ABSTRACT:

Limonene, a monocyclic monoterpenal, is present in orange peel and other plants and has been shown to have chemopreventive activities. (+)- and (−)-Limonene enantiomers were incubated with human liver microsomes and the oxidative metabolites thus formed were analyzed using gas chromatography-mass spectrometry. Two kinds of metabolites, (+)- and (−)-trans-carveol (a product by 6-hydroxylation) and (+)- and (−)-perillyl alcohol (a product by 7-hydroxylation), were identified, and the latter metabolites were found to be formed more extensively, the former ones with liver microsomes prepared from different human samples. Sulfoxonazole, flavoxamine, and antibodies raised against purified liver microsomes of untreated rats and by CYP2B1 in those of 62 human samples, whereas these activities did not correlate with contents of CYP2C19 and activities of S-mephenytoin 4-hydroxylation. Of 11 recombinant human P450 enzymes (expressed in Trichoplusia ni cells), CYP2C8, 2C9, 2C18, 2C19, and CYP3A4 catalyzed oxidations of (+)- and (−)-limonenes to respective carveols and perillyl alcohol. Interestingly, human CYP2B6 did not catalyze limonene oxidations, whereas rat CYP2B1 had high activities in catalyzing limonene oxidations. These results suggest that both (+)- and (−)-limonene enantiomers are oxidized at 6- and 7-positions by CYP2C9 and CYP2C19 in human liver microsomes. CYP2C9 may be more important than CYP2C19 in catalyzing limonene oxidations in human liver microsomes, since levels of the former protein are more abundant than CYP2C19 in these human samples. Species-related differences exist in the oxidations of limonenes in CYP2B subfamily in rats and humans.

The monocyclic monoterpenal (+)- and (−)-limonene enantiomers have been shown to be present in orange peel and other plants and are used as fragrances in household products and components of artificial essential oils (Crowell and Gould, 1994). The (+)-limonene isomeric form is more abundantly present in these plants than the racemic mixture and (−)-isomeric form (Haudenschild et al., 2000). It has previously been shown that (+)-limonene has chemopreventive activities in experimental animal models including rats and mice (Crowell and Gould, 1994; el-Bayoumy, 1994). Some of the hydroxylated metabolites of (+)-limonene, such as sobrerol, carveol, and uroterpine, have been reported to be more potent than the parent compound in preventing mammary tumors caused by 7,12-dimethylbenz[a]anthracene (Crowell et al., 1992). To better understand the basis of mechanisms of chemopreventive action of limonene, it is of interest to examine the metabolism of limonene in experimental animals and humans.

Metabolism of limonene enantiomers has been studied extensively in experimental animal models in rats, mice, rabbits, guinea pigs, and dogs both in vivo and in vitro (Igimi et al., 1974; Kodama et al., 1974, 1976; Regan and Bjeldanes, 1976; Watabe et al., 1981). Cytochrome P450 P450(1) enzymes in liver microsomes of these animal species have been shown to oxidize limonene to several oxidation products such as 1,2- and 8,9-epoxides, carveol (a product by 6-hydroxylation), perillyl alcohol (a product by 7-hydroxylation) (Watabe et al., 1980, 1981; Jager et al., 1999). Recently we reported that limonene enantiomers are oxidized to carvols and perillyl alcohols by CYP2C11 in liver microsomes of untreated rats and by CYP2B1 in those of phenobarbital-treated rats (Miyazawa et al., 2002). Interestingly, a female specific CYP2C12 did not catalyze oxidations of (+)- and (−)-limonene enantiomers by liver microsomes at significant levels. However, it remains unclear which P450 enzymes catalyze limonene oxidations in human liver microsomes.

In this study, we examined oxidations of (+)- and (−)-limonene by P450 enzymes in liver microsomes prepared from different human samples. The metabolites thus formed were analyzed on GC-MS. To determine which P450s are the major enzymes in the oxidations of (+)- and (−)-limonene enantiomers, we used specific P450 inhibitors and antibodies raised against purified human liver P450 enzymes and P450s isolated from Escherichia coli membranes to which human P450 isoform cDNAs have been introduced. Catalytic rates with 11
forms of human P450 enzymes expressed in Trichoplusia ni cells in the oxidation of limonene enantiomers are also reported.

