: role of the enzyme in the metabolic activation of aflatoxin B_1. *Mol. Carcinogen.*, **3,** 5–8.

Crespi,C.L., Gonzalez,F.J., Steimel,D.T., Turner,T.R., Gelboin,H.V., Penman,B.W. and Langenbach,R. (1991a) A metabolically competent cell line expressing five cDNAs encoding procarcinogen-activating enzymes: applications to mutagenicity testing. *Chem. Res. Toxicol.*, **4,** 566–572.

Crespi,C L., Gonzalez,F.J., Steimel,D.T., Turner,T.R., Gelboin,H.V., Penman,B.W. and Langenbach,R. (1991b) A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including the polymorphic cytochrome P4502D6. *Carcinogenesis*, **12,** 1197–1201.

Crespi,C.L., Penman,B.W., Steimel,D.T., Gelboin,H.V. and Gonzalez,F.J. (1991c) The development of a human cell line stably expressing human CYP3A4: role in the metabolic activation of aflatoxin B_1 and comparison to CYP1A2 and CYP2A3. *Carcinogenesis*, **12,** 355–359.

Crespi,C.L., Steimel,D.T., Penman,B.W, Korzekwa,K.R., Fernandezsalguero,P., Buters,J.T.M., Gelboin,H.V., Gonzalez,F.J., Idle,J.R. and Daly,A.K. (1995) Comparison of substrate metabolism by wild type CYP2D6 protein and a variant containing methionine, not valine, at position 374. *Pharmacogenetics*, **5,** 234–243.

Crofton-Sleigh,C., Doherty,A., Ellard,S., Parry,E.M. and Venitt,S. (1993) Micronucleus assays using cytochalasin-blocked MCL-5 cells, a proprietary human cell line expressing five human cytochromes P-450 and microsomal epoxide hydrolase. *Mutagenesis*, **8,** 363–372.

DeLuca,J.G., Dysart,G.R., Rasnick,D. and Bradley,M.O. (1988) A direct, highly sensitive assay for cytochrome P-450 catalyzed *O*-deethylation using a novel coumarin analog. *Biochem. Pharmacol.*, **39,** 1731–1739.

Eaton,D.L., Gallagher,E.P., Bammler,T.K. and Kunze,K.L. (1995) Role of cytochrome P4501A2 in chemical carcinogenesis—implications for human variability in expression and enzyme activity. *Pharmacogenetics*, **5,** 259–274.

Forrester,L.M., Neal,G.M., Judah,D.J., Glancey,M.J. and Wolf,C.R (1990) Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B_1 metabolism in human liver. *Proc. Natl. Acad. Sci. USA*, **87,** 8306–8310.

Gonzalez,F.J. (1989) The molecular biology of cytochrome P450s. *Pharmacol. Rev.*, **40,** 243–288.

Gonzalez,F.J. and Gelboin,H.V. (1994) Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab. Rev.*, **26,** 165–183.

Greenlee,W.F. and Poland,A. (1978) An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Pharmacol. Exp. Ther.*, **205,** 596–605.

Guengerich,F.P. (1988) Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.*, **48,** 2946–2954.

Hochuli,E. (1990) *Genetic Engineering*. In Setlow,J.K. (ed.), Plenum Press, New York, pp. 87–98.

Kronbach,T., Mathys,D., Gut,J., Catin,T. and Meyer,U.A. (1987) High performance liquid chromatographic assays for bufuralol 1′-hydroxylation, debrisoquine 4-hydroxylation and dextromethorphan *O*-demethylation in microsomes and purified cytochrome P-450 isozymes of human liver. *Analyt. Biochem.*, **162,** 24–32.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680–685.

Leeman,T., Transon,C. and Dayer,P. (1993) Cytochrome P450TB (CYP2C): a major monooxygenase catalyzing diclofenac 4′-hydroxyation in human liver. *Life Sci.*, **52,** 29–34.

Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,F.J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **62,** 315–323.

Nebert,D.W. and Gelboin,H.V. (1968) Substrate-inducible microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. *J. Biol. Chem.*, **243,** 6242–6249.

Omura,T. and Sato,R. (1964) The carbon monoxide-binding pigment of liver microsomes II: solubilization, purification and properties. *J. Biol. Chem.*, **239,** 2379–2385.

Otto,S., Marcus,C., Pidgeon,C. and Jefcoate,C.R. (1991) A novel adrenocorticotropin-inducible cytochrome P450 from rat adrenal microsomes catalyzes polycyclic aromatic hydrocarbon metabolism. *Endocrinology*, **129,** 970–982.

Patten,C.J., Smith,T.J., Wang,M.-H., Tynes,R.E., Lee,J., Koch,P. and Yang,C.S. (1996) Kinetic analysis of activation of 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone by heterologously expressed human P450 enzymes and the effect of P450 specific chemical inhibitors on this activation in human liver microsomes. *Arch. Biochem. Biophys.*, **333,** 127–138.

