ROLE OF CYP2E1 IN DERAMCICLANE METABOLISM

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ABSTRACT:
The aim of our study was to identify the form(s) of cytochrome P450 responsible for the metabolism of deramciclane, a new anxiolytic drug candidate. The main routes of biotransformation in hepatic microsomes were side chain modification (N-demethylation or total side chain cleavage) and hydroxylation at several points of the molecule. Although several cytochrome P450 forms were involved in the metabolism, the role of CYP2E1 should be emphasized, since it catalyzed almost all steps. Production of deramciclane metabolites was significantly inhibited by diethylthiocarbamate and was elevated in liver microsomes of isoniazid-treated rats. Furthermore, cDNA-expressed rat CYP2E1 generated the metabolites formed by side chain modification and hydroxylation. Neither deramciclane nor its primary metabolite, N-desmethyl deramciclane were able to influence directly the activity of CYP2E1. However, during the biotransformation, one or more metabolites must have been formed which were potent inhibitors of CYP2E1.

Variability in drug response is often due to interindividual differences in drug metabolism. A patient’s individual response to a drug depends on the activities of drug-metabolizing enzymes that determine the fate and elimination of the drug from the body. One of the reasons for variable drug metabolism is the polymorphic expression of these enzymes, mainly cytochromes P450 (P450s). Although poor metabolizers often have a low frequency in the population, being a poor metabolizer can cause severe side effects if a drug is metabolized through a polymorphic enzyme, particularly if it is the only pathway of the elimination (Tamási et al., 2003). Another aspect of interindividual variation involves enzyme induction and inhibition (Rendic and Di Carlo, 1997; Maurel, 1998). Not only drugs, but smoking, alcohol consumption, or foodstuffs can act as inducers or inhibitors of drug-metabolizing enzymes. Environmental influences (including drug interactions) and clinical consequences can be predicted from the identification of the enzyme(s) responsible for the metabolic steps. The procedures to determine the P450 form(s) involved in drug metabolism can use P450 form-selective inhibitors as well as microsomes containing cDNA-expressed P450 proteins (Ono et al., 1996).

Deramciclane, developed by EGIS Pharmaceuticals Ltd. (Budapest, Hungary), is a new non-benzodiazepine-type anxiolytic agent that is more effective than diazepam or chlordiazepoxide (Gacsaýi et al., 1990). Its anticonvulsant activity is exerted via inhibition of synaptosomal γ-aminobutyric acid uptake (Kovács et al., 1989). Our previous work (Monostory et al., 2005) identified the structures of the metabolites formed during the biotransformation of deramciclane in rat, mouse, rabbit, dog, and human primary hepatocytes. Deramciclane underwent side chain modification and oxidation at several positions of the molecule. The side chain modification led to the formation of N-desmethyl deramciclane and phenylborenone. The oxidation of deramciclane resulted in several hydroxy-, carboxy-, and N-oxide derivatives. The hydroxylation took place at primary or secondary carbon atoms of the camphor ring as well as at the side chain. Furthermore, the side chain-modified metabolites were also oxidized to hydroxy- or carboxy-derivatives. Our present work has made an attempt to identify the P450 form(s) participating in deramciclane metabolism.

Materials and Methods

Chemicals. Deramciclane and the presumable metabolites N-desmethyl deramciclane, 9-hydroxy-deramciclane, N-desmethyl 9-hydroxy-deramciclane, and phenylborenone were provided by EGIS Pharmaceuticals Ltd. (Budapest, Hungary). [14C]Deramciclane labeled on the phenyl-ring (1.3394 GBq/mmol) was synthesized at the Chemical Research Center of the Hungarian Academy of Sciences (Budapest, Hungary). d-Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, diethyl-dithiocarbamate, dichloromethane, acetonitrile, perchloric acid, and ethyl acetate were purchased from Merck (Darmstadt, Germany). Triethylamine was obtained from Loba Finechemie (Fischamend, Austria). α-Naphthoflavone, quinidine, treleandomycin, sulfaphenazole, isoniazid, and bovine serum albumin were the products of Sigma Chemie GmbH (Deisenhofen, Germany). Chlorozoxazone was purchased from...
Aldrich Chemical Co. (Steinheim, Germany). 6-Hydroxy-chloroxazone was obtained from Ultrafine Chemicals (Manchester, UK). Methanol and butanol were obtained from ChemoLab (Budapest, Hungary) as chromatography-grade products. All other chemicals were obtained from Reanal (Budapest, Hungary).

