OXIDATION OF 1,8-CINEOLE, THE MONOTERPENE CYCLIC ETHER ORIGINATED FROM EUCALYPTUS POLYBRACTEA, BY CYTOCHROME P450 3A ENZYMES IN RAT AND HUMAN LIVER MICROSOMES

MITSUO MIYAZAWA, MASAKI SHINDO, AND TSUTOMU SHIMADA

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka, Osaka Japan (M.M., M.S.); and Osaka Prefectural Institute of Public Health, Nakamichi 1-chome, Higashinari-ku, Osaka, Japan (T.S.)

(Received May 10, 2000; accepted October 10, 2000)

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

ABSTRACT:

1,8-Cineole, the monoterpenic cyclic ether known as eucalyptol, is one of the components in essential oils from Eucalyptus polybractea. We investigated the metabolism of 1,8-cineole by liver microsomes of rats and humans and by recombinant cytochrome P450 (P450 or CYP) enzymes in insect cells in which human P450 and NADPH-P450 reductase cDNAs had been introduced. 1,8-Cineole was found to be oxidized at high rates to 2-exo-hydroxy-1,8-cineole by rat and human liver microsomal P450 enzymes. In rats, pregenolone-16α-carbonitrile (PCN) and phenobarbital induced the 1,8-cineole 2-hydroxylation activities by liver microsomes. Several lines of evidence suggested that CYP3A4 is a major enzyme involved in the oxidation of 1,8-cineole by human liver microsomes: 1) 1,8-cineole 2-hydroxylation activities by liver microsomes were inhibited very significantly by ketoconazole, a CYP3A inhibitor, and anti-CYP3A4 immunoglobulin G; 2) there was a good correlation between CYP3A4 contents and 1,8-cineole 2-hydroxylation activities in liver microsomes of eighteen human samples; and 3) of various recombinant human P450 enzymes examined, CYP3A4 had the highest activities for 1,8-cineole 2-hydroxylation; the rate catalyzed by CYP3A5 was about one-fourth of that catalyzed by CYP3A4. Kinetic analysis showed that K_m and V_max values for the oxidation of 1,8-cineole by liver microsomes of human sample HL-104 and rats treated with PCN were 50 μM and 91 nmol/min/nmol P450 and 20 μM and 12 nmol/min/nmol P450, respectively. The rates observed using human liver microsomes and recombinant CYP3A4 were very high among other CYP3A substrates reported so far. These results suggest that 1,8-cineole, a monoterpenoid present in nature, is one of the effective substrates for CYP3A enzymes in rat and human liver microsomes.

1,8-Cineole (Fig. 1), known as eucalyptol, is one of the components present in essential oils from Eucalyptus polybractea. This component has characteristic fresh and camphoraceous fragrance and pungent taste and is used for pharmaceutical preparations as an external applicant, a nasal spray, a disinfectant, an analgesic, or a food flavoring. It is also for cosmetics. Furthermore, it has been reported that 1,8-cineole is used to treat cough, muscular pain, neurosis, rheumatism, asthma, and urinary stone (Geremia, 1955; Margret, 1999). The metabolite of 1,8-cineole in humans has not yet been reported. A variety of components in numerous species of plants have been reported to be oxidized by multiple forms of cytochrome P450 (P450 or CYP) in laboratory animals and humans (Gonzalez and Nebert, 1990; Gonzalez et al., 1991; Khojasteh-Bakht et al., 1999). P450 enzymes have been shown to detoxify and/or toxify these compounds to more polar, and sometimes more reactive, metabolites (Thomassen et al., 1988; Gonzalez, 1989; Guengerich and Shimada, 1991; Guengerich, 1992; Khojasteh-Bakht et al., 1999). For example, pulegone, the major constituent of pennyroyal oil and a hepatotoxin in humans (Anderson et al., 1996), is catalyzed by P450 enzymes to a proximate hepatotoxic metabolite, menthofuran, by P450 enzymes (Thomassen et al., 1988; McClanahan et al., 1989). Several human P450 enzymes, such as CYP2E1, CYP1A2, and CYP2C9, have recently been shown to catalyze oxidation of pulegone (Khojasteh-Bakht et al., 1999).

The aim of the current study was to investigate metabolism of 1,8-cineole by human liver microsomes and by recombinant human P450 enzymes expressed in insect cells in which both human P450 and NADPH-P450 reductase cDNAs had been introduced (Inoue et al., 1999; Shimada et al., 1999). We also determined the 1,8-cineole...
2-hydroxylation activities by liver microsomes of untreated rats and rats treated with several P450 inducers.

