Cytochrome P450 mediated bioactivation of methyleugenol to 1′-hydroxymethyleugenol in Fischer 344 rat and human liver microsomes

Iain Gardner[1], Hiroshi Wakazono[1], Pauline Bergin[1], Isabelle de Waziers[2], Phillippe Beaune[2], J.Gerald Kenna[1,3] and John Caldwell[1]

[1]Pharmacology and Toxicology, Imperial College School of Medicine at St. Mary's, Norfolk Place, London, W2 1PG, UK and [2]Biochimie Pharmacologique et Metabolique, Universite Rene Descartes-INSERM U 75, Paris, France

[3]To whom correspondence should be addressed

Cytochrome P450 mediated metabolism of methyleugenol to the proximate carcinogen 1′-hydroxymethyleugenol has been investigated *in vitro*. Kinetic studies undertaken in liver microsomes from control male Fischer 344 rats revealed that this reaction is catalyzed by high affinity (K_m of 74.9 ± 9.0 µM, V_{max} of 1.42 ± 0.17 nmol/min/nmol P450) and low affinity (apparent K_m several mM) enzymic components. Studies undertaken at low substrate concentration (20 µM) with microsomes from livers of rats treated with the enzyme inducers phenobarbital, dexamethasone, isosafrole and isoniazid indicated that a number of cytochrome P450 isozymes can catalyze the high affinity component. In control rat liver microsomes, 1′-hydroxylation of methyleugenol (assayed at 20 µM substrate) was inhibited significantly ($P < 0.05$) by diallylsulfide (40%), *p*-nitrophenol (55%), tolbutamide (30%) and α-naphthoflavone (25%) but not by troleandomycin, furafylline, quinine or cimetidine. These results suggested that the reaction is catalyzed by CYP 2E1 and by another as yet unidentified isozyme(s) (most probably CYP 2C6), but not by CYP 3A, CYP 1A2, CYP 2D1 or CYP 2C11. Administration of methyleugenol (0–300 mg/kg/day for 5 days) to rats *in vivo* caused dose-dependent auto-induction of 1′-hydroxylation of methyleugenol *in vitro* which could be attributed to induction of various cytochrome P450 isozymes, including CYP 2B and CYP 1A2. Consequently, high dose rodent carcinogenicity studies are likely to overestimate the risk to human health posed by methyleugenol. The rate of 1′-hydroxylation of methyleugenol *in vitro* in 13 human liver samples varied markedly (by 37-fold), with the highest activities being similar to the activity evident in control rat liver microsomes. This suggests that the risk posed by dietary ingestion of methyleugenol could vary markedly in the human population.

Introduction

Methyleugenol (3,4-dimethoxyallylbenzene) is a natural flavour present in a number of herbs and spices (e.g. basil, sweet bay, cloves and lemon grass) which are consumed at low levels in the human diet and also is approved for commercial use as a fragrance and flavouring agent. Human exposure to methyleugenol is of toxicological concern because this compound has been shown to be a genotoxic rodent carcinogen (as have the structurally related allylbenzene compounds safrole and estragole) (1,2).

Although the allylbenzenes themselves are not genotoxic, they undergo two-step bioactivation to yield chemically reactive electrophiles. The first step is hydroxylation at the 1′ position of the allyl side chain, which is catalyzed by the cytochrome P450 enzyme system (3). The 1′-hydroxy metabolites are then sulfated, yielding 1′-sulfooxy metabolites (4) which decompose spontaneously in an aqueous environment to electrophilic carbonium ions that bind covalently to DNA (2,5) and to other cellular macromolecules, including proteins (6).

Currently, little is known concerning the kinetics of the 1′-hydroxylation of allylbenzenes or of the cytochrome P450 isozymes which catalyze the reaction. This is an important issue since individual cytochrome P450 isozymes show marked species differences, have different substrate specificities and also have different modes of regulation (reviewed in 7). Variability in expression of P450s in the human population could lead to marked inter-individual variability in susceptibility to methyleugenol induced toxicity. Moreover, a rational, mechanism-based assessment of the risk posed to the human population by ingestion of low levels of allylbenzenes will only be possible once the similarities and/or differences between bioactivation of methyleugenol in rodents and humans have been established.

In the present study, the kinetics of 1′-hydroxylation of methyleugenol in rat liver microsomes *in vitro* have been investigated, as has the nature of the isozymes of cytochrome P450 which catalyze the reaction and the influence of treatment of rats for 5 days with methyleugenol on this bioactivation process. In addition, the ability of a panel of 13 human liver microsomal preparations to catalyze formation of 1′-hydroxymethyleugenol has been investigated. The results obtained suggest that the rodent lifetime carcinogenicity bioassay undertaken recently, by the National Toxicology Program, could markedly overestimate the risk to human health posed by dietary ingestion of methyleugenol, and furthermore that this risk could vary markedly between individuals.

Materials and methods

Materials

p-Nitrophenol (PNP*), tolbutamide, testosterone, NADP⁺, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (Poole, UK). Methyleugenol and 11α-hydroxyprogesterone were purchased from Aldrich Chemical Co. (Gillingham, UK). 16α-, 6β-, 7α- and 2α-hydroxytestosterone and 4-MA (17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one) were gifts from Dr A.G.Parkinson (Kansas University Medical Center, USA). 7-Ethoxy-, 7-methoxy- and 7-pentoxyresorufin were purchased from Cambridge Bioscience (Cambridge, UK). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce and Warriner, (Chester, UK). Furafylline was purchased from Ultrafine Chemicals (Manchester, UK). 1′-Hydroxymethyleugenol and 1′-hydroxysafrole were synthesised according to Borchert *et al.* (8).

Table I. Induction of liver microsomal 1'-hydroxylation of methyleugenol by pre-treatment of male Fischer 344 rats with compounds which induce cytochromes P450

Inducer	Cytochrome P450 content (nmol/mg protein)	1'hydroxymethyleugenol formation	
		(nmol/min/mg protein)	(nmol/min/nmol P450)
Saline control	0.70 ± 0.05	0.36 ± 0.03	0.51 ± 0.04
Phenobarbitone	$1.82 \pm 0.09^*$	$2.11 \pm 0.14^*$	$1.16 \pm 0.08^*$
Isoniazid	$0.86 \pm 0.07^*$	$0.73 \pm 0.09^*$	$0.85 \pm 0.10^*$
Tricaprylin control	0.73 ± 0.09	0.31 ± 0.02	0.42 ± 0.03
Dexamethasone	$1.33 \pm 0.15^*$	$2.03 \pm 0.10^*$	$1.52 \pm 0.08^*$
Isosafrole	$1.16 \pm 0.04^*$	$1.91 \pm 0.26^*$	$1.65 \pm 0.22^*$

Activities are mean $\pm$ SD of at least four replicate determinations and were determined at 20 μM substrate. *Significantly different from control values, $P < 0.05$.

Animals and treatments with inducers of cytochromes P450

Male Fischer 344 rats (200–250 g), or age matched female Fischer 344 rats, were purchased from Harlan-Olac (Oxford, UK). The animals had access to water and laboratory chow *ad libitum*. For the experiment shown in Table I, inducers of cytochromes P450 were administered i.p.: sodium phenobarbital (100 mg/kg/day for 3 days; $n = 3$) and isoniazid (100 mg/kg/day for 10 days; $n = 6$) were administered in saline, while dexamethasone (80 mg/kg/day for 3 days; $n = 3$) and isosafrole (150 mg/kg/day for 3 days; $n = 3$) were administered in tricaprylin. Control animals received equivalent volumes (<1 ml/kg per rat) of the saline or tricaprylin dose vehicles alone.

