(R)-(+-)-MENTHOFRAN IS A POTENT, MECHANISM-BASED INACTIVATOR OF HUMAN LIVER CYTOCHROME P450 2A6

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ABSTRACT:

(R)-(+-)-Menthofuran is a potent, mechanism-based inactivator of human liver cytochrome P450 (CYP or P450) 2A6. Menthofuran caused a time- and concentration-dependent loss of CYP2A6 activity. The inactivation of CYP2A6 was characterized by a $K_i$ of 2.5 $\mu$M and a $k_{inact}$ of 0.22 min$^{-1}$ for human liver microsomes and a $K_i$ of 0.84 $\mu$M and a $k_{inact}$ of 0.25 min$^{-1}$ for purified expressed CYP2A6. Addition of various nucleophiles, a chelator of iron, or scavengers of reactive oxygen species or extensive dialysis failed to protect CYP2A6 from inactivation. An antibody to metallothionein conjugates of a suspected reactive metabolite of menthofuran was used to detect reactive menthofuran metabolite adducts with CYP2A6. These adducts were formed only in the presence of NADPH-P450 reductase and NADPH. Glutathione, methoxylamine, and semicarbazide did not prevent adduction of reactive menthofuran metabolites to CYP2A6, however. The menthofuran metabolite formation/CYP2A6 inactivation partition ratio was determined to be 3.5 ± 0.6 nmol/nmol of P450. Menthofuran was unable to inactivate CYP1A2, CYP2D6, CYP2E1, or CYP3A4 in a time- and concentration-dependent manner.

Pennyroyal oil is a volatile oil obtained from the leaves of the plants Mentha pulegium and Hedeoma pulegoides (Guenther, 1949), and it is thought to induce abortion (Gleason et al., 1969). However, it does so at lethal or near-lethal doses, so that its action is unpredictable and dangerous (Anderson et al., 1996; Bakerink et al., 1996). (R)-(+-)-Pulegone is the major constituent of pennyroyal oil, and it is responsible for pennyroyal hepatotoxicity (Gordon et al., 1982). Pulegone is metabolized to several metabolites, of which menthofuran appears to be the major proximate toxin, based on toxicokinetic studies (Thomasen et al., 1988) (fig. 1). We now report that menthofuran is a potent, mechanism-based inactivator of human liver CYP2A6.1

Materials and Methods

MATERIALS. N-Acetylcycteine, N-acetyllsine, caffeine, catalase, chloroxazone, deferoxamine mesylate, dextromethorphan, 7-hydroxycoumarin, glutathione, NADP+, and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium cyanide and methoxylamine hydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Semicarbazide hydrochloride was purchased from Eastman Kodak Co. (Rochester, NY). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase (yeast, grade II) were purchased from Boehringer-Mannheim (Indianapolis, IN). The peroxidase rabbit IgG staining kit (Immunopure ABC kit) and diaminobenzidine/peroxidase kit were purchased from Boehringer-Mannheim (Indianapolis, IN). The peroxidase rabbit IgG staining kit (Immunopure ABC kit) and diaminobenzidine/peroxidase kit were purchased from Boehringer-Mannheim (Indianapolis, IN). The cDNA for human CYP2A6 was kindly provided by Dr. Frank J. Gonzalez, National Institutes of Health (Bethesda, MD).

CYP2A6 Inactivation Assays. Menthofuran (0–10 $\mu$M) was incubated with human liver microsomes (HL109) or purified CYP2A6 and the NADPH-generating system to determine its ability to inhibit CYP2A6 in a mechanism-based manner. After exposure of the enzyme to menthofuran and NADPH, aliquots of the mixture were transferred to a vial containing coumarin and the NADPH-generating system, to determine the amount of enzyme activity remaining. Coumarin 7-hydroxylase activity was used as a marker for CYP2A6 activity (Miles et al., 1990).