Experimental Procedures

Chemicals. (+)- and (−)-Limonene enantiomers, (+)- and (−)-trans-carveols and (+)- and (−)-perillyl alcohols, were purchased from Wako Pure Chemical Co. (Osaka, Japan) and were used without further purification; the purities of these compounds were judged to be >99% on analysis with GC-MS. NADP+/glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). Other reagents and chemicals used were obtained from sources as described previously or of highest qualities commercially available (Shimada et al., 1999, 2001; Miyazawa et al., 2001).

Enzymes. Human liver samples were obtained from 27 Japanese and 35 Caucasians; the latter samples were the generous gifts of Dr. F. P. Guengerich (Vanderbilt University). All of the liver samples were collected from portions of the livers without particular pathological changes (Inoue et al., 2000; Shimada et al., 2001). Liver microsomes were prepared as described and suspended in 10 mM Tris-Cl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (Guengerich, 1994; Shimada et al., 1994). Recombinant CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2E1, and 3A4 expressed in T. ni infected with a baculovirus containing rat P450 and NADPH-P450 reductase cDNA inserts were obtained from Gentest Corp. (Woburn, MA); the P450 contents in these systems were used as described in the data sheets provided by the manufacturer.

CYP2C9 was purified to electrophoretic homogeneity from human liver microsomes as described previously (Shimada et al., 1996). Rabbit antihuman CYP2C9 antibodies were prepared as described (Kaminsky et al., 1981; Shimada et al., 1986). Rabbit antiserum raised against purified CYP2C8, 2C9, and 2C19 were the generous gifts from Nihon Nosan Kogyo KK (Yokohama, Japan).

Oxidation of (+)- and (−)-Limonene by Human Liver Microsomes and by Human P450 Enzymes. Oxidations of (+)- and (−)-limonene enantiomers by human P450 enzymes were determined as follows. Standard reaction mixture contained liver microsomes (0.05 mg of protein/ml) or recombinant P450 (3 pmol/ml) and 200 μM (+)- or (−)-limonene in a final volume of 0.50 ml of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP+, 5 mM glucose 6-phosphate, and 0.5 units of glucose 6-phosphate dehydrogenase/ml) (Shimada et al., 1996). Incubations were carried out at 37°C for 30 min and terminated by adding 1.0 ml of dichloromethane. The extracts (organic layer) were collected by centrifugation at 3000 rpm for 5 min and were used for analysis with GC-MS for identification of the metabolites.

A Hewlett-Packard model 5890A gas chromatograph (Hewlett-Packard, Atlanta, GA) equipped with a split injector was combined with a direct coupling to Hewlett-Packard 5972 mass spectrometer. The metabolites were separated by a TC-WAX FFS (GL Sciences, Tokyo, Japan) silica capillary column (0.25 mm × 60 m) using helium (at 1 ml/min) as a carrier gas, as described previously (Miyazawa et al., 2002). A GC-MS system equipped with Wiley 138K Mass Spectral Database software (John Wiley & Sons Inc., New York, NY) was used for identification of products.

Other Assays. P450 and protein contents were estimated by the methods described elsewhere (Lowry et al., 1951; Omura and Sato, 1964).

Statistical Analysis. Kinetic parameters for (+)- and (−)-limonene oxidations by human P450 enzymes were estimated using a computer program (KaleidaGraph; Synergy Software, Reading, PA) designed for nonlinear regression analysis.