In vivo administration of methyleugenol

After seven days acclimatisation, male Fischer 344 rats were assigned to one of five treatment groups ($n = 5$ per group). These animals were weighed, on a daily basis, for three days then dosed by i.p. injection with either 10, 30, 100 or 300 mg methyleugenol/kg/day (dissolved in tricaprylin, which was administered as a constant dose of 1 ml/kg) for each of five days. Control animals ($n = 5$) received tricaprylin alone.

Effect of pre-treatment with dexamethasone on bioactivation of methyleugenol in vivo

Male Fischer 344 rats ($n = 6$ per group) were dosed i.p. with dexamethasone (80 mg/kg/day, for 3 days) dissolved in tricaprylin, or an equivalent volume of tricaprylin vehicle alone. Twenty-four hours after the last dose, the animals received either a single i.p. dose of methyleugenol (100 mg/kg) dissolved in tricaprylin (3 animals from the control group and 3 animals from the dexamethasone pretreatment group) or an equivalent volume of tricaprylin (the remaining animals) and were killed after a further 4 h.

Preparation of hepatic microsomes

Animals were killed by cervical dislocation and the livers were removed and placed in ice cold sucrose buffer (0.25 M sucrose, 15 mM Tris−HCl, 0.1 mM EDTA, pH 6.8). All subsequent steps were performed at 4°C. Livers were blotted to remove excess buffer, weighed, then minced thoroughly with scissors. Five volumes of fresh sucrose buffer were added and homogenates were prepared using eight strokes of a Potter homogenizer, at 1000 rpm. The homogenates were strained through muslin and centrifuged at $10\,000 \times g$ for 20 min, the pellets were resuspended in fresh sucrose buffer and re-centrifuged. The combined $10\,000 \times g$ supernatants were centrifuged at $100\,000 \times g$ for 60 min to sediment the microsomal fraction. Microsomal pellets were resuspended in buffer (100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA), then snap frozen in liquid nitrogen and stored at $-80°C$ until use.

Human liver microsomes

Human liver samples were obtained from kidney donors, in compliance with French legal requirements and with permission from the relevant ethical committee. Liver samples were obtained within 1 h of circulatory arrest and small portions (~1 cm³) were cut, snap-frozen by immersion in liquid nitrogen, then stored at $-80°C$. Subsequently, liver samples were thawed and microsomal fractions were isolated as described previously (9). The following clinical details were available: HL1 (male, 35 yr, death by head trauma, treated with dopamine); HL2 (male cirrhotic; gunshot suicide); HL3 (female, 45 yr, heavy alcohol intake, death by vascular accident); HL4 (male, 13 yr, death by head trauma, treated with dopamine, nembutal and phenytoin); HL5 (male, 64 yr, heavy alcohol intake, death by head trauma, treated with phenylephrine and dopamine). Specific cytochrome P450 contents were determined in 10 of the human liver preparations (see Results for details).

Determination of microsomal cytochrome P450 and protein content

Microsomal cytochrome P450 content was determined by the reduced carbon monoxide difference spectrum method of Omura and Sato (10) using the reported extinction coefficient of 91 mM/cm. Protein content was determined using a bicinchonic acid assay kit (Pierce and Warriner, Chester, UK) with bovine serum albumin as standard.

Measurement of 1'-hydroxymethyleugenol formation

Methyleugenol (1–2000 μM) was incubated with microsomal suspensions (0.5 mg protein/ml) from untreated rats in the presence of an NADPH generating system (1 mM NADP⁺, 10 mM glucose-6-phosphate and 0.5 unit glucose-6-phosphate dehydrogenase). Control experiments consisted of incubations performed with (i) methyleugenol and microsomes in the absence of the NADPH generating system, (ii) methyleugenol and the generating system without microsomes and (iii) microsomes and generating system without methyleugenol. Reactions were stopped after 5–30 min by addition of 1 ml of ice cold 0.05 M sodium phosphate buffer, pH 4.5. Following addition of the internal standard (1'-hydroxysafrole, 10 μg) the incubation mixture was shaken mechanically for 60 min with 5 ml ether. After centrifugation at $1700 \times g$ for 10 min, the ether layer was removed by aspiration and was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl acetonitrile and derivatised by heating at 60°C for 10 min with 200 μl BSTFA. Aliquots (2 μl) were then analysed by GC-MS using a HP5890 series II gas chromatograph connected to a HP5971 Mass Selective Detector (ionization energy 70 eV). The column was 30 m×0.25 mm i.d., 0.25 μm film thickness, HP5MS (Hewlett-Packard Ltd, Bracknell, UK) and helium carrier gas flow was 1 ml/min. The GC temperature programme was held at 80°C for 3 min and then increased at 20°C/min to 270°C. Monitoring was performed in the single ion mode at m/z 250 (for 1'-hydroxysafrole-TMS) and m/z 266 (for 1'-hydroxymethyleugenol-TMS). 1'-Hydroxymethyleugenol was quantitated using a calibration curve prepared by adding known amounts of 1'-hydroxymethyleugenol to boiled microsomes, then processing these samples as described above. Initial experiments verified that reaction rates were linear with respect to protein concentration and time.

Inhibition studies

These experiments were performed at 20 μM methyleugenol, using the concentration of the inhibitors listed in Table II. Troleandomycin, furafylline and cimetidine are mechanism-based inhibitors (11–13) and so were pre-incubated with microsomes, in the presence of the NADPH generating system described above, for 15 min at 37°C prior to addition of methyleugenol. All other inhibitors (PNP, diallylsulfide, α-naphthoflavone, quinine and tolbutamide) were incubated with methyleugenol and microsomes at 37°C for 5 min, then reactions were started by addition of the NADPH generating system.

Determinations of other microsomal catalytic activities

Microsomal incubations were performed in a final volume of 1 ml and contained 0.5 mg of microsomal protein plus the NADPH generating system described previously. Preliminary experiments ensured that reaction rates for each assay were linear with respect to time and protein concentration. Hydroxylation of PNP to 4-nitrocatechol was determined spectrophotometrically according to Koop (14), using a substrate concentration of 100 μM.

Dealkylation of 7-ethoxyresorufin (5 μM), 7-methoxyresorufin (1.25 μM) and 7-pentoxyresorufin (5 μM) were determined by fluorometric measurement of resorufin according to Lake (15). Testosterone hydroxylation was determined according to Arlotto *et al.* (16), at a substrate concentration of 0.25 mM, in the presence of the 5-α-steroid reductase inhibitor 4-MA (10 μM). In these experiments, microsomes were incubated with testosterone for 5 min and reactions were started by addition of NADPH generating system, then stopped after 10 min by addition of 6 ml dichloromethane. 11-α-Hydroxyprogesterone (2.5 μg) was added as internal standard. Following extraction and reconstitution in mobile phase A (methanol: water: acetonitrile, 39:60:1), testosterone

Table II. Influence of isozyme-selective inhibitors of cytochromes P450 on bioactivation of methyleugenol in microsomes from livers of untreated rats

Inhibitor	1′-hydroxymethyleugenol formation (percent of control activity)
p-Nitrophenol (50 μM)	78 ± 5*
p-Nitrophenol (200 μM)	46 ± 12*
Diallylsulfide (200 μM)	88 ± 10
Diallysulfide (1 mM)	63 ± 0*
Tolbutamide (200 μM)	83 ± 10
Tolbutamide (500 μM)	73 ± 7*
Tolbutamide (1 mM)	60 ± 7*
α-naphthoflavone (1 μM)	88 ± 7
α-naphthoflavone (10 μM)	76 ± 2.5*
Troleandomycin (50 μM)	111 ± 9
Troleandomycin (200 μM)	89 ± 10
Furafylline (25 μM)	100 ± 3
Furafylline (100 μM)	89 ± 5
Quinine (1 μM)	100 ± 0.2
Quinine (10 μM)	98 ± 1
Cimetidine (50 μM)	90 ± 9.8