Materials and Methods

Chemicals. Ketoconazole, NADP⁺, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). 1,8-Cineol was purchased from Taiyo Koryo Co. (Osaka, Japan). 2-exo-Hydroxy-1,8-cineole was isolated and purified from cultured medium on incubation of 1,8-cineole with *G. cingulata* (Miyazawa et al., 1991). Other reagents and chemicals used in this study were obtained from sources as described previously or of the highest quality commercially available (Shimada et al., 1994, 1998).

Enzyme Preparation. Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously (Mimura et al., 1993; Shimada et al., 1994). 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). Recombinant CYP1A1, -1A2, -2A6, -2B6, -2C9, -2C19, -2D6, -2E1, -3A4, and -3A5 expressed in microsomes of *Trichoplusia ni* cells infected with a baculovirus containing human 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.

Preparation of Rat Liver Microsomes. Male Sprague-Dawley rats (weighing about 200 g) obtained from Nihon Clea Co. (Osaka, Japan) were treated with β-naphthoflavone and isosafrole (50 mg/kg, daily for 3 days), phenobarbital (80 mg/kg, daily for 3 days), or pregnenolone-16α-carbonitrile (PCN, 100 mg/kg, daily for 3 days). Ethanol was given to rats in drinking water.
Results

Identification of 2-Hydroxylated Metabolite of 1,8-Cineole on Incubation with Human Liver Microsomes. 1,8-Cineole was incubated with liver microsomes of human sample HL-104 in the presence of an NADPH-generating system. We detected only one metabolite in this assay condition using GC-MS equipped with EI-MS as described under Materials and Methods (Fig. 2). The formation of 2-exo-hydroxylated metabolite of 1,8-cineole was suggested with the following lines of evidence from analysis with GC and GC-MS: 1) the molecular mass of the metabolite was increased from 154 to 170; 2) dehydration peak (M⁺ – H₂O) was changed from 170 to 152 by this fragment, indicating that a hydroxyl group was introduced into the molecule at the C-2 position of 1,8-cineole (Nishimura et al., 1982); and 3) the position and relative stereochemistry of a hydroxyl group were determined by relative abundance of mass fragments and retention time with GC. These values were consistent with those of authentic 2-exo-hydroxy-1,8-cineole, which has been isolated from biotransformation products of 1,8-cineole with G. cingulata (Miyaizawa et al., 1991).

Fig. 3. Dependence on incubation time (A), P450 contents (B), and 1,8-cineole concentration (C) of 1,8-cineole 2-hydroxylation activities catalyzed by liver microsomes of human sample HL-104.

A. concentrations of 1,8-cineole and P450 were 200 μM and 0.2 nmol P450/ml, respectively. B. incubation time and concentration of 1,8-cineole were 30 min and 200 μM, respectively. C. incubation time and concentration of P450 were 30 min and 0.2 nmol P450/ml, respectively.

for 6 days at a final concentration of 15% (v/v). Rats were starved overnight before being killed, and liver microsomes were prepared.

1,8-Cineole 2-Hydroxylation Assay. 1,8-Cineole 2-hydroxylation activities by P450 enzymes were determined as follows. Standard reaction mixture contained rat and human liver microsomes (0.025 mg of protein/ml) or recombinant P450 (50 pmol/ml) with 200 μM 1,8-cineole in a final volume of 0.50 ml of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system consisting of 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, terminated by adding 1.0 ml of ethyl acetate, and mixed vigorously. The extracts (organic layer) were collected by centrifugation at 3000 rpm for 10 min and transferred to an insert tray for analysis by electron impact (EI)-MS.

A Hewlett-Packard (Atlanta, GA) model 5890A gas chromatograph equipped with a split injector was combined by direct coupling to a Hewlett-Packard 5972 mass spectrometer. The metabolites were separated with a TC-WAX FFS (GL Sciences, Tokyo, Japan) silica capillary column (0.25 mm × 30 m) using helium (2 ml/min) as a carrier gas. The column temperature was programmed from 80°C to 180°C at the rate of 4°C/min and then held at 180°C for 10 min. The injector temperature was set at 270°C. The detector interface temperature was set at 280°C, with the actual temperature in the MS source reaching approximately 180°C and an ionization voltage of 70 eV. The EI mode was used.

Other Assays. P450 contents were estimated spectrophotometrically by the original method (Omura and Sato, 1964). The contents of human CYP3A proteins in human liver microsomes were estimated by coupled SDS-polyacrylamide gel electrophoresis/immunoblotting (Western blotting) (Guengerich et al., 1982). Rabbit anti-serum raised against purified human liver CYP3A4 was prepared as described previously (Mimura et al., 1993). The intensities of the immunoblots were measured with an Epson (Tokyo, Japan) GT-8000 Scanner equipped with NIH Image/Gel Analysis program adapted for Macintosh computers. Protein concentrations were estimated by the method of Lowry et al. (1951).

