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Human Cytochrome P450 Enzymes of Importance for the Bioactivation of Methyleugenol to the Proximate Carcinogen 1′-Hydroxymethyleugenol

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In vitro studies were performed to elucidate the human cytochrome P450 enzymes involved in the bioactivation of methyleugenol to its proximate carcinogen 1′-hydroxymethyleugenol. Incubations with Supersomes, expressing individual P450 enzymes to a high level, revealed that P450 1A2, 2A6, 2C9, 2C19, and 2D6 are intrinsically able to 1′-hydroxylate methyleugenol. An additional experiment with Gentest microsomes, expressing the same individual enzymes to roughly average liver levels, indicated that P450 1A2, 2C9, 2C19, and 2D6 contribute to methyleugenol 1′-hydroxylation in the human liver. A study, in which correlations between methyleugenol 1′-hydroxylation in human liver microsomes from 15 individuals and the conversion of enzyme specific substrates by the same microsomes were investigated, showed that P450 1A2 and P450 2C9 are important enzymes in the bioactivation of methyleugenol. This was confirmed in an inhibition study in which pooled human liver microsomes were incubated with methyleugenol in the presence and absence of enzyme specific inhibitors. Kinetic studies revealed that at physiologically relevant concentrations of methyleugenol P450 1A2 is the most important enzyme for bioactivation of methyleugenol in the human liver showing an enzyme efficiency (k_{cat}/K_m) that is ∼30, 50, and >50 times higher than the enzyme efficiencies of, respectively, P450 2C9, 2C19, and 2D6. Only when relatively higher methyleugenol concentrations are present P450 2C9 and P450 2C19 might contribute as well to the bioactivation of methyleugenol in the human liver. A 5-fold difference in activities was found between the 15 human liver microsomes of the correlation study (range, 0.89−4.30 nmol min^{-1} nmol $P450^{-1}$). Therefore, interindividual differences might cause variation in sensitivity toward methyleugenol. Especially lifestyle factors such as smoking (induces P450 1A) and the use of barbiturates (induces P450 2C) can increase the susceptibility for adverse effects of methyleugenol.

Introduction

Methyleugenol is an alkenylbenzene compound that is a natural ingredient of several herbs, including basil, nutmeg, tarragon, star anise, and fennel. Furthermore, methyleugenol is used as a flavoring substance in a wide variety of dietary products, like cookies, ice cream, and nonalcoholic beverages (*1*). Dietary herb-based ingredients are natural compounds and are often regarded as safe because of their safe history of use. Methyleugenol was also classified as GRAS (Generally Recognized as Safe) by the Flavor and Extract Manufacturers Association (FEMA) in 1965 (*2*). However, the National Toxicology Program (NTP) of the United States selected methyleugenol for an investigation, because of its widespread use and its structural resemblance to safrole, a known carcinogen (*3*). The NTP reported carcinogenic activity of methyleugenol in male and female F344/N rats and in male and female $B6C3F_1$ mice (*3*). In 2002, the FEMA reevaluated the data available for methyleugenol and concluded again that present exposure to methyleugenol, resulting from food consumption, does not pose a significant cancer risk (*4*). This conclusion was based on evidence of a nonlinear relationship between dose and profiles of metabolism, metabolic activation, and covalent binding of methyleugenol to protein and DNA. According to the FEMA, the harmful effects of methyleugenol are expected to be minimal at a dose rate of 1−10 mg/kg bw/day, which is 100−1000 times the average human daily intake estimated by the FEMA (*4*). Recently, the European Union Scientific Committee on Food evaluated methyleugenol and the related herb-based alkenylbenzenes estragole and safrole. They concluded that these compounds are carcinogenic and genotoxic and that their use should be restricted (*1*, *5*, *6*). Moreover, they estimated a much

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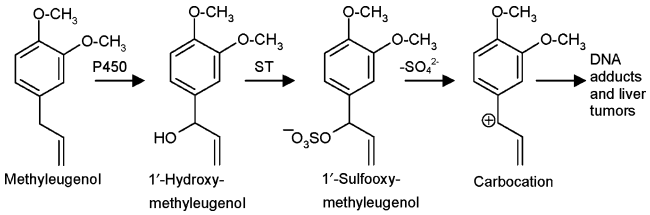

Figure 1. Bioactivation of methyleugenol (P450, cytochrome P450 enzymes; ST, sulfotransferase enzymes).

higher average daily intake of methyleugenol (0.19 mg/kg bw/day) than the FEMA did (0.01 mg/kg bw/day) (*1*, *4*).