Results were obtained using 20 μM substrate and are percentages (± SD) of the rate of metabolism of methyleugenol in the absence of inhibitors (which was 0.41 ± 0.04 nmol 1′-hydroxymethyleugenol/min/nmol P450).
*Significantly different from control value, $P < 0.05$.

metabolites were quantified by HPLC with reference to calibration curves prepared with standard reference compounds. HPLC analysis was performed using a gradient elution system consisting of two Shimadzu LC6A pumps, a Shimadzu SIL-6A autoinjector and a Shimadzu SPD-6A UV spectrophotometric detector (254 nm) (Shimadzu, Kyoto, Japan). Analysis was performed on a 5 μm C18 reverse phase column (Hichrom, Reading, UK). The samples were eluted with an initial mobile phase composition of 30% mobile phase B (methanol:water:acetonitrile, 80:18:2), 70% mobile phase A, for 15 min, increasing linearly to 35% B at 22 min, then 50% B at 27 min and 90% B at 30 min.

SDS-PAGE and immunoblotting

Microsomal protein samples were diluted in distilled water to the required protein concentration, then diluted 1:1 with sample buffer (8% w/v SDS; 20% w/v glycerol; 0.1% w/v bromophenol blue in 0.25 M Tris−HCl buffer, pH 6.8) and boiled for 5 min in the presence of 6 mg/ml dithiothreitol prior to loading onto polyacrylamide slab gels. The stacking gels contained 4% acrylamide and the resolving gels contained 10% acrylamide. The discontinuous buffer system of Laemmli (17) was employed and electrophoresis was performed at 30 mA per gel and 15°C. Resolved proteins were transferred electrophoretically to nitrocellulose at 200 V and 4°C for 1 h (6). Nitrocellulose membranes were blocked overnight at room temperature in buffer comprising 2.5% (w/v) casein, 0.15 M NaCl, 0.5 mM Thimerosal and 10 mM Tris−HCl, pH 7.6.

The antisera and techniques used to detect methyleugenol-modified polypeptides have been described in detail previously (6). Immunodetection of cytochrome P450 isozymes was performed by incubating the blocked nitrocellulose for 3 h at room temperature with primary antisera (anti-CYP 3A, anti-CYP 2B1/2, anti-CYP1A1 or anti-CYP 1A2, from Amersham International Plc, Bucks., UK) diluted 1:1000 in wash buffer (10 mM Tris−HCl, pH 7.4 containing 0.5% w/v casein, 0.15 M NaCl and 0.5 mM Thimerosal). The nitrocellulose was washed for 5 min in wash buffer containing 0.1% (w/v) SDS and 0.5% (w/v) Triton X−100 in wash buffer, then in wash buffer alone for 2×10 min. Subsequently, nitrocellulose was incubated for 2 h in secondary antiserum (goat anti rabbit IgG, HRP conjugate; Serotec, UK) diluted in wash buffer (1:10 000). After further thorough washing in wash buffer (3×10 min), then 0.2 M NaCl, 50 mM Tris−HCl pH 7.4 (3×5 min), bound secondary antibodies were detected by enhanced chemiluminescence (ECL), using commercial ECL Western blotting detection reagents (from Amersham International Plc, Bucks, UK). Finally, nitrocellulose membranes were wrapped in Saran wrap (from ROTEC, Bucks, UK) and exposed to X-ray film (Hyperfilm, from Amersham International Plc) in an autoradiography cassette for between 10 s and 5 min. X-ray films were developed according to the manufacturer's instructions and relative intensities of immunoblot signals were determined by densitometric scanning using a CS-930 Dual Wavelength Scanner (Shimadzu, Kyoto, Japan) coupled to a DR-2 data recorder (Shimadzu, Kyoto, Japan).
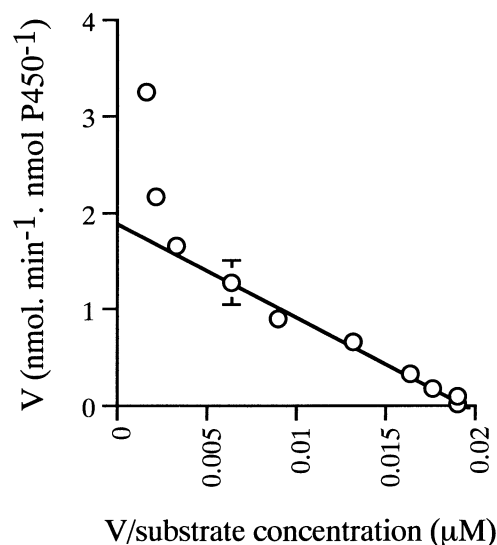

Fig. 1. Kinetics of 1′-hydroxylation of methyleugenol in control rat liver microsomes. Methyleugenol (1–2000 μM) was incubated with microsomes from livers of male Fischer 344 rats in the presence of an NADPH generating system. Data points are mean ± SD of at least 3 different microsome preparations. The line represents the estimates of the high affinity, low K_m site as predicted by NON-LIN.

Statistics

Kinetic analysis was performed using PC-Non Lin with a weighting of $1/y^2$. K_i was determined mathematically and graphically from Dixon plots. Statistical significance was assessed either by two-way analysis of variance followed by the least significant difference test or by the non-parametric Mann–Whitney U-test, as appropriate. The level of statistical significance was set at $P < 0.05$.

Results

Kinetics of formation of 1′-hydroxymethyleugenol

Analysis of the kinetics of 1′-hydroxylation of methyleugenol formation in control male F344 rat liver microsomes yielded a non-linear Eadie-Hofstee plot (Figure 1), indicating the existence of distinct high and low affinity enzymic reactions. The high affinity activity exhibited a K_m of 74.9 ± 9.0 μM and a V_{max} of 1.42 ± 0.17 nmol/min/mol P450 (mean ± SD of three experiments undertaken with different microsomal preparations). Due to the low solubility of methyleugenol it was not possible to define the kinetics of the low affinity reaction accurately, but it had an apparent $K_m > 2$ mM. The rate of formation of 1′-hydroxymethyleugenol exhibited a small but statistically significant increase ($P < 0.05$) in liver microsomes prepared from female rats, when assayed at a low substrate concentration (20 μM) (female, 0.43 ± 0.03 nmol/min/nmol P450; male, 0.34 ± 0.03). This difference was not evident when activities were determined at a high substrate concentration (500 μM) (female, 1.54 ± 0.03 nmol/min/nmol P450; male, 1.54 ± 0.08 nmol/min/nmol P450).

