ROLES OF HUMAN CYP2A6 AND 2B6 AND RAT CYP2C11 AND 2B1 IN THE 10-HYDROXYLATION OF (−)-VERBENONE BY LIVER MICROSOMES

MITSUO MIYAZAWA, ATSUSHI SUGIE, AND TSUTOMU SHIMADA

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka, Osaka, Japan (M.M., A.S.); and Osaka Prefectural Institute of Public Health, Osaka, Japan (T.S.)

(Received February 4, 2003; accepted April 25, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

(−)-Verbenone, a monoterpenic bicyclic ketone, is a component of the essential oil from rosemary species such as Rosmarinus officinalis L., Verbena triphylla, and Eucalyptus globulus and is used for an herb tea, a spice, and a perfume. In this study, (−)-verbenone was found to be converted to 10-hydroxyverbenone by rat and human liver microsomal cytochrome P450 (P450) enzymes. The product formation was determined by high-performance liquid chromatography with UV detection at 251 nm. There was a good correlation between activities of comumarin 7-hydroxylation and (−)-verbenone 10-hydroxylation catalyzed by liver microsomes of 16 human samples, indicating that CYP2A6 is a principal enzyme in (−)-verbenone 10-hydroxylation in humans. Human recombinant CYP2A6 and CYP2B6 catalyzed (−)-verbenone 10-hydroxylation at V_{max} values of 15 and 21 nmol/min/nmol P450 with apparent K_{m} values of 16 and 91 μM, respectively. In contrast, rat CYP2A1 and 2A2 did not catalyze (−)-verbenone 10-hydroxylation at all, suggesting that there were species-related differences in the catalytic properties of human and rat CYP2A enzymes in the metabolism of (−)-verbenone. In the rat, recombinant CYP2C11, CYP2B1, and CYP3A2 catalyzed (−)-verbenone 10-hydroxylation with V_{max} and K_{m} ratios (ml/min/nmol P450) of 0.73, 0.20, and 0.03, respectively. Male-specific CYP2C11 was a major enzyme in (−)-verbenone 10-hydroxylation by untreated rat livers, and CYP2B1 catalyzed this reaction in liver microsomes of phenobarbital-treated rats. Rat CYP2C12, a female-specific enzyme, did not catalyze (−)-verbenone 10-hydroxylation. These results suggest that human CYP2A6 and rat CYP2C11 are the major catalysts in the metabolism of (−)-verbenone by liver microsomes and that there are species-related differences in human and rat CYP2A enzymes and sex-related differences in male and female rats in the metabolism of (−)-verbenone.

Verbenone, a monoterpenic bicyclic ketone, has been shown to be a component of the essential oil from rosemary species such as Rosmarinus officinalis L., Verbena triphylla, and Eucalyptus globulus (Ravid et al., 1997; Giorgio et al., 2002). (−)-Verbenone enantiomer has a spicy odor and camphoraceous fragrance and has been used for an herb tea, a spice, and a perfume (Ravid et al., 1997; Giorgio et al., 2002). It has not previously been reported whether verbenone enantiomers or their metabolites have any biological and toxicological potencies in mammals when these chemicals are ingested into animals and humans.

Our previous studies have demonstrated that several terpenes, 1,4-cineole, 1,8-cineole, and (+)- and (−)-limonenes are catalyzed by cytochrome P450 (P450) enzymes to their respective oxidation products in rat and human liver microsomes (Miyazawa et al., 2001a,b). 1,4-Cineole and 1,8-cineole have been determined to be oxidized at the 2-position of the molecules both by rat and human CYP3A enzymes (Miyazawa et al., 2001a,b). Limonene enantiomers are metabolized to their respective carvones and perillyl alcohols in liver microsomes of mice, rats, guinea pigs, rabbits, dogs, monkeys, and humans (Miyazawa et al., 2002a,b). There are species-related differences in the metabolism of carveols to carvone in which dogs, rabbits, and guinea pigs catalyze this reaction, although other species of animals examined do not (Shimada et al., 2002b). It has also been reported that there are sex-related differences in the metabolism of limonene enantiomers in rats, in which male-specific CYP2C11 catalyzes limonene metabolism, although female-specific CYP2C12 does not (Miyazawa et al., 2002a).