Results

Oxidation of (+)- and (−)-Limonene Enantiomers by Human Liver Microsomes. On incubation of (+)-limonene with human liver microsomes in the presence of an NADPH-generating system, we detected two metabolites, namely (+)-trans-carveol (a product by 6-hydroxylation) and (+)-perillyl alcohol (a product by 7-hydroxylation) (Fig. 1). Liver samples from two Japanese and two Caucasians were used for analysis, and we found that human liver microsomes were more active in oxidizing at 7-position than at 6-position (Fig. 2). Formation of these oxidative metabolites increased with incubation time up to 30 min and protein concentration up to 0.5 mg/ml incubation mixture (Fig. 2, A and B). Dependence of formation rates on substrate concentrations of (+)- and (−)-enantiomers of limonene by human liver microsomes were similar (Fig. 2C). Using five human liver samples, we found that Km and Vmax values for the oxidation of limonenes to respective carveols and perillyl alcohol were very similar (Table 1). Enzyme efficiencies (Vmax/Km ratio) were 3.5- and 4.0-fold higher in the formation of perillyl alcohol than that of carveol.

Effects of Flavoxamine, Sulfaphenazole, Ketoconazole, and Anti-P450 Antibodies on Oxidations of Limonenes by Human Liver Microsomes. Flavoxamine and sulfaphenazole, known inhibitors of CYP2C-dependent catalytic activities (Guengerich and Shimada, 1991; Inoue et al., 1997), significantly inhibited 6- and 7-hydroxylations of limonene enantiomers catalyzed by human liver microsomes (Fig. 3). However, ketoconazole, a potent inhibitor of CYP3A enzymes (Guengerich and Shimada, 1991), weakly inhibited limonene oxidation activities by human liver microsomes.
Antibodies raised against purified human liver CYP2C9 that inhibit both CYP2C9- and CYP2C19-dependent catalytic activities (Shimada et al., 1986), were found to inhibit significantly limonene oxidation activities catalyzed by liver microsomes of HL-104 (Fig. 4). We also used antibodies raised against purified CYP2C8, 2C9, and 2C19 (generous gifts from Nihon Nosan Kogyo KK) that were isolated from membranes of E. coli in which respective P450 cDNAs have been introduced (Fig. 4). The data sheets provided by the manufacturer suggested that both anti-CYP2C8 and anti-CYP2C19 inhibited taxol reduction and 7-mephenytoin 4-hydroxylation, respectively, by liver microsomes of a human sample (data not shown).

Recently, we found that CYP2B1 is a principal enzyme in catalyzing oxidations of limonenes by liver microsomes of phenobarbital-treated rats, although CYP2C11 is a major enzyme in liver microsomes of untreated male rats (MIYAZAWA et al., 2002). Since our present study suggested that CYP2B6 did not have any considerable activities for the oxidations of limonene enantiomers, we examined the effects of concentrations of rat CYP2B1 and human CYP2B6 on the oxidation of (+)-limonene in recombinant (T. ni) cells (Fig. 6). The results showed that CYP2B6 did not catalyze oxidations of (+)-limonene at considerable rates, whereas CYP2B1 catalyzed limonene oxidations at higher rates for the formation of carveol than those of perillyl alcohol (data not shown).

Kinetic analysis were performed in recombinant CYP2C9, 2C19, and 3A4 in the oxidations of (+)- and (−)-limonene enantiomers (Fig. 6). Km values for the formation of carveols and perillyl alcohols by these P450 enzymes were around 0.3 mM, except that Km value for the formation of carveol by CYP2C9 was somewhat higher (0.57 mM) in these human samples (data not shown). Vmax values were higher in CYP2C19 than those in CYP2C9 and 3A4 in the oxidations of (+)- and (−)-limonene enantiomers. As a result, enzyme efficiencies (Vmax/Km ratio) were always higher in CYP2C19 than those of CYP2C9 and 3A4 (Table 3).

**Discussion**

In this study, we found that (+)- and (−)-limonene enantiomers were oxidized to respective carvone (6-hydroxylation) and perillyl alcohol (7-hydroxylation) metabolites by CYP2C9 and CYP2C19 in human liver microsomes. Although enzyme efficiencies (Vmax/Km ratio) were much higher in CYP2C19 than those of CYP2C9 in the formation of carvone and perillyl alcohols from limonene enantiomers by recombinant P450 systems. CYP2C9 was found to be more active than CYP2C19 in catalyzing limonene enantiomers by human liver microsomes with the following lines of evidence. First, sulfa-

**TABLE 1**

Oxidation of (+)- and (−)-Limonene Enantiomers to respective carveol and perillyl alcohol derivatives by liver microsomes of five human samples