**Microsomes.** Male Wistar rats (Charles River, Budapest, Hungary), weighing 150 to 200 g, were pretreated with isoniazid (50 mg/kg i.p.) for 3 consecutive days. Controls were administered the same volume of saline. After an overnight fast, the rats were killed by decapitation, and the livers were excised. Human liver tissues from kidney transplant donors were obtained from Transplantation and Surgery Clinic, Semmelweis University (Budapest, Hungary). The permission of the Local Research Ethics Committee was obtained to use human tissues. Liver microsomes were prepared as described by van der Hoeven and Coon (1974). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Microsomes from human lymphoblastoid cells expressing rat CYP2E1 and NADPH-P450 reductase were purchased from BD Gentest (Woburn, MA).

**Inhibition of Deramiclane Biotransformation.** The incubation mixture (in a final volume of 1 ml) consisted of 0.1 M Tris-HCl buffer (pH 7.4), NADPH-generating system (0.4 mM NADP, 4 mM glucose 6-phosphate, 2 mM MgCl₂, 1 unit/ml glucose-6-phosphate dehydrogenase), 1.5 mg/ml microsomal protein, and 50 μM [¹⁴C]deramiclane (266.4 MBq/mmol). In inhibition studies, selective P450 inhibitors (10 μM α-naphthoflavone, 10 μM sulfaphenazole, 2 μM quinidine, 100 μM diethyl-dithiocarbamate, or 50 μM troleandomycin) were also added to the incubation mixtures (Miners et al., 1988; Kobayashi et al., 1988; Guengerich et al., 1991; Roos et al., 1993; Siess et al., 1995; Maurel, 1996). Diethyl-dithiocarbamate and troleandomycin were preincubated with microsomes and NADPH-generating system for 15 min. Deramiclane biotransformation was carried out aerobically at 37°C for 30 min. Deramiclane and its metabolites were extracted from the aqueous phase three times in 5-ml dichloromethane, the organic phase was evaporated to dryness, and the residue was dissolved in 0.1 ml of methanol. After extraction, the total radioactivity of organic and aqueous phases was determined by liquid scintillation techniques (LKB 1217 Rackbeta type; PerkinElmer Wallac, Turku, Finland). Samples (10 μl) of the organic phases were analyzed by thin-layer chromatography on 0.2-mm-thick DC-Alufolien Kieselgel 60 F254 thin-layer plates (Fig. 1). Deramiclane underwent side chain modification, which led to the production of N-desmethyl deramiclane (M4, the main metabolite) and phenylborneol (M8). Hydroxylation of deramiclane resulted in the formation of 9-hydroxy-deramiclane (M5) and hydroxy-deramiclane II (M6). N-Desmethyl 9-hydroxy-deramiclane (M2) and N-desmethyl hydroxy-deramiclane II (M3), the corresponding hydroxy-derivatives of M4, were also detected. The rate of total deramiclane metabolism by rat liver microsomes was 0.453 ± 0.013 nmol/mg protein/min.

Microsomal fractions from human liver tissues were also incubated with deramiclane. The total metabolism by human microsomes was lower (0.139 ± 0.013 nmol/mg protein/min) than that by rat microsomes. Human microsomal incubations produced primarily side chain-modified metabolites: N'-desmethyl deramiclane (M4) as a major metabolite, and phenylborneol (M8).