Effect of cytochrome P450 inducers on 1′-hydroxylation of methyleugenol

When assayed at a low concentration of methyleugenol (20 μM), formation of 1′-hydroxymethyleugenol was increased significantly ($P < 0.05$) in microsomes prepared from the livers of rats pre-treated with phenobarbital (2.3-fold greater than control, when expressed per nmol P450), dexamethasone (3-fold), isosafrole (3.2-fold) and isoniazid (1.7-fold) (Table I). These treatments were shown to result in the expected pattern of isozyme selective cytochrome P450 catalytic activities.
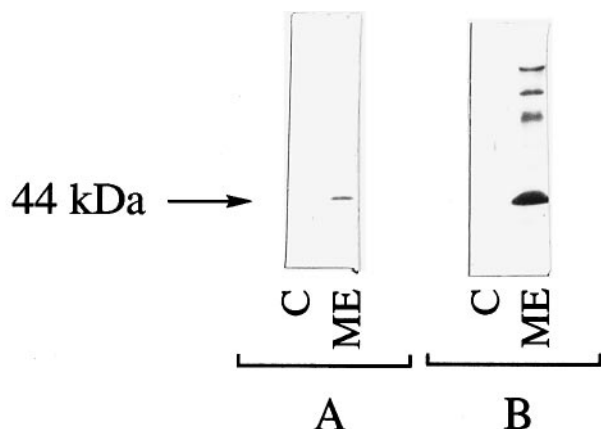
Fig. 2. Influence of dexamethasone pre-treatment on methyleugenol protein adduct formation *in vivo*, in livers of male Fischer 344 rats. Rats ($n = 3$ per group) were pre-treated i.p. with dexamethasone (80 mg/kg/day) in tricaprylin for 3 days (**B**), or tricaprylin vehicle alone (**A**), then given single i.p. doses of methyleugenol (100 mg/kg) in tricaprylin (ME), or tricaprylin alone (C), and killed after a further 4 h. Liver microsomal samples from these animals were analyzed by immunoblotting, using an anti-(methyleugenol adduct) antiserum. Protein loading was 40 μg per lane.

Fig. 3. Inhibition by methyleugenol of metabolism of *p*-nitrophenol to 4-nitrocatechol in liver microsomes from male Fischer 344 rats. Microsomes were pre-incubated at 37°C for 5 min with *p*-nitrophenol and methyleugenol (ME, at the concentrations indicated), then activities (nmol f-nitrocatechol formed/min/mg/protein) were assayed after addition of an NADPH generating system. Each data point is the mean ± SD of 4 replicate determinations.

When compared with the activities measured in control microsomes and expressed per nmol P450, phenobarbital produced a 21.6-fold increase in 7-pentoxyresorufin-*O*-depentylase (PROD) activity (catalyzed by CYP 2B1/2), while dexamethasone increased 6β-hydroxytestosterone activity by 7.4 fold (CYP 3A1/2), isosafrole increased methoxyresorufin-*O*-demethylase (MROD) activity by 7.2-fold (CYP 1A1/2) and isoniazid increased 4-nitrocatechol formation from PNP by 4.2-fold (CYP 2E1). The control activities (means ± SD of 4 replicate determinations, expressed as pmol product/min^{-1}/nmol P450^{-1}) were: PROD 8.7 ± 1.4; testosterone 6β-hydroxylase 240 ± 45.7; MROD 14.3 ± 1.4; PNP hydroxylase 700 ± 57.1.

Effect of pre-treatment with dexamethasone on the formation of methyleugenol protein adducts in vivo.

Previously we have reported that livers from rats treated i.p. with methyleugenol express novel, reactive metabolite-modified protein adducts, which can be detected using methyleugenol adduct-specific rabbit antisera (6). Generation of these adducts, which were not expressed in livers from control rats, was found to require bioactivation of methyleugenol via the 1′-hydroxymetabolite (6). Livers from rats treated i.p. with methyleugenol (100 mg/kg), after pre-treatment for 3 days with dexamethasone (80 mg/kg/day), exhibited markedly higher levels of these adducts than livers of rats given methyleugenol without dexamethasone pre-treatment (Figure 2).

Effect of isozyme selective cytochrome P450 inhibitors on 1′-hydroxylation of methyleugenol

Assays undertaken at a low methyleugenol substrate concentration (20 μM) revealed that formation of 1′-hydroxymethyleugenol was inhibited significantly ($P < 0.05$) and in a concentration-dependent manner by PNP, diallylsulfide, tolbutamide and α-naphthoflavone, but not by troleandomycin, furafylline, quinine or cimetidine (Table II). PNP was the most potent inhibitor, causing 54% inhibition, followed by tolbutamide (40%), diallylsulfide (37%) and α-naphthoflavone (24%). Furthermore, methyleugenol was a potent competitive
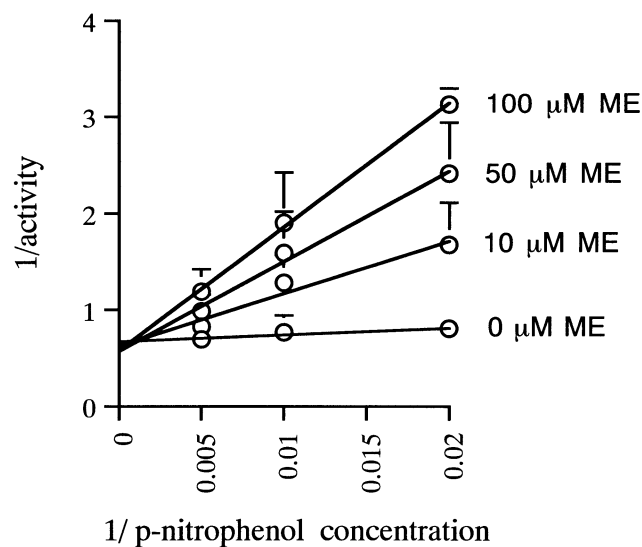
inhibitor of the metabolism of PNP to 4-nitrocatechol (apparent K_i 33 μM; Figure 3).

Auto-induction of bioactivation in rats treated in vivo with methyleugenol

Rats which had been treated i.p. with methyleugenol for 5 days exhibited dose-dependent auto-induction of 1′-hydroxylation of the compound (Table III). This auto-induction was statistically significant even at doses as low as 30 mg methyleugenol/kg/day ($P < 0.01$)), while the greatest increase (1.7-fold over control) was at the highest dose level tested (300 mg/kg/day). However, no auto-induction was evident following treatment of rats with methyleugenol at 10 mg/kg/day (Table III).

The group of rats dosed with methyleugenol at 300 mg/kg/day for 5 days exhibited significantly lower body weights and absolute liver weights than control animals (Table III). These changes were not evident in animals given lower doses of the compound (10, 30 or 100 mg/kg/day). Although no statistically significant change in total liver microsomal cytochrome P450 content was detected following administration of methyleugenol to the rats (Table III), dose-dependent alterations in several isozyme-selective catalytic activities were observed, while immunoblotting studies performed using specific antisera revealed induction of expression of two isozymes. Of the activities assayed, PROD exhibited the greatest maximal induction (5-fold over control, when expressed per nmol P450; Table IV). Indeed, PROD activity was increased significantly even in livers of rats given the lowest dose of methyleugenol ($P < 0.01$). Significant ($P < 0.01$) dose-dependent induction of EROD (1.9-fold greater than control) and testosterone 7α- (1.6-fold) and 6β- (2.5-fold) hydroxylase activities by methyleugenol was also observed, as was a very modest but significant ($P < 0.05$) induction of MROD activity (1.1-fold). A 1.2-fold induction of PNP hydroxylation was evident at the highest dose of methyleugenol (300 mg/kg/day). In addition, a very modest (1.1-fold) but significant induction of testosterone 16α- and 2α-hydroxylase was evident in microsomes from

Table III. Autoinduction of bioactivation of methyleugenol in rats treated with the compound *in vivo* for 5 days

Parameter	Dose of methyleugenol (mg/kg/day)				
	0	10	30	100	300
Rate of 1′-hydroxylation of methyleugenol (nmol/min/nmol P450)	0.42 ± 0.03	0.43 ± 0.04	0.58 ± 0.07**	0.61 ± 0.09**	0.70 ± 0.13**
Cytochrome P450 content (nmol/mg protein)	0.73 ± 0.09	0.72 ± 0.09	0.73 ± 0.09	0.74 ± 0.03	0.86 ± 0.10
Final liver weight (g)	11.8 ± 1.3	11.5 ± 0.5	11.6 ± 1.0	10.9 ± 1.5	8.9 ± 0.7**
Final body wt (g)	268 ± 18	263 ± 9	268 ± 11	254 ± 17	210 ± 11**
Ratio, final liver wt/100 g body wt	4.40	4.37	4.31	4.28	4.24

Rats ($n = 5$ per group) were treated i.p. with methyleugenol, at the doses indicated, for each of 5 days. Rates of formation of 1′-methyleugenol, which were assayed using 20 μM methyleugenol, and cytochrome P450 contents, were determined using pooled liver microsomes and are means ± SD of at least four replicate determinations. **Significantly different from values in control rats, $P < 0.01$.