Statistical Analysis. Kinetic parameters for 1,8-cineole 2-hydroxylation by rat and human P450 enzymes were estimated using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis. Substrate concentrations used for the analysis of 1,8-cineole (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis.

Other Inducers of 1,8-Cineole 2-Hydroxylation. PCN and phenobarbital were found to induce microsomal 1,8-cineole 2-hydroxylation activities on incubation of 1,8-cineole with human liver microsomes in the presence of an NADPH-generating system, the formation of 2-exo-hydroxy-1,8-cineole metabolite was increased with increasing incubation time, P450 levels in liver microsomes, and substrate concentrations (Fig. 3, A, B, and C, respectively).

Effects of treatment of rats with various chemical inducers on liver microsomal 1,8-cineole 2-hydroxylation activities were determined (Table 1). Of various chemical inducers examined, PCN and phenobarbital were found to induce microsomal 1,8-cineole 2-hydroxylation activities, whereas other chemicals did not.

Correlation between CYP3A4 contents and 1,8-cineole 2-hydroxylation activities was examined using liver microsomes of 18 human subjects (Table 1). The correlation coefficient was 0.66 (P < 0.05) between CYP3A4 contents and 1,8-cineole 2-hydroxylation activities.

<table>
<thead>
<tr>
<th>Treatment of Rats</th>
<th>P450 Content</th>
<th>1,8-Cineole 2-Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.67</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>β-Napthoflavone</td>
<td>1.16</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Isosafrole</td>
<td>1.10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1.48</td>
<td>1.6 ± 0.04</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.96</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PCN</td>
<td>1.41</td>
<td>10.4 ± 0.17</td>
</tr>
</tbody>
</table>
samples (Fig. 4). There was good correlation between two parameters ($r = 0.93$). The average rate of 1,8-cineole 2-hydroxylation by liver microsomes of 18 humans was $24.9 \pm 16.9 \text{ nmol/min/nmol P450}$.

The effects of ketoconazole, an inhibitor of CYP3A-dependent activities, on 1,8-cineole 2-hydroxylation by liver microsomes of rats treated with PCN and of human samples HL-11 and HL-104 were determined (Table 2). Ketoconazole significantly inhibited the 1,8-cineole 2-hydroxylation catalyzed by rat and human liver microsomes.

The anti-CYP3A4 IgG was found to inhibit very significantly the 1,8-cineole 2-hydroxylation activities catalyzed by liver microsomes of HL-11 and HL-104 (Table 3), whereas preimmune IgG did not cause any significant decreases in the activities (results not shown).

### Table 2

<table>
<thead>
<tr>
<th>Ketoconazole</th>
<th>1,8-Cineole 2-Hydroxylation by Liver Microsomes of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL-11</td>
<td>HL-104</td>
</tr>
<tr>
<td>µM</td>
<td>nmol/min/nmol P450</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>34.5 (100)</td>
<td>58.0 (100)</td>
</tr>
<tr>
<td>1</td>
<td>7.6 (22)</td>
<td>15.1 (26)</td>
</tr>
<tr>
<td>2</td>
<td>4.1 (12)</td>
<td>9.9 (17)</td>
</tr>
<tr>
<td>4</td>
<td>3.5 (10)</td>
<td>8.7 (15)</td>
</tr>
<tr>
<td>8</td>
<td>2.0 (6)</td>
<td>5.2 (9)</td>
</tr>
<tr>
<td>16</td>
<td>1.8 (5)</td>
<td>4.6 (8)</td>
</tr>
<tr>
<td>32</td>
<td>1.2 (3)</td>
<td>3.5 (6)</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Anti-CYP3A4 IgG</th>
<th>1,8-Cineole 2-Hydroxylation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mg IgG/nmol P450</td>
<td>nmol/min/nmol P450</td>
<td>%</td>
</tr>
<tr>
<td>0.0</td>
<td>33.6 (100)</td>
<td>56.5 (100)</td>
</tr>
<tr>
<td>4.5</td>
<td>6.0 (18)</td>
<td>6.2 (11)</td>
</tr>
<tr>
<td>9.0</td>
<td>2.4 (7)</td>
<td>3.4 (6)</td>
</tr>
</tbody>
</table>

The kinetic analysis of 1,8-cineole 2-hydroxylation activities catalyzed by liver microsomes of rat and human samples and recombinant CYP3A4 was determined (Fig. 5). The $K_m$ value for 1,8-cineole 2-hydroxylation by liver microsomes of rats treated with PCN was $20 \mu M$, and those of liver microsomes of HL-104 and recombinant CYP3A4 were 50 and 90 µM, respectively. $V_{max}$ values were highest in HL-104 (91 nmol/min/nmol P450) followed by recombinant CYP3A4 (48 nmol/min/nmol P450) and PCN-treated rats (12 nmol/min/nmol P450).