Figure 1 shows the bioactivation pathway of methyleugenol. The cytochrome P450-based conversion of methyleugenol to the proximate carcinogen 1′-hydroxymethyleugenol is the first step in the bioactivation of methyleugenol (*7*, *8*). The sulfate ester of 1′-hydroxymethyleugenol is the likely ultimate carcinogenic species of methyleugenol (*9*). In an aqueous environment, 1′-sulfooxymethyleugenol is unstable. Upon loss of the sulfate group, a carbocation remains. This carbocation can cause DNA adducts and may ultimately cause liver tumors (*9*).

Insight in this bioactivation pathway in the human situation is necessary to extrapolate experimental animal data on the harmful effect of methyleugenol to the situation in man. Identification of the human P450 enzymes involved in the 1′-hydroxylation of methyleugenol is essential to gain insight into groups at a higher risk of the adverse effects of methyleugenol in the human population, due to genetic polymorphisms and life style factors that influence the P450 enzymes involved in the conversion of methyleugenol to its proximate carcinogen.

For the related alkenylbenzene safrole, important roles for the human P450 enzymes 2A6, 2C9, 2D6, and 2E1 were elucidated (*10*, *11*). For methyleugenol, Gardner et al. indicated that P450 2E1 and another enzyme, most likely P450 2C6, are important enzymes in this bioactivation step in rats (*12*), but no human data and data from experiments using recombinant enzymes are available yet. Therefore, the aim of the present study was to identify the human P450 enzymes involved in the 1′-hydroxylation of methyleugenol. Incubations with Supersomes, expressing individual human P450 enzymes to a high level, and incubations with Gentest microsomes, derived from cell lines expressing individual human P450 enzymes to roughly average human liver levels, were performed to investigate which human P450 enzymes are able to 1′-hydroxylate methyleugenol in the human liver. Additionally, a correlation study was performed in which methyleugenol was incubated with a series of 15 human liver microsomes. The 1′-hydroxylation rates obtained were correlated with the activities of these microsomes toward specific substrates for 9 different P450 enzymes. In addition, pooled human liver microsomes were incubated with methyleugenol in the presence and absence of enzyme specific inhibitors. Finally, the kinetics for methyleugenol 1′-hydroxylation of the P450 enzymes involved were investigated.

Materials and Methods

Materials. 1. Chemicals. Ascorbic acid, acetone, and dimethyl sulfoxide were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid and ammonium chloride were from Acros (Geel, Belgium). NADPH was obtained from Boehringer (Mannheim, Germany). Hydrochloric acid (37%) was purchased from Roche Diagnostics (Mannheim, Germany). Methyleugenol was obtained from Aldrich (Milwaukee, WI). Tris(hydroxymethylaminomethane) was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Dimethoxybenzaldehyde (veratraldehyde, purity 99%), tetrahydrofuran (THF), vinylmagnesium bromide (1 M solution in THF), diethyl ether, magnesium sulfate, α-naphthoflavone, coumarin, quinidine, and ketoconazole were purchased from Sigma-Aldrich (Steinheim, Germany). (*S*)-*N*-3-Benzylnirvanol, monoclonal antibody for human 2B6 (MAB 2B6), and monoclonal antibody for human 2C8 (MAB 2C8) were obtained from Gentest (Woburn, MA). Sulfaphenazole was purchased from Ultrafine Chemicals (Manchester, United Kingdom). 1′-Hydroxymethyleugenol was synthesized as described previously for 1′-hydroxysafrole (*10*, *13*), with veratraldehyde as the starting material. All other chemicals were of the highest quality available.

2. Enzymatic Preparations. Supersomes, prepared from baculovirus-infected insect cells expressing the human individual P450 enzymes 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4 were obtained from BD Gentest. In all cells, also human P450 reductase and (except for P450 1A2) cytochrome b5 were coexpressed. Gentest microsomes, prepared from lymphoblastoid cell lines expressing the same human individual P450 enzymes, were obtained from BD Gentest. For P450 2A6, 2C8, 2C9*1, 2D6*1, 2E1, and 3A4, human P450 reductase was coexpressed. For the other enzymes, the catalytic activity was supported by reductase activity endogenous to the cell line. In general, in Gentest microsomes, the activities toward enzyme selective substrates, expressed as nmol min^{-1} mg $protein^{-1}$, are roughly at the same level as the mean activities found in human liver microsomes (the ratio between the activity of the Gentest microsomes and the human liver microsomes is approximately 1 for P450 2A6, 2B6, 2C19, 2C9, and 2E1; 0.5 for P450 1A2; 0.3 for P450 2C8; and 3.3 for P450 2D6), whereas in Supersomes, the enzyme levels are much higher than those in the human liver (described in the Gentest catalog). Microsomes from 15 individual human livers were obtained from Human Biologics (Phoenix, AZ). Pooled human liver microsomes (mixed gender pool) were obtained from Gentest.