Table IV. Cytochrome P450-dependent catalytic activities of microsomes from livers of rats treated with methyleugenol *in vivo* for 5 days, assessed using isozyme-selective substrates

Activity	Dose of methyleugenol (mg/kg/day)				
	0	10	30	100	300
p-Nitrophenol hydroxylase	1274 ± 41	1278 ± 28 (100)	1233 ± 68 (97)	1284 ± 95 (101)	1535 ± 47** (120)
7-Ethoxyresorufin-*O*-dealkylase	15.3 ± 0.4	16.1 ± 0.3 (105)	19.3 ± 0.3** (126)	23.6 ± 0.4**	28.4 ± 0.2** (185)
7-Methoxyresorufin-*O*-dealkylase	28.9 ± 1.4	32.8 ± 3.2 (113)	29.6 ± 2.1 (102)	34.2 ± 2.2** (118)	31.5 ± 1.3* (109)
7-Pentoxyresorufin-*O*-dealkylase	7.4 ± 0.4	8.6 ± 0.4** (116)	10.8 ± 0.1** (146)	21.5 ± 0.4** (291)	37.4 ± 0.6** (506)
Testosterone 7-α-hydroxylase	560 ± 44	568 ± 58 (101)	651 ± 36 (116)	707 ± 26** (126)	902 ± 69** (161)
Testosterone-6β-hyroxylase	474 ± 30	532 ± 60 (112)	614 ± 44** (129)	823 ± 23** (174)	1167 ± 69** (246)
Testosterone 16α-hydroxylase	4701 ± 232	4836 ± 377 (103)	5542 ± 200** (118)	4334 ± 143 (92)	1299 ± 92** (28)
Testosterone 2α-hydroxylase	3873 ± 260	3883 ± 300 (100)	4379 ± 134* (113)	3449 ± 172 (89)	1071 ± 42** (28)

Results (pmol/min/nmol P450) are means ± SD of at least 4 replicate determinations. Numbers in parentheses represent data expressed as a percentage of control values. *Significantly different to activities of control rat liver microsomes, $P < 0.05$; **$P < 0.01$.

Table V. Bioactivation of methyleugenol by human liver microsomes

Human liver sample	Cytochrome P450 content (nmol/mg protein)	1′-hydroxymethyleugenol formation (nmol/min^{-1}/mg protein^{-1})
HL1	0.75	0.34 ± 0.08
HL2	0.32	0.11 ± 0.02
HL3	0.50	0.94 ± 0.12
HL4	n.d.	1.33
HL5	n.d.	0.55
HL6	0.46	0.41 ± 0.01
HL7	0.46	0.38 ± 0.02
HL8	0.45	0.54 ± 0.02
HL9	0.69	0.38 ± 0.00
HL10	0.33	0.05 ± 0.01
HL11	0.38	0.23 ± 0.04
HL12	0.64	0.41 ± 0.00
HL13	n.d.	0.81

Activities are means of duplicate or triplicate determinations (± SD for triplicates), assayed at 200 μM substrate. n.d. = not determined.

livers of rats given methyleugenol at 30 mg/kg/day, while these activities were markedly depressed (by 3.6-fold) following administration of methyleugenol at 300 mg/kg/day (Table IV).

The immunoblotting studies demonstrated dose-dependent induction of CYP 2B1/2 and CYP 1A2 isozymes in livers of the methyleugenol-treated rats. CYP 2B1/2 protein was not detectable in liver microsomes from control animals or rats that had received low doses of methyleugenol (10 or 30 mg/

kg/day), but was clearly evident in the animals given the higher doses (Figure 4A). Densitometric analysis revealed that the levels of CYP 1A2 protein in livers of rats given methyleugenol at 100 and 300 mg/kg/day were increased by 2.3-fold and 3.3-fold over control values, respectively (Figure 4D). However, no induction of CYP 1A1 and CYP 3A isozymes was detected (Figure 4B and C).

1′-Hydroxylation of methyleugenol in human liver microsomes
Investigations of 1′-hydroxylation of methyleugenol in human liver microsomal fractions were undertaken using a concentration of substrate (200 μM) approximately equivalent to the V_{max} of the high affinity enzymic component identified in rat liver microsomes. These studies revealed a 37-fold variation in the rate of the reaction (Table V). The rate of 1′-hydroxylation observed in the most active human liver (HL4; 1328 ± 100 pmol/min/mg protein) was similar to those seen in control male rat liver microsomes, when assayed at 200 μM substrate (1.28 ± 0.23 nmol/min/mg protein). The variable rates of metabolism of methyleugenol by the human liver microsomal samples did not correlate with their cytochrome P450 contents ($r = 0.33$, $p = 0.36$), which for 10 of the preparations were determined by UV difference spectroscopy (see Table V).