Penman,B.W., Reece,J., Smith,T., Yang,C.S., Gelboin,H.V., Gonzalez,F.J. and Crespi,C.L. (1993) Characterization of a human cell line expressing high levels of cDNA-derived CYP2D6. *Pharmacogenetics*, **3,** 28–39.

Penman,B.W., Chen,L.P., Gelboin,H.V., Gonzalez,F.J. and Crespi,C.L. (1994) Development of a human lymphoblastoid cell line constitutively expressing human CYP1A1 cDNA-substrate specificity with model substrates and promutagens. *Carcinogenesis*, **15,** 1931–1937.

Penman,B.W. and Crespi,C.L. (1987) Analysis of human lymphoblast mutation assays by using historical negative control data bases. *Environ. Molec. Mutagen.*, **10,** 35–60.

Peterson,L.A., Mathew,R. and Hecht,S.S. (1991) Quantitation of microsomal α-hydroxylation of the tobacco smoke nitrosamine, 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone. *Cancer Res.*, **51,** 5495–5500.

Phillips,A.H. and Langdon,R.G. (1962) Hepatic triphosphopyridine nucleotide cytochrome C reductase: isolation, characterization and kinetic studies. *J. Biol. Chem.*, **237,** 2652–2660.

Reinke,L.A. and Moyer,M.J. (1985) *Drug Metab. Disp.*, **13,** 548–552.

Savas,U., Bhattacharyya,K.K., Christou,M., Alexander,D.L. and Jefcoate,C.R. (1994) Mouse cytochrome P-450EF, representative of a new 1B subfamily of cytochrome P-450s–cloning, sequence determination and tissue expression. *J. Biol. Chem.*, **269,** 14905–14911.

Sesardic,D., Pasanen,M , Pelkonen,O. and Boobis,A.R. (1990) Differential expression and regulation of members of the cytochrome P4501A gene subfamily in human tissues. *Carcinogenesis*, **11,** 1183–1188.

Shen,Z.Y., Liu,J.M., Wells,R.L. and Elkind,M.M. (1994) cDNA cloning, sequence analysis and induction by aryl hydrocarbons of a murine cytochrome P450 gene, CYP1B1. *DNA Cell Biol.*, **13,** 763–769.

Shimada,T., Yun,C.-H., Yamazaki,H., Gautier,J.-C , Beaune,P.H. and Guengerich,F.P. (1992) Characterization of human lung microsomal cytochrome P-4501A1 and its role in the oxidation of chemical carcinogens. *Mol. Pharmacol.*, **41,** 856–684.

Shimada,T., Hayes,C.L., Yamazaki,H., Amin,S., Hecht,S.S., Guengerich,F.P. and Sutter,T.R. (1996) Activation of chemically diverse procarcinogens by human cytochrome P450 1B1. *Cancer Res.*, **56,** 2979–2984.

Shimada,T. and Guengerich,F.P. (1989) Evidence for cytochrome P-450nf, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Sci. USA*, **86,** 462–465.

Smith,T.J., Guo,Z., Gonzalez,F.J., Stoner,G.C. and Yang,C.S. (1992) Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human lung and liver microsomes and cytochromes P-450 expressed in hepatoma cells. *Cancer Res.*, **52,** 1757–1763.

Spink,D.C., Hayes,C.L., Young,N.R., Christou,M., Sutter,T.R., Jefcoate,C.R. and Gierthy,J.F. (1994) The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on estrogen metabolism in MCF-7 breast cancer cells: evidence for induction of a novel 17(beta)-estradiol 4-hydroxylase. *J. Steroid Biochem. Mol. Biol.*, **51,** 251–258.

Sutter,T.R., Tang,Y.M., Hayes,C.L., Wo,Y.Y.P., Jabs,E.W., Li,X., Yin,H., Cody,C.W. and Greenlee,W.F. (1994) Complete cDNA sequence if a human dioxin-inducible mRNA identities a new gene subfamily of cytochrome P450 that maps to chromosome 2. *J. Biol. Chem.*, **269,** 13092–13099.

Towbin,H., Staehelin,T. and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. procedures and some applications. *Proc. Natl. Acad. Sci. USA*, **76,** 4350–4354.

Walker,N.J , Gastel,J.A., Costa,L.T., Clark,G.C., Lucier,G.W. and Sutter,T.R. (1995) Rat CYP1B1: an adrenal cytochrome P450 that exhibits sex-dependent expressions in livers and kidneys of TCDD-treated animals. *Carcinogenesis*, **16,** 1319–1327.

Wrighton,S.A., Stevens,J.C., Becker,G.W. and VandenBranden,M. (1993) Isolation and characterization of human liver cytochrome P450 2C19: correlation between 2C19 and S-mephenytoin 4′-hydroxylase. *Arch. Biochem. Biophys.*, **306,** 240–245.

Yamano,S., Tatsumo,J. and Gonzalez,F.J. (1990) The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry*, **29,** 1322–1329.

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