Methods. 1. Synthesis of 1′-Hydroxymethyleugenol. 1′-Hydroxymethyleugenol was synthesized as described previously for 1′-hydroxysafrole (*10*, *13*), with veratraldehyde as the starting material. Structural confirmation was obtained from the UV and MS data of the compound [*m*/*z* (rel int. %) 194 (M^+, 99), 167 (20), 165 (30), 163 (61), 151 (32), 139 (100), 138 (22), 124 (20), 91 (21), 77 (25), and 55 (55)]. The purity of 1′-hydroxymethyleugenol was more than 98%, according to GC and HPLC analysis.

2. In Vitro Incubations. 2.1. Incubations with Recombinant Enzymes. Microsomal incubations with methyleugenol, using Supersomes or Gentest microsomes expressing one single P450 enzyme, were performed in a 100 μL incubation mixture containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 0.3 nmol P450/mL Supersomes or 1 mg protein/mL microsomes in 0.2 M Tris-HCl, pH 7.4. Under these circumstances, 1′-hydroxymethyleugenol formation was linear with protein concentration, P450 concentration, and time. The reaction was started by adding the substrate methyleugenol (200 μM final concentration, added from a 20 mM stock solution in DMSO) and was performed at 37 °C. The reaction was terminated after 20 min by adding 25 μL of ice-cold acetonitrile. The substrate concentration (200 μM) is approximately the K_m for methyleugenol in human liver microsomes (K_m 0.2 ± 0.04 mM). All incubations were performed in triplicate, and control incubations without NADPH or microsomes were performed. Samples were centrifuged for 5 min at 2750*g* and stored at −20 °C until HPLC analysis.

2.2. Correlation Study. The human liver microsomes from Human Biologics were characterized with respect to 7-ethoxyresorufin *O*-dealkylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase, diclofenac 4′-hydroxylase, *S*-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, chlorzoxazone 6-hydroxylase, paclitaxel 6α-hydroxylase, and testosterone 6β-hydroxylase activities, as described previously (*10*). Data on protein and P450 content were provided by the supplier. Incubations with human liver microsomes from Human Biologics were performed identical to the incubations with Gentest microsomes described above, using a substrate concentration of 200 μM and microsomes

in a concentration of 1 mg protein/mL (range of 0.18−0.82 nmol P450/mL). Under these circumstances, 1′-hydroxymethyleugenol formation was linear with protein concentration, P450 concentration, and time. Incubations were performed in duplicate.

2.3. Inhibition Study. Microsomal incubations, using pooled human liver microsomes (1 mg/mL; 0.36 nmol P450/mL), were performed in 100 μL incubations containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 0.2 M Tris-HCl, pH 7.4. To these incubations, 1 μL of a 100 times concentrated stock solution of one of the chemical inhibitors in methanol was added as follows: α-naphthoflavone (final concentration, 1 μM), coumarin (final concentration, 10 μM), sulfaphenazole (final concentration, 10 μM), (*S*)-*N*-3-benzylnirvanol (final concentration, 5 μM), quinidine (final concentration, 5 μM), ketoconazole (final concentration, 1 μM), and acetone (final concentration, 1% v/v). For P450 2B6 and P450 2C8, 5 μL of their respective antibody was added (5 μL/100 μg microsomal protein). The selection of the specific chemical inhibitors and their concentrations is based on either literature data (*14*−*16*) or data of the manufacturer [for (*S*)-*N*-3-benzylnirvanol, MAB 2B6, and MAB 2C8, see Gentest catalog]. After 5 min of preincubation, 1 μL of 20 mM methyleugenol (final concentration, 200 μM) was added. The reactions were terminated after 20 min of incubation by adding 25 μL of acetonitrile. All incubations were performed in triplicate, and control incubations without NAPDH and without chemical inhibitor/antibody were performed. Samples were centrifuged for 5 min at 2750*g* and stored at −20 °C until HPLC analysis.

2.4. Kinetic Studies. For Gentest microsomes expressing P450 1A2, 2C9, 2C19, and 2D6, the K_m and k_{cat} values were determined by incubating these microsomes with substrate concentrations ranging from 0 to 200 μM (for P450 1A2) or from 0 to 500 μM (for P450 2C9, 2C19, and 2D6) (in triplicate). The incubation conditions were similar to the incubation conditions with Gentest microsomes described above. The data were fitted to the standard Michaelis−Menten equation $v = V_{max}/\{1 + (K_m/[S])\}$, where [S] = substrate concentration, using the LSW data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.) and the parameters k_{cat}, K_m, and k_{cat}/K_m were determined for all enzymes.