Discussion

The current studies have shown that 1′-hydroxylation of methyleugenol, which previously has been implicated in formation of DNA and protein adducts and thereby in the rodent hepatotoxicity and carcinogenicity of the compound, is cata-
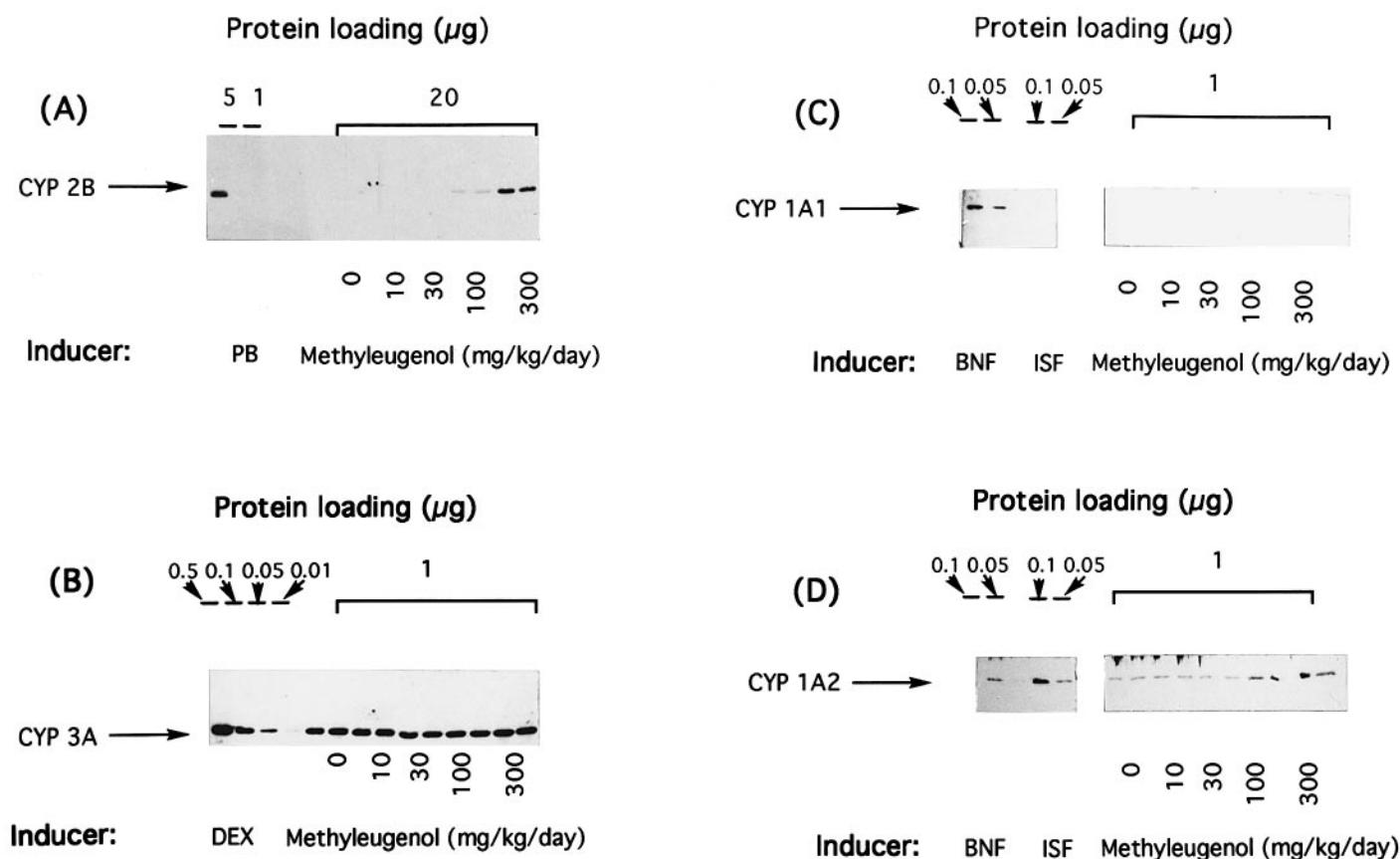
Fig. 4. Expression of cytochrome P450 isozymes in livers from male Fischer 344 rats treated with methyleugenol for 5 days. Liver microsomal fractions were analyzed by immunoblotting, using antisera specific for CYP 2B1/2 (**A**), CYP 3A (**B**), CYP 1A1 (**C**) and CYP 1A2 (**D**) isozymes, respectively. Microsomes from livers of male Fischer 344 rats that had been treated with phenobarbital (PB) (panel A), dexamethasone (DEX) (panel B), β-naphthoflavone (BNF) and isosafrole (ISF) (panels C and D) were included as positive controls. Liver microsomes from methyleugenol-treated rats were analyzed using protein loadings of 40 μg per lane.

lyzed *in vitro* by distinct high affinity (apparent K_m of 74.9 ± 9.0 μM; apparent V_{max} of 1.42 ± 0.17 nmol/min/nmol P450) and low affinity (apparent K_m several mM) enzymic components in microsomes from livers of untreated male Fischer 344 rats (Figure 1). The NTP carcinogenicity bioassay on methyleugenol, which is under evaluation currently, involves lifetime treatment of rats and mice with the compound at 0, 37, 75 or 150 mg/kg/day. In these chronic toxicity studies, bioactivation of methyleugenol should be catalyzed predominately by the high affinity, low K_m enzymic component. A recent study has shown that concentrations of methyleugenol in plasma of rats given the compound orally, at doses of 37 and 150 mg/kg, are ~1–7 μg/ml (5.6–39 μM), while peak plasma concentrations of 20 μg/ml (112 μM) are reached following i.v. dosing with methyleugenol at 37 mg/kg (18).

The marked increase in the rate of 1′-hydroxylation of methyleugenol that was evident in microsomes from rats treated *in vivo* with phenobarbital, dexamethasone, isosafrole or isoniazid, when assayed at a low substrate concentration (20 μM) (Table I), implies that several cytochrome P450 isozymes can catalyze the high affinity reaction. This is because phenobarbital has been shown to induce CYP 2B1/2, CYP 2C and CYP 3A isozymes (19,20), while dexamethasone is an inducer of CYP 3A and 2B isozymes (21), isosafrole induces CYP 1A1 and CYP 1A2 isozymes (22), and isoniazid is a selective CYP 2E1 inducer (23,24). However, the results

obtained using various isozyme-selective chemical inhibitors indicate that, when assayed at low substrate concentration, 1′-hydroxylation of methyleugenol in liver microsomes from untreated rats is catalyzed predominantly by CYP 2E1 and by one or more additional isozymes (most probably CYP 2C6).

Involvement of CYP 2E1 in 1′-hydroxymethyleugenol formation is consistent with the inhibition of the reaction seen in the presence of diallylsulfide and PNP (37% and 54% inhibition, respectively), which selectively inhibit this isozyme (25,26). This interpretation is further supported by the observation that methyleugenol competitively inhibited hydroxylation of PNP to 4-nitrocatechol (Figure 3), which is a reaction catalyzed by CYP 2E1 (27). CYP 2E1 has been implicated previously in the bioactivation and metabolism of a number of other hepatotoxins and carcinogens, including paracetamol, benzene, nitrosamines and carbon tetrachloride (28,29).

Involvement of CYP 2C6 in 1′-hydroxylation of methyleugenol seems likely in view of the significant (40%) inhibition of the reaction by tolbutamide, which has been shown to inhibit CYP 2C6 mediated reactions (30) and by α-naphthoflavone (24% inhibition). α-Naphthoflavone has been shown to inhibit tolbutamide hydroxylation (31) and to inhibit the N[4]-hydroxylation of sulfamethoxazole, which is catalyzed by CYP 2C6 (30). Although α-naphthoflavone is known to be a potent inhibitor of CYP 1A isozymes (32), 1′-hydroxylation of methyleugenol was not inhibited significantly by the potent

CYP 1A2 inhibitor furafylline (12,33). This excludes the possibility that CYP 1A2 makes a significant contribution to catalysis of the high affinity component of 1′-hydroxymethyleugenol formation in livers of untreated rats. The relatively modest induction of 1′-hydroxymethyleugenol formation evident in microsomes from livers of rats treated with phenobarbital (2.3-fold) is also consistent with induction of CYP 2C6 (20,21,30,31). This may be contrasted with the very marked (21.6-fold) induction of PROD activity, which is catalyzed by CYP 2B1/2 isozymes (34), that was evident in the phenobarbital-induced microsomes. Moreover, it should be noted that CYP 2B1/2 isozymes are expressed only at very low levels in livers of untreated rats (34). Involvement of CYP 3A isozymes in the high affinity component of 1′-hydroxylation of methyleugenol in untreated rat liver microsomes may be excluded since troleandomycin, which is a potent CYP 3A inhibitor (35), did not inhibit the reaction. The other compounds which did not inhibit formation of 1′-hydroxymethyleugenol were the CYP 2D1 inhibitor quinine (36) and cimetidine, which under the conditions used in the present study (50 μM, with 15 min pre-incubation) is a selective inhibitor of CYP 2C11 (11).

Since 1′-hydroxylation is the first step in metabolic bioactivation of methyleugenol, any factor increasing the fraction of a dose of methyleugenol metabolized by this pathway is likely to increase the amount of electrophilic metabolite bound to cellular DNA and protein, and thus increase the potency of methyleugenol as a carcinogen and hepatotoxin. This interpretation is supported by the marked increase in methyleugenol-protein adduct formation that was evident when rats were pre-treated with dexamethasone, to induce cytochrome P450-dependent bioactivation, before administration of methyleugenol (Figure 2). Methyleugenol-protein adduct formation has been shown to proceed via the 1′-hydroxy metabolite (8), and so is a useful surrogate marker of bioactivation of the compound *in vivo* in the target organ for toxicity (the liver). This finding confirms and extends previous investigations, which showed that co-administration of cytochrome P450 inducers and the related allylbenzene safrole to rats resulted in increased production of 1′-hydroxysafrole and in increased numbers of hepatic tumours, when compared with rats given safrole alone (37,38). We did not investigate the effects of pre-treatment of rats with isoniazid and isosafrole on methyleugenol-protein adduct formation *in vivo* because both compounds bind to the active sites of the cytochrome P450 isozymes that they induce (7,39,40).