Recently we have established that human liver microsomes catalyze 10-hydroxylation of (−)-verbenone enantiomers by analysis with gas chromatography-mass spectrometry (Miyazawa et al., 2002c). Since P450 enzymes are likely to be involved in the 10-hydroxylation of (−)-verbenone, we examined which P450 species are the major enzymes in the metabolism of (−)-verbenone in liver microsomes of rats and humans. (−)-Verbenone was incubated in the presence of an NADPH-generating system with liver microsomes of rats treated with different chemical inducers and with liver microsomes of different human liver samples. Recombinant rat and human P450 enzymes expressed in Trichoplusia ni cells were also used. The product formation was measured with HPLC using a C_{18} 5-μm analytical column.

Materials and Methods

Chemicals. (−)-Verbenone and its 10-hydroxylated metabolite were synthesized as described previously (Miyazawa et al., 2002c). 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 were of the highest quality commercially available (Wang et al., 1980; Shimada et al., 1994, 2002a, 2002b; Miyazawa et al., 2002a,b).

**Enzymes and Antibodies.** Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously (Shimada et al., 1994, 1999). Liver microsomes were prepared as described and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (Guengerich, 1994; Shimada et al., 2002a). Recombinant CYP1A1, 1A2, 1B1, 2A6, 2B6, 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 BD Gentest (Woburn, MA); the P450 contents in these systems were tested as described in the data sheets provided by the manufacturer.

Male Sprague-Dawley rats (weighing about 200 g) obtained from Nihon Clea Co. (Osaka, Japan) were used throughout this study. Liver microsomes from untreated rats and rats treated with PB (80 mg/kg, daily for 3 days), β-naphthoflavone (50 mg/kg, daily for 3 days), and pregnenolone 16α-carbonitrile (80 mg/kg, daily for 3 days) were prepared as described and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (Guengerich, 1994; Shimada et al., 2002a). Rabbit anti-human CYP2A6 antibodies were prepared as described (Yun et al., 1991).

**HPLC Analysis of (−)-Verbenone 10-Hydroxylation.** Standard incubation mixtures consisted of rat and human liver microsomes (0.10 mg of protein/ml) or recombinant rat and human P450s (10 pmol) and 200 μM (−)-verbenone (dissolved in 2 μl of dimethyl sulfoxide) 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 unit of glucose-6-phosphate dehydrogenase/ml). Incubations were carried out at 37°C for 20 min and terminated by adding 1.5 ml of CH3Cl2. The mixture was mixed vigorously and centrifuged at 2,000 rpm for 4 min. The organic layer was evaporated to dryness under nitrogen atmosphere, and the residues were dissolved in small amounts of 40% methanol containing 20 mM NaClO4 (pH 2.5).

An aliquot (usually 25 μl) of the extracts described above was used for HPLC analysis with a LC-CCPS system (Tosoh Co., Tokyo, Japan) with a spectrophotometer (FS-2000; Tosoh Co.). Separation was done with a C18 5-μm analytical column (Mightsil RP-18, 150 × 4.6 mm; Kanto Chemical Co., Tokyo, Japan) equipped with a C18 5-μm guard column (Mightsil RP-18, 5–4.6 mm; Kanto Chemical Co.). The eluent consisted of a mixture of 40% methanol containing 20 mM NaClO4 (pH 2.5). The flow rate was 1.0 ml/min, and the UV detection was done at 251 nm (Fig. 1). Peak areas thus obtained were integrated with a Chromatopac Instrument (C-R6A Chromatopac; Shimadzu, Kyoto, Japan).

**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 verbenone 10-hydroxylation by rat and human liver microsomes and recombinant rat and human P450s were estimated using a computer program (KaleidaGraph program from Synergy Software, Reading, PA) designed for nonlinear regression analysis of a hyperbolic Michaelis-Menten equation.

**Results**

**HPLC Analysis of (−)-Verbenone 10-Hydroxylation by Liver Microsomes of Rats and Humans.** (−)-Verbenone was incubated with liver microsomes of human sample HL-J22 and of rats treated with PB, and the products were extracted with dichloromethane and then analyzed with HPLC as described under Materials and Methods (Fig. 1). Human liver microsomes produced small amounts of 10-hydroxyverbenone (Fig. 1A), whereas liver microsomes of PB-treated rats formed (−)-verbenone at much higher rates (Fig. 1B). No other metabolites of (−)-verbenone were detected in these liver microsomes on analysis with HPLC. Formation of 10-hydroxyverbenone increased linearly with incubation time up to 20 min (Fig. 1C) and with liver microsomes of human sample HL-J22 and of rats treated with PB, and the products were extracted with dichloromethane and then analyzed with HPLC as described under Materials and Methods (Fig. 1). Human liver microsomes produced small amounts of 10-hydroxyverbenone (Fig. 1A), whereas liver microsomes of PB-treated rats formed (−)-verbenone at much higher rates (Fig. 1B). No other metabolites of (−)-verbenone were detected in these liver microsomes on analysis with HPLC. Formation of 10-hydroxyverbenone increased linearly with incubation time up to 20 min (Fig. 1C) and with liver microsomes of PB-treated rats up to 0.2 mg/ml of incubation (Fig. 1D).