3. Sample Analyses. 3.1. HPLC Analysis. Aliquots (10 μL) of each sample were analyzed using an HPLC (Waters M600 liquid chromatography system) equipped with an Alltima C18 column, 150 mm × 4.6 mm (Alltech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 1.0 mL/min. A linear gradient from 10 to 30% (v/v) acetonitrile in water was applied during 12 min. The percentage of acetonitrile was kept at 30% (v/v) for 2 min, increased to 100% in 3 min, and was kept at 100% for 2 min. The retention time of 1′-hydroxymethyleugenol was 16.4 min under these conditions. Detection was carried out by a Waters 996 photodiode array detector at 280 nm. The amount of 1′-hydroxymethyleugenol was quantified by means of a calibration curve made using synthesized 1′-hydroxymethyleugenol. Activities were expressed as nmol 1′-hydroxymethyleugenol min^{-1} mg $protein^{-1}$ and/or nmol 1′-hydroxymethyleugenol min^{-1} nmol $P450^{-1}$.

3.2. GC-MS Analysis. GC-MS analysis was performed to identify the 1′-hydroxymetabolite of methyleugenol formed during the microsomal incubations. An incubation mixture of methyleugenol with Supersomes expressing P450 1A2 and a reference mixture containing methyleugenol and synthesized 1′-hydroxymethyleugenol in buffer were extracted with CH_2Cl_2. The organic layers were transferred into new vials, and the solutions were concentrated under a stream of nitrogen. Aliquots (2 μL) of each sample were analyzed using an HP6890 gas chromatograph, equipped with a J&W DB-5 column (30 m × 0.25 mm, 0.25 μm film) and an HP5973 mass selective detector. The GC was programmed for a 45 min run with a temperature gradient from 50 to 250 °C at 5 °C/min. The inlet temperature was 260 °C, the split ratio was 15:1, and the pressure of the helium carrier gas was 12.0 psi. The mass spectrometer was run in the electron impact mode with electron energy at 70 eV with a mass range of *m*/*z* 30−550 and a source temperature of 280 °C.

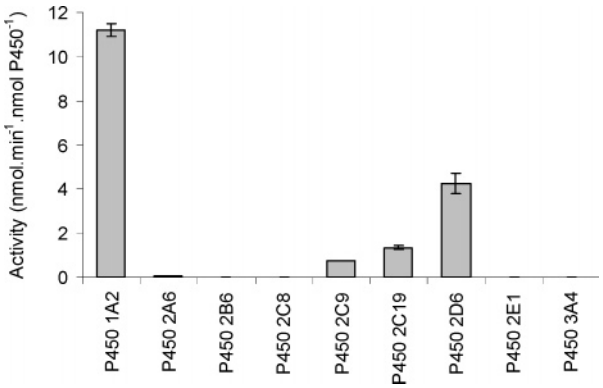

Figure 2. Methyleugenol 1′-hydroxylation activity by Supersomes at a substrate concentration of 200 μM. Bars indicate average activities of triplicate measurements ± SD.

3.3. Statistical Analysis. For correlations between the metabolism of methyleugenol and the metabolism of P450 marker substrates, enzyme activities expressed as nmol min^{-1} nmol $P450^{-1}$ were used, because in this way, correlation analysis will be independent of the amount of P450 present in the various samples. Pearson correlation tests were performed to investigate correlations between the metabolism of individual P450 marker substrates and the 1′-hydroxylation of methyleugenol. To investigate the relationship between the metabolism of multiple P450 marker substrates and the methyleugenol 1′-hydroxylation, multiple linear regressions were performed using an inclusion approach in which all activities were tested. These statistical analyses were performed with SPSS 10.1 for Windows (SPSS Inc, Chicago, IL).

Results

Formation of 1′-Hydroxmethyleugenol by Recombinant Enzymes. Incubations with Supersomes were performed to define the human P450 enzymes capable of methyleugenol 1′-hydroxylation. HPLC analysis of a typical incubation of Supersomes expressing P450 1A2 with methyleugenol showed formation of 1′-hydroxymethyleugenol, which could be identified based on the similarity of its retention time (16.4 min), UV spectrum, and mass spectrum to those of the chemically synthesized reference compound. Figure 2 shows the results obtained with the Supersomes. P450 1A2 showed the highest methyleugenol 1′-hydroxylating activity, followed by P450 2D6. In addition, P450 2C9 and P450 2C19 showed some methyleugenol 1′-hydroxylating activity. Hardly any activity was observed for P450 2A6 (∼0.04 nmol min^{-1} nmol $P450^{-1}$), whereas no activity was detected for P450 2B6, 2C8, 2E1, and 3A4 (detection limit ∼ 0.02 nmol min^{-1} nmol $P450^{-1}$).