### Discussion

Various monoterpenoids, including 1,8-cineole, have been reported to be biotransformed by several microorganisms (Miyazawa, 1997). We have previously shown that 1,8-cineole is metabolized by G. cingulata, a pathogenic fungus, to (−)-2-hydroxy-1,8-cineole as a major metabolite, as well as by some minor metabolites (Miyazawa et al., 1991). Additional studies have indicated that A. niger converts 1,8-cineole to three alcohols and two ketones, such as 2-exo-hydroxy-1,8-cineole, (±)-endo-hydroxy-1,8-cineole, (±)-3-exo-hydroxy-1,8-cineole.
1,8-cineole, 2-oxo-1,8-cineole, and 3-oxo-1,8-cineole (Nishimura et al., 1982). In mammalian species, it has been reported that 1,8-cineole is metabolized to four alcohols, namely 2-oxo-hydroxy-1,8-cineole, 2-endo-hydroxy-1,8-cineole, 3-oxo-hydroxy-1,8-cineole, and 3-endo-hydroxy-1,8-cineole, in rabbits (Miyazawa et al., 1989). However, it remains unclear whether 1,8-cineole is metabolized in humans.

The following lines of evidence suggested that 1,8-cineole is actually oxidized at the 2-position forming 2-hydroxy-1,8-cineole by CYP3A4 in human liver microsomes. First, on incubation of 1,8-cineole with human liver microsomes, 2-hydroxylated metabolite of 1,8-cineole was found to be formed on analysis with GC-MS-EI. In this study, we found that ketoconazole more potently inhibited 1,8-cineole 2-hydroxylation activities catalyzed by CYP3A4/5 enzymes in human liver microsomes than the CYP3A enzymes in rat liver microsomes. Mechanisms underlying such different inhibitory action of ketoconazole in rat and human CYP3A enzymes are not known at present.

Kinetic analysis suggested that the $V_{\text{max}}$ value for 1,8-cineole 2-hydroxylation catalyzed by liver microsomes of human sample HL-104 was extremely high (91 nmol/min/nmol P450). This human sample has been shown to have a high level of CYP3A enzymes in liver microsomes; immunoblotting analysis determined the level of CYP3A proteins in this sample to be about 60% of total P450 (Shimada et al., 1994, 1999). The $V_{\text{max}}$ value for 1,8-cineole 2-hydroxylation by recombinant CYP3A4 expressed in insect cells was determined to be 48 nmol/min/nmol P450; the value was similar to, but lower than, that of liver microsomes of HL-104.

Recombinant human CYP2B6 and CYP2A6 had activities for 1,8-cineole 2-hydroxylation at rates of 2.3 and 1.9 nmol/min/nmol P450, respectively. The levels of these proteins in human liver microsomes have been determined to be about 1 and 4%, respectively, of total P450 (Shimada et al., 1994), indicating that these P450 forms may have minor roles in the oxidation of 1,8-cineole in human liver microsomes.

It has been reported that there are at least four CYP3A enzymes in rats, and among them CYP3A1 and -3A2 are shown to be the major enzymes in rat liver microsomes (Guengerich and Shimada, 1991; Maurel, 1996). Both CYP3A1 and -3A2 have been reported to be induced by several chemical inducers, such as PCN and phenobarbital, although the latter chemical has also been known to be a typical inducer of CYP2B enzymes in rat livers (Maurel, 1996; Yamazaki et al., 1996). The 1,8-cineole 2-hydroxylation activities by liver microsomes were induced in rats by both PCN and phenobarbital, suggesting that CYP3A enzymes are involved in the oxidation of 1,8-cineole in rats. The $K_{\text{m}}$ value for 1,8-cineole 2-hydroxylation by liver microsomes of rats treated with PCN was lower than that of liver microsomes of HL-104, whereas the $V_{\text{max}}/K_{\text{m}}$ ratio of 1,8-cineole 2-hydroxylation was found to be 3-fold higher in HL-104 than in PCN-treated rats.

In conclusion, the present study showed that 1,8-cineole is highly catalyzed by CYP3A enzymes to form 2-oxo-hydroxy-1,8-cineole in rat and human liver microsomes. CYP3A4 is suggested to be a principal enzyme in catalyzing 1,8-cineole 2-hydroxylation by human liver microsomes; the rate catalyzed by liver microsomes of 18 human samples was 26.9 ± 16.9 nmol/min/nmol P450. In rats, 1,8-cineole 2-hydroxylation was also suggested to be catalyzed by CYP3A enzymes that are induced by PCN and phenobarbital in liver microsomes. The rate catalyzed by liver microsomes of PCN-treated rats was about 10 nmol/min/nmol P450 for 1,8-cineole 2-hydroxylation.

### References