**Role of Human CYP2A6 in the 10-Hydroxylation of (−)-Verbenone.** Liver microsomes of 16 human samples were used for the determination of 10-hydroxylation of (−)-verbenone and 7-hydroxylation of coumarin, and correlation of these activities was compared (Fig. 2). There was good correlation between these activities (r = 0.89).

Recombinant human P450 enzymes expressed in T. ni cells were tested for their catalytic activities to catalyze (−)-verbenone (Fig. 3). Among 10 human P450 enzymes determined so far, CYP2A6 and 2B6 were found to have the highest activities to hydroxylate (−)-verbenone at turnover rates of 18 and 16 nmol/min/nmol P450, respectively. Activities (nmol/min/nmol P450) with other recombi-
nant human P450s were 0.39 for CYP3A4, 0.38 for CYP2D6, 0.28 for CYP2C9, 0.26 for CYP1A1, 0.16 for CYP1A2, 0.19 for CYP1B1, 0.16 for CYP1A2, 0.09 for CYP2E1, and 0.02 for CYP2C19.

Anti-CYP2A6 IgG significantly inhibited the (−)-verbenone 10-hydroxylation catalyzed by human liver microsomes, HL-J22 (Fig. 4A). (R)-(−)-Menthofuran, an inhibitor of CYP2A6 (Khojasteh-Bakht et al., 1998), inhibited (−)-verbenone 10-hydroxylation catalyzed by human liver microsomes (Fig. 4B) and by recombinant CYP2A6 (Fig. 4C). Triethylenethiophosphoramide (thioTEPA), an inhibitor of human CYP2B6 (Rae et al., 2002), inhibited (−)-verbenone 10-hydroxylation catalyzed by liver microsomes slightly (Fig. 4B) and by recombinant CYP2B6 significantly (Fig. 4D).

Roles of Rat CYP2C11 and 2B1 in the 10-Hydroxylation of (−)-Verbenone. (−)-Verbenone 10-hydroxylation activities were determined in recombinant rat P450 enzymes expressed in T. ni cells (Fig. 5). CYP2B1 was the highest in the verbenone hydroxylation followed by CYP2C11, CYP2A2, and CYP3A1. Other rat P450s were very low in the 10-hydroxylation of (−)-verbenone. Particularly, female-specific CYP2C12 did not catalyze the hydroxylation at all.

Kinetic Analysis of (−)-Verbenone 10-Hydroxylation by Human and Rat P450 Enzymes. Kinetic analysis of (−)-verbenone 10-hydroxylation catalyzed by liver microsomes of human HL-J22 and C12 showed that apparent $K_{m}$ values were 91 and 67 μM, and $V_{max}$ values were 3.5 and 0.4 nmol/min/nmol P450, respectively (Table 1). The $K_{m}$ values of recombinant human CYP2A6 and 2B6 were 16 and 91 μM, respectively. The ratio of $V_{max}$ and $K_{m}$ was about 4-fold higher in CYP2A6 than in CYP2B6. In the rat, the $K_{m}$ values of (−)-verbenone 10-hydroxylation by liver microsomes of untreated rats and of PB-treated rats were 198 and 61 μM, respectively. Liver microsomes of rats treated with β-naphthoflavone and pregnenolone-16α-carbonitrile had high $K_{m}$ values for (−)-verbenone 10-hydroxylation. The apparent $K_{m}$ value of recombinant CYP2B6 was lower than that of CYP2C11 in the verbenone 10-hydroxylation, and the $K_{m}$ values of CYP3A1 and 3A2 were 900 and 1850 μM, respectively.