Incubations of methyleugenol with Gentest microsomes were performed to investigate which enzymes show methyleugenol 1′-hydroxylation, when tested at roughly average liver levels. Figure 3 shows the results of these incubations. In Figure 3A, activities are expressed in nmol min^{-1} mg $protein^{-1}$. In general, in Gentest microsomes, the activities toward enzyme selective substrates, expressed in nmol min^{-1} mg $protein^{-1}$, are roughly comparable to the activities found in human liver microsomes (see Gentest catalog). These results indicate that the P450 enzymes 1A2, 2C9, 2C19, and 2D6 may be involved in methyleugenol 1′-hydroxylation in the human liver (detection limit ∼ 0.01 nmol min^{-1} mg $protein^{-1}$). In Figure 3B, activities are expressed in nmol min^{-1} nmol $P450^{-1}$. The different activities show the same pattern as in Figure 3A.

Correlation Study. The average rate of 1′-hydroxylation of methyleugenol in liver microsomes from 15 individuals was 2.45 ± 0.34 nmol 1′-hydroxymethyleugenol min^{-1} nmol $P450^{-1}$. A

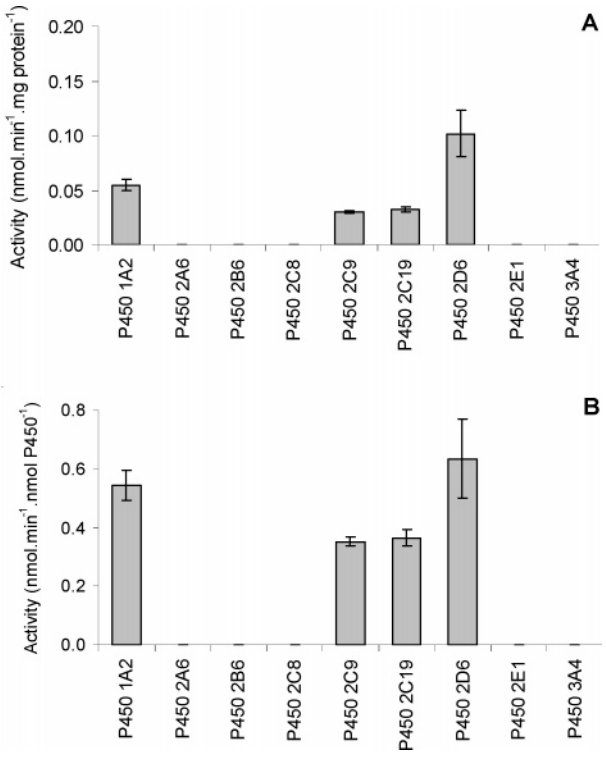

Figure 3. Methyleugenol 1′-hydroxylation activity expressed in (A) nmol min^{-1} mg $protein^{-1}$ and (B) nmol min^{-1} nmol $P450^{-1}$ by Gentest microsomes at a substrate concentration of 200 μM. Bars indicate average activities of triplicate measurements ± SD.

Table 1. Correlations among the Activities toward P450 Enzyme Selective Substrates and the Formation of 1′-Hydroxymethyleugenol[a]

marker substrate	mean activity ± SD in 15 human liver microsomes (nmol min^{-1} nmol $P450^{-1}$)	P450 enzyme	correlation coefficient
EROD	0.15 ± 0.08 (range 0.02−0.30)	P450 1A2	0.51*
COUM	3.19 ± 2.64 (range 0.01−10.4)	P450 2A6	0.02
7-ETC	0.70 ± 0.23 (range 0.27−1.12)	P450 2B6	0.35
PACL	0.55 ± 0.22 (range 0.18−0.91)	P450 2C8	−0.05
DICLF	5.79 ± 2.82 (range 2.50−12.1)	P450 2C9	0.47
MEPH	0.03 ± 0.06 (range 0.00−0.23)	P450 2C19	0.45
BUFU	0.38 ± 0.33 (range 0.05−1.22)	P450 2D6	0.22
CLZOX	6.41 ± 4.13 (range 1.74−15.0)	P450 2E1	−0.04
TEST	10.5 ± 5.16 (range 2.85−20.7)	P450 3A	0.05

[a] Statistical significance: *P < 0.05. EROD, 7-ethoxyresorufin *O*-dealkylation; COUM, coumarin 7-hydroxylation; 7-ETC, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation; PACL, paclitaxel 6α-hydroxylation; DICLF, diclofenac 4′-hydroxylation; MEPH, *S*-mephenytoin 4′-hydroxylation; BUFU, bufuralol 1′-hydroxylation; CLZOX, chlorzoxazone 6-hydroxylation; and TEST, testosterone 6β-hydroxylation.