Consequently, our observation of auto-induction of 1′-hydroxylation of methyleugenol in microsomes from livers of rats given the compound *in vivo* for 5 days, at doses ranging from 30–300 mg/kg/day, but not in rats given methyleugenol for 5 days at a dose of 10 mg/kg/day, has important toxicological implications. The high doses of methyleugenol administered to rodents in the NTP carcinogenicity assay which is under evaluation currently (37, 75 and 150 mg/kg/day) will have caused significant auto-induction of bioactivation. Such auto-induction will not occur in the human population, where levels of dietary ingestion of the compound are of the order of μg/kg/day. Thus, the results of the NTP carcinogenicity bioassay are likely to significantly overestimate the risk to humans posed by methyleugenol. The possibility that auto-induction of bioactivation occurs during high dose rodent carcinogenicity studies should be considered for other allylbenzenes, and indeed other classes of compounds.

The auto-induction of bioactivation of methyleugenol evident in rats can be attributed to induction of expression of cytochromes P450. The profile of induced cytochrome P450 apoproteins that was detected by immunoblotting, and of altered cytochrome P450-dependent catalytic activities, indicates that multiple mechanisms are involved. The elevated expression of CYP 2B apoprotein, the elevated activities of PROD and testosterone 7α- and 6β-hydroxylation, and the depression of testosterone 16α- and 2α-hydroxylation, are all consistent with a weak phenobarbital-like pattern of enzyme induction (16,34,41,42). Moreover, the elevated MROD and EROD activities indicate dose-dependent induction of CYP 1A (34), as does the elevated levels of CYP1A2 apoprotein, while the elevated PNP hydroxylase activity that was evident in livers of rats treated with the highest dose of methyleugenol (300 mg/kg/day) implies induction of CYP 2E1 (23,26), which perhaps is related to the weight loss seen in these animals.

Recently it has been shown that long term (13 weeks) administration of methyleugenol to rodents leads to increased liver size (43), which is a characteristic of phenobarbital administration. Perhaps methyleugenol, in addition to being a genotoxic carcinogen, can exert epigenetic tumour promoting effects when given to rodents at high doses for prolonged periods, as has been shown for phenobarbital (44,45). It should be noted that phenobarbital does not appear to be a human hepatocarcinogen, even though it has been shown to induce human cytochromes P450 (46). In future work, it will be important to investigate the contribution of epigenetic mechanisms to the overall carcinogenic potential of methyleugenol and to assess whether such mechanisms operate in human, as well as rodent, tissues.

The primary objective of the studies undertaken using human liver microsomes was to assess whether the cytochromes P450 expressed in a panel of human livers had the capability to catalyze 1′-hydroxylation of methyleugenol. The results obtained demonstrate that the first step in metabolic bioactivation of methyleugenol is catalyzed by the human enzymes. These studies were undertaken using 200 μM substrate. If used in the studies of rat liver microsomes, this substrate concentration would have revealed catalytic activities that were attributable primarily to the low K_m enzymic component and approximated to the V_{max} of this component. Consequently, the 37-fold variation in the rate of 1′-hydroxymethyleugenol formation observed in the 13 human liver preparations raises the possibility that the toxic potential of the compound is subject to marked inter-individual variability in the human population. Whether this is due to variable expression of CYP 2E1, CYP 2C isozymes, or other isozymes of cytochrome P450 is unclear. Although rat and human CYP 2E1 exhibit a high degree of similarity in amino acid sequence and substrate specificity (47), the structures and catalytic activities of CYP 2C family members and of many other P450 isozymes exhibit marked species variabilities (47,48). In future experiments it will be important to define the human isozymes (cytochromes P450 and sulfotransferases) which catalyze bioactivation of methyleugenol. In combination with toxicity data which avoids the problems posed by *in vivo* auto-induction of bioactivation, such information should greatly aid the assessment of the risk to human health posed by dietary ingestion of this compound.

References

1. Miller,E.C., Swanson,A.B., Phillips,D.H., Fletcher,T.L., Liem,A. and Miller,J.A. (1983) Structure activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.*, **43**, 1124–1134.

2. Randerath,K., Haglund,R.E., Phillips,D.H. and Reddy,M.V. (1984) ^{32}P-Post labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis*, **5**, 1613–1622.

3. Swanson,A.B., Miller,E.C. and Miller,J.A. (1981) The side-chain epoxidation and hydroxylation of the hepatocarcinogens safrole and estragole and some related compounds by rat and mouse liver microsomes. *Biochim. Biophys. Acta*, **673**, 504–516.

4. Boberg,E.W., Miller,E.C., Miller,J.A., Poland,A. and Liem,A. (1983) Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfooxysafrole is the major electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver. *Cancer Res.*, **43**, 5163–5173.

5. Chan,V.S.W. and Caldwell,J. (1992) Comparative induction of unscheduled DNA synthesis in cultured rat hepatocytes by allylbenzenes and their 1′-hydroxy metabolites. *Fd. Chem. Toxicol.*, **30**, 831–836.

6. Gardner,I., Bergin,P., Stening,P., Kenna,J.G. and Caldwell,J. (1996) Immunochemical detection of covalently modified protein adducts in the livers of rats treated with methyleugenol. *Chem. Res. Toxicol.*, **9**, 713–721.

7. Guengerich,F.P. (1987) Enzymology of rat liver cytochromes P-450. In Guengerich,F.P. (ed.) *Mammalian Cytochromes P-450*. CRC Press, Boca Raton, Florida, vol. 1, pp. 1–54.

8. Borchett,P., Wislocki,P.G., Miller,J.A. and Miller,E.C. (1973) Metabolism of the naturally occurring hepatocarcinogen safrole to 1′-hydroxysafrole and the electrophilic reactivity of 1′-acetoxysafrole. *Cancer Res.*, **33**, 575–589.

9. Kremers,P., Beaune,P.H., Cresteil,T., De Graeve,J., Columelli,L., Le Roux,J.P. and Gielen,J.E. (1981) Cytochrome P450 monoxygenase activities in human and rat liver microsomes. *Eur. J. Biochem.*, **1181**, 599–606.

10. Omura,T. and Sato,R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its haemoprotein structure. *J. Biol. Chem.*, **239**, 2370–2378.

11. Chang,T., Levine,M. and Bellward,G.D. (1992) Selective inhibition of rat hepatic microsomal cytochrome P450 II. Effect of the *in vitro* administration of cimetidine. *J. Pharm. Exp. Ther.*, **260**, 1450–1455.

12. Kunze,K.L. and Trager,W.F. (1992) Isoform selective mechanism based inhibition of human cytochrome P450 1A2 by furafylline. *Chem. Res. Toxicol.*, **6**, 649–656.

13. Kato,R. and Yamazoe,Y. (1992) Sex-specific cytochrome P450 as a cause of sex and species related differences in drug toxicity. *Toxicol. Lett.*, **64/65**, 661–665.

14. Koop,D.R. (1990) Inhibition of ethanol inducible cytochrome P450IIE1 by 3-amino-1,2,4-triazole. *Chem. Res. Toxicol.*, **3**, 377–383.