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Formation of Carveol</th>
<th>Formation of Perillyl Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (mM)</td>
<td>Vmax (nmol/min/mg protein)</td>
</tr>
<tr>
<td>(+)-Limonene</td>
<td>0.47 ± 0.19</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>(−)-Limonene</td>
<td>0.45 ± 0.12</td>
<td>0.18 ± 0.10</td>
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</table>

**Fig. 3.** Effects of flavoxamine (A), sulfaphenazole (B), and ketoconazole (C) on oxidations of (+)- and (−)-limonene enantiomers to respective carveol (○) and perillyl alcohol (■) by liver microsomes of a human sample (data not shown).
phenazole, a specific CYP2C9 inhibitor, significantly suppressed the activities of oxidations of limonene enantiomers catalyzed by human liver microsomes. Second, anti-CYP2C9 IgG, which was obtained from rabbits immunized with purified human liver CYP2C9 (Shimada et al., 1986), completely inhibited limonene oxidations by liver microsomes. Third, antisera raised against CYP2C9 (purified from membranes of \textit{E. coli}) inhibited very significantly the oxidations of (+)- and (−)-limonene enantiomers by human liver microsomes. It should, however, be mentioned that antibodies raised against CYP2C19 (purified from membranes of \textit{E. coli}) inhibited more effectively than anti-CYP2C9 for the oxidation of (+)-limonene by liver microsomes (Fig. 4). The data sheets provided by the manufacturer (Nippon Nosan Kogyo Co.) suggested that anti-CYP2C19 (at 2 μl sera) inhibited S-mephenytoin 4-hydroxylation by more than 80%, whereas anti-

![Graph 1](image1.png)

**Fig. 1.** Effects of preimmune sera (○), anti-2C9 sera (△), anti-CYP2C8 sera (●), anti-CYP2C9 sera (□), and anti-CYP2C19 sera (■) on the formation of (+)- and (−)-carveols (A and C) and (+)- and (−)-perillyl alcohols (B and D) from (+)- and (−)-limonene, respectively, by human liver microsomes of HL-104.

Activities are represented as percent of controls (in the absence of antibody).

![Graph 2](image2.png)

**Fig. 2.** Correlation between contents of CYP2C9 (A and B) and CYP2C19 (C and D) and catalytic activities of liver microsomes of 62 human samples toward oxidation of (+)-limonene.

In A and B, contents of CYP2C9 were compared with formation of (+)-carveol and (+)-perillyl alcohol, respectively, in these human samples; in C and D, contents of CYP2C19 were compared with formation of (+)-carveol and (+)-perillyl alcohol, respectively, in these human samples.

**TABLE 2**

<table>
<thead>
<tr>
<th>P450</th>
<th>Oxidation of (+)-Limonene</th>
<th>Oxidation of (−)-Limonene</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(+)-Carveol</td>
<td>(+)-Perillyl alcohol</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<tr>
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<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.11 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>CYP2C18</td>
<td>0.08 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>CYP2C19</td>
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</tr>
<tr>
<td>CYP2E1</td>
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<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.09 ± 0.04</td>
<td>0.25 ± 0.03</td>
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</table>

Oxidations of (+)- and (−)-limonene enantiomers by recombinant human P450 enzymes were determined by the methods as described under Experimental Procedures. Substrate concentrations used were 200 μM. Data are means of duplicate determinations and range.
CYP2C9 sera (at 2 μl) inhibited by only 40% diclofenac 4’-hydroxylation catalyzed by human liver microsomes. Finally, limonene oxidation activities were found to correlate with contents of CYP2C9, but not CYP2C19, in liver microsomes of 62 human samples. The reasons why CYP2C9 is more active than CYP2C19 in the oxidations of limonenes by liver microsomes may be related to higher contents of this P450 species in human livers. Our estimate suggested that the mean levels of CYP2C9 in liver microsomes of 62 human samples were about 15-fold higher than those of CYP2C19, probably resulting in more important roles of CYP2C9 than CYP2C19 in catalyzing limonene oxidations by human liver microsomes.