FIG. 5. (−)-Verbenone 10-hydroxylation by recombinant rat P450 enzymes expressed in T. ni cells. Other details are the same as in the legend to Fig. 3.
Our results showed that human and rat liver microsomes catalyze 10-hydroxylation of (−)-verbenone, a monoterpenic bicyclic ketone and a component of the essential oil from rosemary species such as *Rosmarinus officinalis* L., *Verbena triphylla*, and *Eucalyptus globulus* (Ravid et al., 1997; Giorgio et al., 2002). CYP2A6 was identified to be a principal enzyme involved in the reaction in human liver microsomes with the following lines of evidence. First, there was good correlation between 10-hydroxylation of (−)/H11002)-verbenone and 7-hydroxylation of coumarin, a typical substrate of CYP2A6 (Shimada et al., 1994; Pelkonen et al., 2000), in liver microsomes of 16 human samples. Second, anti-CYP2A6, coumarin, and (R)-(−)/H11001)-menthofuran significantly inhibited (−)/H11002)-verbenone 10-hydroxylation by human liver microsomes. Third, recombinant human CYP2A6 expressed in *Trichoplusia ni* cells had very high turnover rates in the hydroxylation of (−)/H11002)-verbenone. Finally, the efficiency of catalysis (V_{max}/K_{m} value) of CYP2A6 was found to be 4-fold higher than that of CYP2B6, having apparent K_{m} values for CYP2A6 and 2B6 of 16 and 91 μM, respectively. Although recombinant CYP2B6 had a high turnover rate for (−)-verbenone 10-hydroxylation, the contribution of this enzyme in}

### TABLE 1

<table>
<thead>
<tr>
<th>Kinetic analysis of the 10-hydroxylation of verbenone by rat and human liver microsomes and by recombinant rat and human P450 enzymes expressed in <em>Trichoplusia ni</em> cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
</tr>
<tr>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>HL-J22</td>
</tr>
<tr>
<td>HL-C12</td>
</tr>
<tr>
<td>Recombinant human P450s</td>
</tr>
<tr>
<td>CYP2A6</td>
</tr>
<tr>
<td>CYP2B6</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
</tr>
<tr>
<td>Male (untreated)</td>
</tr>
<tr>
<td>Female (untreated)</td>
</tr>
<tr>
<td>Male (PB-treated)</td>
</tr>
<tr>
<td>Male (BNF-treated)</td>
</tr>
<tr>
<td>Male (PCN-treated)</td>
</tr>
<tr>
<td>Recombinant rat P450s</td>
</tr>
<tr>
<td>CYP2B1</td>
</tr>
<tr>
<td>CYP2C11</td>
</tr>
<tr>
<td>CYP3A1</td>
</tr>
<tr>
<td>CYP3A2</td>
</tr>
<tr>
<td>N.D., not detectable; BNF, β-naphthoflavone; PCN, pregnenolone 16a-carbonitrile.</td>
</tr>
</tbody>
</table>

**Discussion**

Our results showed that human and rat liver microsomes catalyze 10-hydroxylation of (−)-verbenone, a monoterpenic bicyclic ketone and a component of the essential oil from rosemary species such as *Rosmarinus officinalis* L., *Verbena triphylla*, and *Eucalyptus globulus* (Ravid et al., 1997; Giorgio et al., 2002). CYP2A6 was identified to be a principal enzyme involved in the reaction in human liver microsomes with the following lines of evidence. First, there was good correlation between 10-hydroxylation of (−)-verbenone and 7-hydroxylation of coumarin, a typical substrate of CYP2A6 (Shimada et al., 1994; Pelkonen et al., 2000), in liver microsomes of 16 human samples. Second, anti-CYP2A6, coumarin, and (R)-(−)-menthofuran significantly inhibited (−)-verbenone 10-hydroxylation by human liver microsomes. Third, recombinant human CYP2A6 expressed in *T. ni* cells had very high turnover rates in the hydroxylation of (−)-verbenone. Finally, the efficiency of catalysis (V_{max}/K_{m} value) of CYP2A6 was found to be 4-fold higher than that of CYP2B6, having apparent K_{m} values for CYP2A6 and 2B6 of 16 and 91 μM, respectively. Although recombinant CYP2B6 had a high turnover rate for (−)-verbenone 10-hydroxylation, the contribution of this enzyme in

**FIG. 6.** Metabolism of (−)-verbenone, 1,4-cineole, 1,8-cineole, and (+)-limonene by human and rat P450 enzymes.
human liver microsomes may be minor, due to the fact that CYP2B6 has been shown to be present at low levels in liver microsomes of human samples (Shimada et al., 1994).

There were clear species-related differences in the metabolism of (-)-verbenone by liver microsomes of humans and rats. As described above, human CYP2A6 was a major enzyme, and CYP2B6 was catalyzed partially in the 10-hydroxylation of (-)-verbenone by liver microsomes. In contrast, rat CYP2A1 and 2A2 were devoid of catalytic activities in rat liver microsomes. Such differences in the catalytic roles of CYP2A enzymes in rats and humans have already been reported in the metabolism of testosterone and coumarin (Yamazaki et al., 1994; Pelkonen et al., 2000). Human CYP2A6 catalyzed coumarin hydroxylation at high rates, whereas rat CYP2A enzymes did not (Yamazaki et al., 1994). It has also been reported that testosterone is catalyzed by rat CYP2A enzymes at the 7α-position, although human CYP2A6 does not catalyze this reaction (Yamazaki et al., 1994). Thus the present results suggest that (-)-verbenone is another substrate that shows differences in the metabolism in rat and human CYP2A enzymes. However, it remains unclear whether such species-related differences in the metabolism of (-)-limonene by CYP2A enzymes affect the biological and toxicological activities of this monoterpene compound in rats and humans.