15. Lake,B.G. (1987) Preparation and characterisation of microsomal fractions for studies on xenobiotic metabolism. In Snell,K. and Mullock,B. (eds) *Biochemical Toxicology*. IRL Press, London, pp. 183–215.

16. Arlotto,M.P., Trani,J.M. and Estabrook,R. (1991) Measurement of steroid hydroxylation reactions by high performance liquid chromatography as indicator of P450 identity and function. *Methods Enzymol.*, **206**, 454–462.

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

18. Graves,S.W. and Runyon,S. (1995) Determination of methyleugenol in rodent plasma by high performance liquid chromatography. *J. Chromatogr. B*, **663**, 255–262.

19. Omiecinski,C.J. (1986) Tissue specific expression of rat mRNAs homologous to cytochromes P450b and P450e. *J. Biol. Chem.*, **260**, 3247–3250.

20. Waxman,D.J. and Azaroff,L. (1992) Phenobarbital induction of cytochrome P450 gene expression. *Biochem. J.*, **281**, 577–592.

21. Meehan,R.R., Forrester,L.M., Stevenson,K., Hastie,N.D., Buchmann,A., Kunz,H.W. and Wolf,C.R. (1988) Regulation of phenobarbital-inducible cytochrome P450s in rat and mouse liver following dexamethasone administration and hypophysectomy. *Biochem. J.*, **259**, 789–797.

22. Soucek,P. and Gut,I. (1992) Cytochromes P-450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica*, **22**, 83–103.

23. Koop,D.R., Crump,B.L., Nordbloom,G.D. and Coon,M.J. (1985) Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P450 of liver microsomes by diverse agents: ethanol, imidazole, trichloroethylene, acetone, pyrazole and isoniazid. *Proc. Natl Acad. Sci. USA*, **82**, 4065–4069.

24. Wortelboer,H.M., De Kruif,C.A., Van Iersel,A.A.J., Falke,H.E., Noordhoek,J. and Blaauboer,B.J. (1991) Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures. The effects of model inducers on apoproteins and biotransformation activities. *Biochem. Pharmacol.*, **42**, 381–390.

25. Brady,J.F., Ishizaki,H., Fukoto,J.M., Lin,M.C., Fadel,A., Gapac,J.M. and Yang,C.S. (1991) Inhibition of cytochrome P450 2E1 by diallylsulfide and its metabolites. *Chem. Res. Toxicol.*, **4**, 642–647.

26. Koop,D.R. (1986) Hydroxylation of *p*-nitrophenol by rabbit ethanol-inducible P450 isozyme 3a. *Mol. Pharmacol.*, **29**, 399–404.

27. Tassaneeyakul,W., Veronese,M.E., Birkett,D.J., Gonzalez,F.J. and Miners,J.O. (1993) Validation of 4-nitrophenol as an *in vitro* substrate probe for human liver CYP 2E1 using cDNA expression and microsomal kinetic techniques. *Biochem. Pharmacol.*, **46**, 1975–1981.

28. Guengerich,F.P., Kim,D.-H. and Iwasaki,M. (1991) Role of human cytochrome P450 HE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.*, **4**, 168–179.

29. Patten,C.J., Thomas,P.E., Guy,R.L., Lee,M.J., Gonzalez,F.J., Guengerich,F.P. and Yang,C.S. (1993) Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem. Res. Toxicol.*, **6**, 511–518.

30. Cribb,A.E., Spielberg,S.P. and Griffin,G.P. (1995) N^4-Hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome 2C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug. Met. Disp.*, **23**, 406–414.

31. Veronese,M., McManus,M.E., Laupattarakasem,P., Miners,J.O. and Birkett,D.J. (1990) Tolbutamide hydroxylation by human, rabbit and rat liver microsomes and by purified forms of cytochrome P450. *Drug. Met. Disp.*, **18**, 356–361.

32. Shimada,T. and Okuda,Y. (1988) Metabolic activation of environmental carcinogens and mutagens by human liver microsomes: role of cytochrome P-450 homologous to a 3-methylcholanthrene-inducible isozyme in rat liver. *Biochem. Pharmacol.*, **37**, 459–464.

33. Sesardic,B., Boobis,A., Murray,B., Murray,S., Segura,J., De La Torre,R. and Davies,D.S. (1990) Furafylline is a potent and selective inhibitor of cytochrome P450 1A2 in man. *Br. J. Clin. Pharmacol.*, **29**, 651–653.

34. Burke,M.D., Thompson,S., Weaver,R.J., Wolf,C.R. and Mayer,R.T. (1994) Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. *Biochem. Pharmacol.*, **48**, 923–936.

35. Pessayre,D., Tinel,M., Larrey,D., Cobert,B., Funck-Brentano,C. and Bobany,G. (1983) Inactivation of cytochrome P-450 by a troleandomycin metabolite. Protective role of glutathione. *J. Pharm. Exp. Ther.*, **224**, 685–691.

36. Kobayashi,S., Murray,S., Watson,D., Sesardic,D., Davies,D.S. and Boobis,A.R. (1989) The specificity of inhibition of debrisoquine-4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochem. Pharm.*, **38**, 2795–2799.

37. Borchert,P., Miller,J.A., Miller,E.C. and Shires,T.K. (1973) 1′-Hydroxysafrole a proximate carcinogenic metabolite of safrole in the rat and mouse. *Cancer Res.*, **33**, 590–600.

38. Wislocki,P.G., Miller,E.C., Miller,J.A., McCoy,E.C. and Rosenkranz,H. (1977) Carcinogenic and mutagenic activities of safrole, 1′-hydroxysafrole, and some known or possible metabolites. *Cancer Res.*, **37**, 1883–1891.

39. Ryan,D.E., Thomas,P.E. and Levin,W. (1980) Hepatic microsomal cytochrome P450 from rats treated with isosafrole: purification and characterization of four enzymic forms. *J. Biol. Chem.*, **255**, 7941–7955.

40. Persson,J.-O., Terelius,Y. and Ingelman-Sundberg,M. (1990). Cytochrome P450 dependent formation of reactive oxygen radicals: isozyme specific inhibition of P450 mediated reduction of oxygen and carbon tetrachloride. *Xenobiotica*, **20**, 887–890.

41. Guengerich,F.P., Dannan,G.A., Wright,S.T., Martin,M.V. and Kaminsky,L.S. (1982) Purification and characterization of rat liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry*, **21**, 6019–6030.

42. Arlotto,M.P. and Parkinson,A. (1989) Identification of cytochrome P450a (P450IIA1) as the principal testosterone 7α-hydroxylase in rat liver microsomes and its regulation by thyroid hormones. *Arch. Biochem. Biophys.*, **270**, 458–471.

43. National Toxicology Program (1989) Subchronic study of methyleugenol (C60991). Contract No. NO1-ES-6-5158.

44. Schulte-Hermann,R. (1985) Tumor promotion in the liver. *Arch. Toxicol.*, **57**, 147–158.

45. Jirtle,R.J. and Meyer,S.A. (1991) Liver Tumor Promotion: Effect of phenobarbital on EGF and protein kinase C signal transduction and transforming growth factor β-1 expression. *Dig. Dis. Sci.*, **36**, 659–668.

46. Olsen,J.H., Boice,J.D., Jensen,J.P.A. and Fraumeni,J.F. (1989) Cancer among epileptic patients exposed to anticonvulsant drugs. *J. Natl Cancer Inst.*, **81**, 803–808.
47. Gonzalez,F.J. (1992) Human cytochromes P450: problems and prospects. *Trends Pharmacol. Sci.*, **13**, 346–352.
48. Smith,D.A. (1991) Species differences in metabolism and pharmacokinetics: are we close to an understanding? *Drug. Met. Rev.*, **23**, 355–373.