METABOLISM OF (±)- AND (−)-LIMONENES TO RESPECTIVE CARVEOLS AND PERILLYL ALCOHOLS BY CYP2C9 AND CYP2C19 IN HUMAN LIVER MICROSOMES

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ABSTRACT:

Limonene, a monocyclic monoterpenes, 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. Sulforaphene, flavoxamine, and antibodies raised against purified liver cytochrome P450 (P450) 2C9 that inhibit both CYP2C9 and 2C19-dependent activities, significantly inhibited microsomal oxidations of (±)- and (−)-limonene enantiomers. The limonene oxidation activities correlated well with contents of CYP2C9 and activities of tolbutamide methyl hydroxylation in liver microsomes 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) tested, 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 monoterpenes (+) 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 uroterpe-

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1 Abbreviations used are: P450, cytochrome P450; GC-MS, gas chromatography-mass spectrometry.
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., 1986). Rabbit antihuman CYP2C9 antibodies were prepared as described (Kaminsky et al., 1981; Shimada et al., 1986). Rabbit antisera 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., 1986). 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 3,000 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 *K*ₘ and *V*ₘₐₓ values for the oxidation of limonene to respective carveols and perillyl alcohol were very similar (Table 1). Enzyme efficiencies (*V*ₘₐₓ/*K*ₘ 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 hydroxylation and 5-mephenytoin 4-hydroxylation, respectively, by more than 80% at 2 μM, 100/100 μg of liver microsomal protein. However, anti-CYP2C9 sera was not so potent to inhibit activities, since the antibodies inhibited by only 40% diclofenac 4'-hydroxylation catalyzed by human liver microsomes (data not shown). Among these three antibodies examined, anti-CYP2C19 inhibited (+)-limonene oxidations more strongly than anti-CYP2C9 did, whereas both antibodies were equally inhibitory for the oxidations of (-)-limonene enantiomer by human liver microsomes. Anti-CYP2C8 slightly inhibited the oxidations of limonene enantiomers (Fig. 4).

Correlation between Contents of CYP2C9 and CYP2C19 and Activities of Limonene Oxidations by Liver Microsomes of 62 Human Samples. These above results suggest that CYP2C9 and 2C19 are the important enzymes in the oxidations of limonene enantiomers by human liver microsomes. Correlation between contents of CYP2C9 and CYP2C19 and rates of formation of carveol and perillyl alcohol of (+)-limonene was compared in liver microsomes of 62 human samples (Fig. 5). The data sheets provided by the manufacturer suggested that both antibodies were equally inhibitory for the oxidations of limonene enantiomers (Table 3). The data sheets provided by the manufacturer suggested that both antibodies were equally inhibitory for the oxidations of limonene enantiomers (Table 3). The data sheets provided by the manufacturer suggested that both antibodies were equally inhibitory for the oxidations of limonene enantiomers (Table 3). The data sheets provided by the manufacturer suggested that both antibodies were equally inhibitory for the oxidations of limonene enantiomers (Table 3). The data sheets provided by the manufacturer suggested that both antibodies were equally inhibitory for the oxidations of limonene enantiomers (Table 3).

**Discussion**

In this study, we found that (+)- and (-)-limonene enantiomers were oxidized to respective carveol (6-hydroxylation) and perillyl alcohol (7-hydroxylation) metabolites by CYP2C9 and CYP2C19 in human liver microsomes. Although enzyme efficiencies (V<sub>max</sub>/K<sub>m</sub> ratio) were always higher in CYP2C19 than those of CYP2C9 and 3A4 in the oxidations of (+)- and (-)-limonene enantiomers. As a result, enzyme efficiencies (V<sub>max</sub>/K<sub>m</sub> ratio) were always higher in CYP2C19 than those of CYP2C9 and 3A4 (Table 3).

**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>K&lt;sub&gt;m&lt;/sub&gt; (mM)</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; (nM/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>
</tr>
</tbody>
</table>
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 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 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-

![Image](https://example.com/image.png)

**Fig. 4.** 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).

![Image](https://example.com/image.png)

**Fig. 5.** 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>
</tr>
</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>
</tr>
<tr>
<td>CYP2A6</td>
<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>
<td>0.47 ± 0.11</td>
<td>1.52 ± 0.08</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.09 ± 0.04</td>
<td>0.25 ± 0.03</td>
</tr>
</tbody>
</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 14-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\textsubscript{max}/K\textsubscript{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). Crowell et al. (1992) have reported that some of the hydroxylated metabolites of (+)-limonene, such as sobrerol, carveol, and uroterpenol, are more potent than the parent compound in preventing mammary tumors caused by 7,12-dimethylbenz[a]anthracene. It has also been shown that (+)-limonene itself does not induce P450 enzymes but induces phase II enzymes such as glutathione S-transferase and UDP-glucuronosyltransferase enzymes (Ariyoshi et al., 1975; 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 carveol 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

![Graph of metabolism of limonenes by rat liver microsomes](image)

**Fig. 6.** Dependence on concentrations of oxidations of (+)-limonene (A and B) and (-)-limonene (C and D) to respective (+)- and (-)-carveol and perillyl alcohol by recombinant human CYP2C9 (●), CYP2C19 (□), and CYP3A4 (○).

**TABLE 3**

<table>
<thead>
<tr>
<th>Oxidation of (+)- and (-)-Limonene</th>
<th>Formation of Carveol</th>
<th>Formation of Perillyl Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K\textsubscript{m}</td>
<td>V\textsubscript{max}</td>
</tr>
<tr>
<td>(+)-Limonene</td>
<td>mM</td>
<td>nmol/min/nmol P450</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.57</td>
<td>0.33</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.27</td>
<td>1.16</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.27</td>
<td>0.16</td>
</tr>
<tr>
<td>(-)-Limonene</td>
<td>0.30</td>
<td>0.39</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.25</td>
<td>0.70</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.26</td>
<td>0.19</td>
</tr>
</tbody>
</table>

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