5-fold variation between different human liver samples was found (range, 0.89−4.30 nmol min^{-1} nmol $P450^{-1}$; median value, 2.25 nmol min^{-1} nmol $P450^{-1}$). Table 1 shows the mean activities of the panel of 15 human liver microsomes toward specific substrates for nine different enzymes. Table 1 also presents the correlation coefficient for the correlation between 1′-hydroxylation of methyleugenol by the 15 human liver microsomes and their activity toward the specific substrates. Correlation analysis showed a significant correlation (p < 0.05) between P450 1A2 activity and methyleugenol 1′-hydroxylation. Because incubations with recombinant P450 enzymes also indicated the involvement of P450 2C9, 2C19, and 2D6 in the metabolism of methyleugenol, the results of the correlation experiment were further investigated with multiple regression analysis. By including both P450 1A2 and P450 2C9 in the model, p values < 0.01 were obtained for both enzymes, demonstrating an important contribution of both P450 1A2 and P450 2C9 in the 1′-hydroxylation of methyleugenol (correlation coefficient model, 0.78). No significant contribution of the other P450 enzymes regarding the metabolism of methyleugenol was found in this correlation study.

Table 2. Inhibition of the Formation of 1′-Hydroxymethyleugenol by Pooled Human Liver Microsomes by Enzyme Specific Inhibitors[a]

inhibitor	percentage of control activity ± SD	P450 enzyme
α-naphthoflavone	54 ± 13	P450 1A2
coumarin	92 ± 16	P450 2A6
MAB-2B6	107 ± 17	P450 2B6
MAB-2C8	102 ± 14	P450 2C8
sulfaphenazole	70 ± 11	P450 2C9
(*S*)-N-3-benzylnirvanol	89 ± 11	P450 2C19
quinidine	97 ± 2	P450 2D6
acetone	122 ± 5	P450 2E1
ketoconazole	99 ± 11	P450 3A

[a] MAB, monoclonal antibody.

Inhibition Study. The average activity of the control incubations of the pooled human liver microsomes was 0.25 ± 0.09 nmol 1′-hydroxymethyleugenol min^{-1} mg $protein^{-1}$ (0.69 ± 0.25 nmol min^{-1} nmol $P450^{-1}$). The percentage activity remaining in the microsomal incubations in the presence of the different enzyme selective chemical inhibitors/antibodies is shown in Table 2. The P450 1A2 inhibitor α-naphthoflavone and the P450 2C9 inhibitor sulfaphenazole were most effective in inhibiting the methyleugenol 1′-hydroxylation by human liver microsomes. In the presence of those inhibitors, activities of only 54 ± 13 and 70 ± 11% of the control 1′-hydroxylation activity were found. For all other inhibitors, the inhibition was less than 12%.

Kinetic Studies. Figure 4 shows the Michaelis−Menten plots for the 1′-hydroxylation of methyleugenol by Gentest microsomes containing, respectively, P450 1A2, 2C9, 2C19, and 2D6, and Table 3 presents the parameters k_{cat}, K_m, and k_{cat}/K_m (enzyme efficiency) derived from these studies. Although the k_{cat} for P450 1A2 is the lowest k_{cat} observed among the four enzymes, the K_m value for P450 1A2 is much lower than the K_m values of the other enzymes. As a result, the enzyme efficiency k_{cat}/K_m of P450 1A2 is ∼ 30, 50, and >50 times higher than the enzyme efficiencies of, respectively, P450 2C9, 2C19, and 2D6.

Discussion

The human P450 enzyme specificity for bioactivation of methyleugenol to its proximate carcinogen 1′-hydroxymethyleugenol was studied with different in vitro test systems, using either recombinant P450 enzymes or human liver micromes. The incubations with Supersomes expressing nine individual enzymes show the intrinsic capacity of especially P450 1A2 to 1′-hydroxylate methyleugenol. Furthermore, methyleugenol was also bioactivated by P450 2D6, 2C9, 2C19, and to a small extent by P450 2A6. Incubations with Gentest microsomes, which express P450 enzymes to roughly average liver levels, indicated that P450 1A2, 2C9, 2C19, and 2D6 contribute to methyleugenol 1′-hydroxylation. For Gentest microsomes expressing P450 2A6, no methyleugenol 1′-hydroxylating activity was detected. In a correlation study, using human liver microsomes, a significant correlation (p < 0.05) between P450 1A2 activity and methyleugenol 1′-hydroxylation was found. By including both P450 1A2 and P450 2C9 in the model, p values < 0.01 were obtained for both enzymes, demonstrating an important contribution of both P450 1A2 and P450 2C9 in the 1′-hydroxylation of methyleugenol (correlation coefficient model, 0.78). In a study