Various form-selective inhibitors for P450s were used to identify the individual isozymes participating in deramiclane metabolism (Table 1). Diethyl-diithiocarbamate was the strongest inhibitor of deramiclane biotransformation. In the presence of this CYP2E1 inhibitor, total deramiclane metabolism was inhibited by 62.3 ± 10.66% and 60.25 ± 6.65% in rat and human microsomal fractions, respectively. The formation of N-desmethyl deramiclane (M4) by rat and human microsomes was reduced by about 50% and 35%, respectively, whereas phenylborneol (M8) could not be detected at all. Addition of diethyl-dithiocarbamate to rat liver microsomal fractions decreased the production of 9-hydroxy-deramiclane (M5) by about 40% and totally abolished the formation of N-desmethyl 9-hydroxy-deramiclane (M2), N-desmethyl hydroxy-deramiclane II (M3), and hydroxy-deramiclane II (M6). Other inhibitors used in the metabolic assays (α-naphthoflavone, sulfaphenazole, quinidine and troleandomycin) exhibited modest or no inhibitory effect on deramiclane metabolism. The production of phenylborneol (M8) decreased in the presence of α-naphthoflavone, sulfaphenazole, or troleandomycin. α-Naphthoflavone was also able to inhibit the formation of N-desmethyl 9-hydroxy-deramiclane (M2) in rat liver microsomes. Interestingly, the amount of N-desmethyl 9-hydroxy-deramiclane (M2) increased significantly in the presence of sulfaphenazole (375 ± 108.6% in rat microsomes).

**Biotransformation of Deramiclane by Microsomes of Isoniazid-Pretreated Rat Liver and cDNA-Expressed Rat CYP2E1.** HPLC analysis of the extracts of rat microsomal incubations detected all deramiclane metabolites that were separated by thin-layer chromatography, but an additional derivative (M9) was also observed (Fig. 2A). Pretreatment of rats with isoniazid (the specific CYP2E1 inducer; Ryan et al., 1985) significantly stimulated the hydroxylation of

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**Results**

**Inhibition of Deramiclane Biotransformation.** Thin-layer chromatography was first used to ascertain the full spectrum of metabolites formed during microsomal metabolism of deramiclane. Six metabolites generated by rat liver microsomes were separated on thin-layer plates (Fig. 1). Deramiclane underwent side chain modification, which led to the production of N-desmethyl deramiclane (M4, the main metabolite) and phenylborneol (M8). Hydroxylation of deramiclane resulted in the formation of 9-hydroxy-deramiclane (M5) and hydroxy-deramiclane II (M6). N-Desmethyl 9-hydroxy-deramiclane (M2) and N-desmethyl hydroxy-deramiclane II (M3), the corresponding hydroxy-derivatives of M4, were also detected. The rate of total deramiclane metabolism by rat liver microsomes was 0.453 ± 0.013 nmol/mg protein/min.

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deramiclane (Fig. 3A). The formation of 9-hydroxy-deramiclane (M5), hydroxy-deramiclane II (M6), N-desmethyl 9-hydroxy-deramiclane (M2), and N-desmethyl hydroxy-deramiclane II (M3) increased by about 40 to 45%. The rate of side chain cleavage was also elevated slightly, but significantly during the incubation with microsomes of isoniazid-treated animals. However, no change was observed in the production of N-desmethyl deramiclane (M4) and M9.

The ability of cDNA-expressed rat CYP2E1 to metabolize deramiclane was also examined. CYP2E1 generated all metabolites that were produced by rat liver microsomes, except for M9 (Fig. 3B).

The Effect of Deramiclane on CYP2E1 Activities. Liver microsomes from isoniazid-treated rats were assayed for p-nitrophenol and chlorozoxazone hydroxylation activities of CYP2E1. Deramiclane was not able to decrease the activities of CYP2E1, even at a concentration of 200 μM. However, some of its metabolites produced during the 15- to 60-min preincubation time caused the reduction in p-nitrophenol and chlorozoxazone hydroxylation rate (Fig. 4A). After 60 min of preincubation with deramiclane, chlorozoxazone 6-hydroxylation activity decreased by 43%, whereas p-nitrophenol hydroxylation was almost totally blocked. The inhibition constant (Kᵢ value) for chlorozoxazone 6-hydroxylation was estimated to be 39.9 ± 4.0 μM, referring to 30 min of preincubation with deramiclane.