Species-related differences were also noted in the catalytic roles of rat and human CYP2C enzymes in the 10-hydroxylation of (-)-verbenone in liver microsomes. Rat CYP2C11 catalyzed (-)-verbenone 10-hydroxylation at a rate of 10 nmol/min/nmol P450, whereas human CYP2C9 and 2C19 had turnover rates of 0.28 and 0.02 nmol/min/nmol P450, respectively. In addition, our present results showed that there were sex-related differences in the roles of rat CYP2C enzymes in the (-)-verbenone 10-hydroxylation, in that female-specific CYP2C12 did not catalyze this reaction. Our recent studies have also shown that male rat-specific CYP2C11, but not female rat-specific CYP2C12, metabolizes (+)- and (-)-limonene enantiomers, monoterpene cyclic ethers, in liver microsomes (Miyazawa et al., 2001a,b, 2002b). Collectively, these studies are of interest, since it has been suggested that CYP2C family enzymes are considered to be diversely developed by catalyzing a variety of lipophilic compounds to inert and polar metabolites, when these are ingested through foods and air (Nebert and Gonzalez, 1987; Gonzalez, 1990).

We have previously reported that 1,4-cineole, 1,8-cineole, and (+)- and (-)-limonene enantiomers, monoterpene compounds present in nature, are metabolized by different forms of P450 enzymes in several animal species including rats and humans (Fig. 6) (Miyazawa et al., 2001a,b, 2002b). 1,4-Cineole and 1,8-cineole are both principally oxidized at the 2-position by CYP3A4 in humans and CYP3A1/2 in rats, and it has also been shown that 1,4-cineole is partially catalyzed by CYP2A6 in humans (Miyazawa et al., 2001a,b). The role of CYP2A6 in the oxidation of 1,8-cineole has been reported to be minor (Miyazawa et al., 2001b). (+)- and (-)-Limonenes are metabolized by liver microsomes to form respective carvones and perillyl alcohols by CYP2C9 and CYP2C19 in humans and CYP2C11 in rats (Miyazawa et al., 2002b; Shimada et al., 2002b). Interestingly, rat CYP2B1 has high catalytic rates for the metabolism of (+)- and (-)-limonene enantiomers, whereas the orthologous human enzyme, CYP2B6, does not oxidize limonene metabolism capacity at all (Miyazawa et al., 2002b; Shimada et al., 2002b). These results collectively indicated that natural terpene compounds in the environment are differentially oxidized by different P450 enzymes in several animal species and that the structures of these chemicals determine which molecules are attacked by P450 enzymes (Korzewka and Jones, 1993; Lewis, 1998; Dai et al., 2000). Further work will be required to determine how these terpene compounds cause biological and toxicological responses in animal species, including humans.

In conclusion, the present results showed that (-)-verbenone, a monoterpene bicyclic ketone, was catalyzed by P450 enzymes to form 10-hydroxylated verbenone in human and rat liver microsomes. Human CYP2A6 had the highest rate in the (-)-verbenone 10-hydroxylation, followed by CYP2B6 in human liver microsomes. In the rat, although CYP2A1/2 was inactive in catalyzing verbenone hydroxylation, CYP2C11, a male-specific enzyme, was a major catalyst in untreated male rats. Female-specific CYP2C12 did not catalyze verbenone 10-hydroxylation at a significant rate. Rat CYP2B1 was a major enzyme in the metabolism of verbenone in liver microsomes of PB-treated rats.

References


Miyazawa M, Shindo M, and Shimada T (2001a) Oxidation of 1,8-cineole, the monoterpene cyclic ether originated from Eucalyptus pollybractea, by cytochrome P450 3A3 enzymes in rat and human liver microsomes. Drug Metab Dispos 29:200–205.

Miyazawa M, Shindo M, and Shimada T (2001b) Roles of cytochrome P450 3A3 enzymes in the 2-hydroxylation of 1,4-cineole, the monoterpene cyclic ether, by rat and human liver microsomes. Xenobiotica 31:713–723.