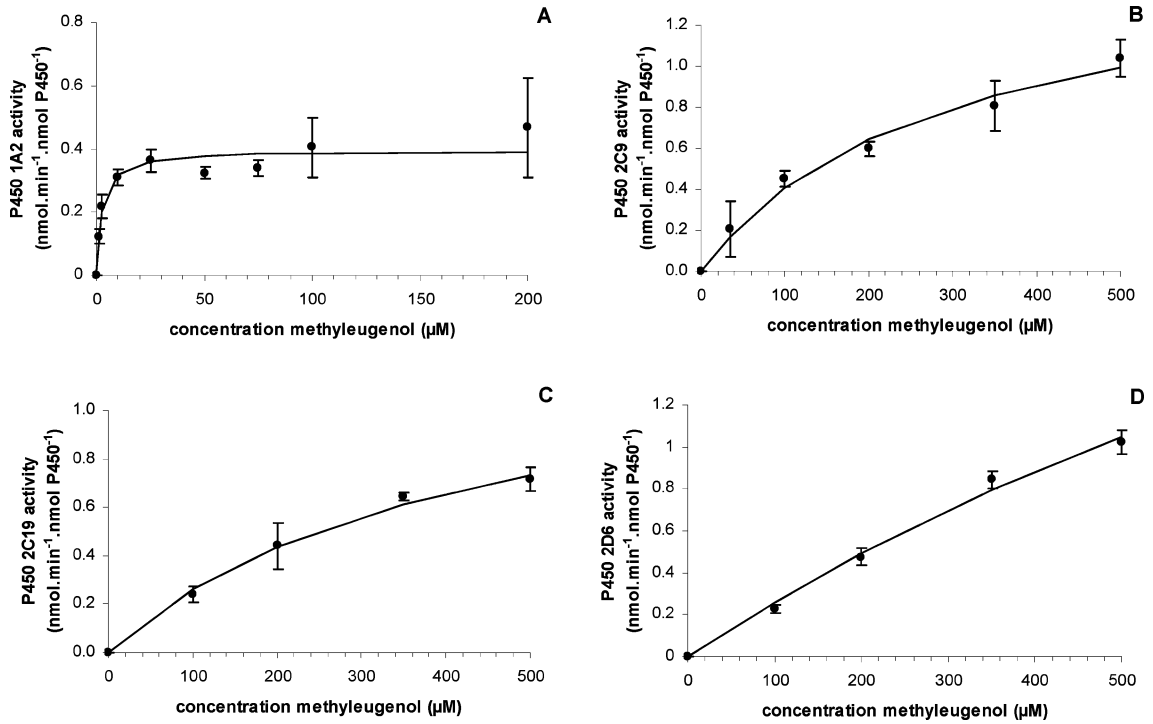

Figure 4. Michaelis−Menten plots for the 1′-hydroxylation of methyleugenol by Gentest microsomes containing (A) P450 1A2, (B) P450 2C9, (C) P450 2C19, and (D) P450 2D6.

Table 3. Kinetic Parameters for Gentest Microsomes Expressing the Enzymes Involved in Methyleugenol 1′-Hydroxylation

enzyme	k_{cat} (nmol min^{-1} nmol P450^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
P450 1A2	0.40 ± 0.03	$2.4 \times 10^{-3} \pm 1.1 \times 10^{-3}$	167
P450 2C9	1.55 ± 0.32	0.28 ± 0.12	5
P450 2C19	1.34 ± 0.31	0.42 ± 0.18	3
P450 2D6	4.22 ± 1.79	>1.5	<3

investigating the effect of enzyme selective inhibitors and antibodies on methyleugenol 1′-hydroxylation in human liver microsomes, the highest inhibition was caused by the P450 1A2 inhibitor α-naphthoflavone and the P450 2C9 inhibitor sulfaphenazole.

Taken together, the different in vitro systems indicate that P450 1A2 and P450 2C9 are important enzymes in the bioactivation of methyleugenol. Furthermore, on the basis of the incubations with Supersomes and Gentest microsomes, P450 2D6 and P450 2C19 may also be involved in methyleugenol 1′-hydroxylation. For those four enzymes, the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were determined using Gentest microsomes. From these studies, it appeared that at physiologically relevant concentrations of methyleugenol, P450 1A2 is the most important enzyme in methyleugenol 1′-hydroxylation with an enzyme efficiency (k_{cat}/K_m) that is ∼30, 50, and >50 times higher than the enzyme efficiencies of, respectively, P450 2C9, 2C19, and 2D6. P450 2D6 has the highest k_{cat} value for the 1′-hydroxylation of methyleugenol, but the K_m is relatively high (>1.5 mM), so at low levels of methyleugenol, this enzyme will not contribute to methyleugenol 1′-hydroxylation. The enzymes P450 2C9 and P450 2C19 might contribute to the bioactivation of methyleugenol only at relatively higher methyleugenol concentrations.