To characterize the nature and the degree of the binding of deramiclane to microsomal P450, we measured the shift in optical spectra induced by its addition to microsomes. The binding of deramiclane in a manner similar to that of chlorozoxazone induced a type I spectral shift of microsomal P450. However, the association constants were estimated to be different. Kᵢ values of 0.0183 and 0.0338 μM⁻¹ were calculated for deramiclane and chlorozoxazone, respectively.

### Biotransformation of N-Desmethyl Deramiclane by Microsomes of Isoniazid-Pretreated Rat Liver and cDNA-Expressed CYP2E1

Liver microsomes (1.5 mg/ml) were incubated with 50 μM deramiclane in the absence or presence of P450 form-specific inhibitors (α-naphthoflavone; SF, sulfaphenazole; QUIN, quinidine; DDC, diethyl-dithiocarbamate; TAO, troleandomycin). The metabolites were separated on a thin-layer plate using ethyl acetate/triethylamine (25:1, v/v). The amounts of metabolites are presented as the percentage of uninhibited activity.

### Table 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>αNF</th>
<th>SF</th>
<th>QUIN</th>
<th>DDC</th>
<th>TAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Desmethyl 9-hydroxy-deramiclane (M2)</td>
<td>63.0</td>
<td>375.2</td>
<td>138.4</td>
<td>72.3</td>
<td>61.6</td>
</tr>
<tr>
<td>N-Desmethyl hydroxy-deramiclane II (M3)</td>
<td>57.5</td>
<td>64.0</td>
<td>85.4</td>
<td>72.3</td>
<td>61.6</td>
</tr>
<tr>
<td>N-Desmethyl deramiclane (M4)</td>
<td>83.2</td>
<td>120.4</td>
<td>136.0</td>
<td>125.3</td>
<td>125.3</td>
</tr>
<tr>
<td>Hydroxy-deramiclane II (M6)</td>
<td>72.1</td>
<td>85.3</td>
<td>106.9</td>
<td>85.2</td>
<td>16.8</td>
</tr>
<tr>
<td>Phenylborneol (M8)</td>
<td>78.5</td>
<td>88.8</td>
<td>130.3</td>
<td>76.7</td>
<td>17.7</td>
</tr>
<tr>
<td>N-Desmethyl deramiclane (M4)</td>
<td>94.6</td>
<td>84.4</td>
<td>116.6</td>
<td>88.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Phenylborneol (M8)</td>
<td>38.0</td>
<td>29.1</td>
<td>75.1</td>
<td>ND**</td>
<td>38.5</td>
</tr>
</tbody>
</table>

ND, not detectable.

* Significant changes (n = 3); **P < 0.05, ***P < 0.01.
Desmethyl hydroxy-deramiciclane II (M3) was elevated significantly, by 63% and 36%, respectively (Fig. 5A). The induction of CYP2E1 by isoniazid had no effect on the production of M10. On the other hand, cDNA-expressed CYP2E1 was able to generate all three metabolites that were formed by rat liver microsomes (Fig. 5B).

**The Effect of N-Desmethyl Deramiciclane on the Activities of CYP2E1.** Similar to deramiciclane, the rate of \( p \)-nitrophenol or chlorzoxazone hydroxylation also failed to be changed by \( N \)-desmethyl deramiciclane alone, even at the highest concentration (200 \( \mu \)M). Although the activities of CYP2E1 were reduced after 15 to 60 min of preincubation with \( N \)-desmethyl deramiciclane, the inhibitory profile was not exactly the same as that of deramiciclane (Fig. 4B). After 30 min of preincubation with \( N \)-desmethyl deramiciclane, chlorzoxazone 6-hydroxylase activity decreased by about 30%, whereas the loss of \( p \)-nitrophenol hydroxylase activity was 37%, and it was blocked by only 43% even after 60 min of preincubation.