Recombinant CYP3A4 was found to be one of the enzymes in catalyzing oxidations of limonene enantiomers. Although the enzyme efficiencies (V_{max}/K_{m} ratio) for the oxidation of limonene enantiomers by recombinant (T. ni cells) CYP3A4 were found to be similar to those by recombinant CYP2C9, the contribution of this P450 isoform seemed be minor in human liver microsomes (Table 3). These were supported by the inability of ketoconazole, a potent inhibitor of CYP3A4 (Guengerich and Shimada, 1991), to inhibit limonene oxidations catalyzed by human liver microsomes.

We have recently shown that there are sex-related differences in the metabolism of limonenes by rat liver microsomes and that the male-specific CYP2C11 has higher catalytic rates for the oxidation of limonenes than female-specific CYP2C12 (Miyazawa et al., 2002). Such differences may, in part, be related to the occurrence of male-specific nephrotoxicity caused by limonenes in rats (Lehman-Mckeeeman et al., 1989; Borghoff et al., 1990; Hard, 1998). In fact, some of the limonene metabolites are shown to be more potent to interact with α2-μ-globulin than the parent compound (Lehman-Mckeeeman et al., 1989; Borghoff et al., 1990). In humans, however, it has been reported that there are few sex-related differences in the contents and catalytic properties of individual P450 enzymes (Shimada et al., 1994).

(-)-Limonene has been shown to have chemopreventive activities in experimental animal models (Crowell et al., 1992; Crowell and Gould, 1994; el-Bayoumy, 1994). One of the mechanisms underlying chemoprevention of limonenes may be related to inactivation of the ultimate carcinogens by inducing phase II enzymes. It is not known at present whether the metabolites of limonenes, such as carvoel and perillyl alcohol, induce phase II enzymes in mammalian tissues.

In this study, we found that CYP2B6 did not catalyze oxidations of limonenes at significant rates. This is in contrast with the results of rat studies in which CYP2B1, a homologous enzyme to CYP2B6, catalyzes limonene oxidations at high rates and anti-CYP2B1 significantly inhibits limonene oxidations by liver microsomes of phenobarbital-treated rats (Miyazawa et al., 2002). Such species-related differences in the metabolism of xenobiotic chemicals by the same family of P450

### TABLE 3

<table>
<thead>
<tr>
<th></th>
<th>Oxidation of (+)- and (-)-Limonene</th>
<th>Formation of Carveol</th>
<th>V_{max}</th>
<th>V_{max}/K_{m}</th>
<th>Formation of Perillyl Alcohol</th>
<th>V_{max}</th>
<th>V_{max}/K_{m}</th>
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<tbody>
<tr>
<td></td>
<td>K_{m} (mM)</td>
<td>V_{max} (nmol/min/nmol P450)</td>
<td>V_{max}/K_{m} (nmol/min/nmol P450)</td>
<td>K_{m} (mM)</td>
<td>V_{max} (nmol/min/nmol P450)</td>
<td>V_{max}/K_{m} (nmol/min/nmol P450)</td>
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<tr>
<td>(+)-Limonene</td>
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<td>CYP2C9</td>
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Kinetic parameters of oxidation of (+)- and (-)-limonene enantiomers by recombinant human P450 were determined by the methods as described under Experimental Procedures.
enzymes are of interest when the biological and pharmacological data are to be extrapolated from experimental animals to humans (Wrighton et al., 1995; Iwatsubo et al., 1997).

In summary, present results showed that both (+)- and (−)-limonene enantiomers are oxidized to respective carveol and perillyl alcohol derivatives by CYP2C9 and 2C19 in human liver microsomes. Our results also showed CYP2C9 plays more important roles than CYP2C19 in catalyzing limonene oxidations. Although recombinant CYP3A4 catalyzed limonene oxidations at considerable rates, contribution of this P450 isoform may be minor, since ketoconazole, a potent CYP3A4 inhibitor, inhibited weakly limonene oxidations catalyzed by liver microsomes. The exact roles of CYP2C enzymes in the biological significance of limonene enantiomers when humans are exposed to these chemicals are unknown at present.

References