For the related alkenylbenzene safrole, we reported that especially the enzymes P450 2A6, 2C9, 2D6, and 2E1 are important in 1′-hydroxylation, whereas P450 2C19 and P450 1A2 also showed intrinsic safrole 1′-hydroxylating activity (*10*). Except for P450 2E1, for which no methyleugenol 1′-hydroxylation was observed, the same enzymes are intrinsically able to 1′-hydroxylate both alkenylbenzenes. However, the relative importance of the different enzymes differs for the two alkenylbenzenes.

Gardner et al. (*12*) have studied the selectivity of P450 enzymes involved in methyleugenol 1′-hydroxylation in rats, in different rat in vivo and in vitro systems. Contrary to our results for man, they found that P450 2E1 is one of the most important enzymes in this bioactivation step in rats (*12*), whereas they excluded P450 1A2 from being involved in methyleugenol 1′-hydroxylation. We did not detect any methyleugenol 1′-hydroxylation activity in Supersomes and Gentest microsomes expressing human P450 2E1, nor did we find support for a role for human P450 2E1 in the correlation and the inhibition study. Therefore, it seems unlikely that this enzyme is an important one in methyleugenol 1′-hydroxylation in the human liver. The exclusion of P450 1A2 by Gardner et al. was based on the results of an inhibitor study, in which rat liver microsomes were incubated with methyleugenol in the presence of the P450 1A2 selective inhibitor furafylline. Although rat and human P450 1A2 are orthologous genes, with 80% sequence similarity (*17*), furafylline is a much weaker inhibitor of 7-ethoxyresorufin *O*-dealkylase activity in rat than in man (*14*, *18*, *19*). This might explain why Gardner et al. found a low inhibition of methyleugenol 1′-hydroxylation in rat microsomes in the presence of furafylline. Furthermore, Gardner et al. found a significant inhibition of methyleugenol 1′-hydroxylation by α-naphthoflavone (*12*), an inhibitor of P450 1A enzymes (*20*), so their results also indicate that P450 1A enzymes might be involved in methyleugenol 1′-hydroxylation.

In the correlation study, 5-fold (range of 0.89−4.30 nmol min^{-1} nmol P450^{-1}) differences were found in the 15 human liver microsomes. Genetic polymorphisms in the enzymes involved in methyleugenol 1′-hydroxylation but also life style factors that influence the activities of these enzymes might cause interindividual differences in the susceptibility for the adverse effects of methyleugenol. For P450 1A2, which accounts for

13% of the total P450 content of the human liver (*21*), polymorphisms are very rare (reviewed in ref *22*), and so far, no allelic variant that is associated with increased enzyme activity has been identified (www.imm.ki.se/cypalleles). Therefore, life style factors that increase the activity of P450 1A2 are more important than genetic polymorphisms for the individual risks of bioactivation of methyleugenol. Cigarette smoking is known to induce P450 1A2 (*23*), and also charbroiled food and cruciferous vegetables can do so (reviewed in ref *24*). For P450 2C9 and P450 2C19, both members of the 2C family that accounts for 20% of the total P450 content of the human liver (*21*), only allelic variants that are associated with impaired enzyme activity in vivo are known so far (www.imm.ki.se/cypalleles). Recently, a P450 2C9*8 allele was discovered, which leads to an increased activity in vitro, but no in vivo data are available yet (*25*). People who use barbiturates, which induce P450 2C enzymes (*26*), might have higher levels of P450 2C enzymes and higher methyleugenol 1′-hydroxylation activity, pointing at increased risk of the adverse effects of methyleugenol.

In conclusion, our results indicate that at low concentrations of methyleugenol, P450 1A2 is the main enzyme involved in the bioactivation of methyleugenol and that at higher substrate concentrations P450 2C9 and 2C19 may also contribute to the bioactivation of methyleugenol. For a risk assessment for the use of methyleugenol, special attention should be paid to interindividual differences in the bioactivation of methyleugenol. In particular, people who smoke or use barbiturates might have a higher methyleugenol 1′-hydroxylation rate. These groups of people might be at higher risk of the adverse effects of exposure to methyleugenol.

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