**Discussion**

The knowledge of the metabolic pathway and the structures of the metabolites is essential for the interpretation of the pharmacological or toxicological behavior of a drug. Additionally, revealing the relative contribution of different P450 isoforms to the metabolism can predict some metabolic drug interactions, which are considered one of the reasons for adverse drug effects. All processes (induction or inhibition of drug-metabolizing enzymes) that can modify the hepatic metabolism constitute the source of drug interactions. Our present study made an attempt to identify P450 isoforms involved in the biotransformation of deramiciclane and to determine its effect on the P450 enzyme, which plays the main role in its metabolism.

Although the results clearly demonstrated the implication of several P450 enzymes in the pathways of deramiciclane metabolism both in rat...
and human, CYP2E1 was the main form involved in almost all metabolic steps. The conclusion was drawn from the following data: 1) inhibition of CYP2E1 by diethyl-dithiocarbamate (CYP2E1 inhibitor) decreased the total deramciclane metabolism and the formation of all metabolites; 2) hepatic microsomes of isoniazid-induced rats exhibited higher (by about 40%) metabolite production; and 3) cDNA-expressed CYP2E1 was able to generate all the metabolites except for M9. It should be mentioned that cDNA-expressed CYP2E1 was active in N-demethylation of deramciclane, but the formation of N-desmethyl deramciclane (M4) did not increase as a result of isoniazid treatment. N-Desmethyl deramciclane (M4), which is considered a primary derivative of the parent compound, can be hydroxylated at various positions (Monostory et al., 2005). We think that CYP2E1 induction may result in some elevation of N-demethylase activity, but hydroxylation as a second step causes consumption of N-desmethyl deramciclane at the same time. Our hypothesis is confirmed by the results of N-desmethyl deramciclane biotransformation: 1) hydroxylation of N-desmethyl deramciclane increased significantly in microsomes of isoniazid-treated rats; and 2) cDNA-expressed rat CYP2E1 was able to produce both hydroxylated derivatives of N-desmethyl deramciclane.

The evidence for the involvement of CYP2E1 in deramciclane metabolism suggested that the effect of deramciclane on the activity of CYP2E1 should be investigated. Although deramciclane was not able to affect directly p-nitrophenol or chlorzoxazone hydroxylation, the activities of CYP2E1, preincubation with the parent compound, resulted in the reduction of CYP2E1 activities. In other words, during deramciclane biotransformation, one or more metabolites must have been produced, which were potent inhibitors of CYP2E1. The fact that N-desmethyl deramciclane did not decrease directly the activity of CYP2E1 precludes the possibility of considering it as an inhibitor of...
CYP2E1. Additionally, the results confirmed that some metabolites with inhibitory effect were also formed after preincubation with N-desmethyl deramciclane, but the inhibitory profile was not the same as that observed in the case of deramciclane. It may be assumed that during the incubation with deramciclane, some other, and/or more potent, inhibitors were produced than those found during the biotransformation of N-desmethyl deramciclane. On the other hand, chlorzoxazone binding to microsomal P450 was found to be twice as strong as that of deramciclane, which was also supposed to contribute to the prevention of direct inhibition of CYP2E1-catalyzed chlorzoxazone 6-hydroxylation. It should also be noted that the $K_a$ value for deramciclane measured in microsomes may cover the binding affinity toward several P450 enzymes catalyzing deramciclane metabolism.

A direct inhibitory effect of deramciclane on CYP2D6 has been reported by Laine et al. (2004). The in vivo drug interaction study demonstrated that the CYP2D6-dependent elimination of desipramine was prolonged during coadministration with deramciclane. Although, from this fact, the binding of deramciclane to CYP2D6 might be supposed, our results indicated that CYP2D6 did not play a specific and significant role in deramciclane metabolism.

Our present work has demonstrated that there is no exclusive P450 isoform in deramciclane biotransformation, but the role of CYP2E1 should be emphasized. CYP2E1 catalyzes almost all steps of deramciclane metabolism, and some of the metabolites are supposed to block the activity of CYP2E1.

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References


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