Dialysis Experiments. Incubations were conducted as described above for CYP2A6 inactivation assays, for 20 min, in the absence or presence of menthofuran (50 $\mu$M). The reaction mixtures were dialyzed against 25 mM potassium phosphate buffer, pH 7.4 (3 x 1 liter), for 4 hr at 4°C, using Slide-A-Lyzer membranes.

NADPH Dependence and Effects of Trapping Agents. The effects of trapping agents were determined by coincubating menthofuran (5 $\mu$M) in the presence of NADPH and trapping agents for 20 min in the preactivation step. Reconstituted CYP2A6. Reconstituted CYP2A6 (100 pmol) was incubated with menthofuran (50 $\mu$M) at 30°C for 60 min, in the absence or presence of a NADPH-generating system. Trapping experiments were performed using reconstituted CYP2A6, menthofuran, and NADPH in the pres-
ence of glutathione (10 mM), N-acetyllysine (10 mM), methoxyamine (10 mM), or semicarbazide (10 mM). The adducted proteins were detected by Western blotting, using an antibody directed against metallothionein conjugated with a γ-ketoenal metabolite of menthofuran (Thomassen et al., 1992; Skiles et al., 1994; Anderson et al., 1996).

**Preparation of Immunogens.** To 5 mg of horse kidney metallothionein (0.735 μmol) in 20 ml of acidic aqueous solution (adjusted to pH 5 with 1 N hydrochloric acid) was added 31.2 mg (147 μmol, 200-fold molar excess) of α,α′-dimethoxydihyromenthofuran [a protected form of the γ-ketoenal 2(2)-(2′-keto-4′-methylocyclohexylene)propanal] in 0.5 ml of ethanol. A 0.2-ml aliquot of the reaction mixture was removed at time 0, 0.5, 3, and 18 hr. To each aliquot, 0.8 ml of 0.1 M phosphate buffer, pH 7.5, was added, and then the total amount of thiols was determined spectrometrically (412 nm) with Ellman’s reagent. After 18 hr of stirring at 4°C, the reaction mixture was transferred to cellulosic dialysis tubing and dialyzed against 10 mM ammonium bicarbonate, pH 7.8 (2 × 3 liters), at 4°C for 48 hr. The dialysate was transferred to plastic vials, lyophilized, and stored at −80°C until immunization. Immunization was performed by injecting modified metallothionein sc into rabbits. Characterization of the antibody will be reported separately.

**Western Blotting.** After 10% resolving sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the separated proteins were blotted to a polyvinylidene fluoride membrane, as described previously (Sambrook et al., 1989). The membrane was blocked with 3% nonfat dry milk in TBS overnight at 4°C with shaking, followed by three 10-min periods of washing with TBS. The membrane was incubated with the primary antibody (diluted 1:500 in TBS) for 3 hr, followed by three 10-min periods of washing with TBS. The secondary antibody used was included in the ABC kit from Pierce. The membrane was incubated with the biotinylated goat anti-rabbit antibody for 30 min, followed by three 10-min periods of washing with TBS. The membrane was then incubated with avidin-biotin complex solution for 30 min, followed by washing (3 × 10 min) with TBS. The membrane was developed with diaminobenzidine/metal-enhanced developer.

**Partition Ratio Experiments.** The partition ratio was determined by quantifying the metabolites of menthofuran (mintlactone and isomintlactone) by GC/MS after a 60-min incubation. A reconstituted system of purified expressed CYP2A6 (100 pmol), rat NADPH-P450 reductase, and human cytochrome b5 was incubated at 30°C in 250 μl (final volume) of an incubation mixture containing 100 mM potassium phosphate buffer, pH 7.4, 0.5 mM NADPH, 10 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and menthofuran. The internal standard was acethylthione (10 μM). The GC/MS analysis has been described previously (Anderson et al., 1996).

**CYP1A2, CYP2D6, CYP2E1, and CYP3A4 Inactivation Assays.** Selected assays were used as probes for the activities of CYP1A2, CYP2D6, CYP2E1, and CYP3A4. Caffeine N-demethylation was used as a probe for CYP1A2 activity (Tassaneeyakul et al., 1992), dextromethorphan O-demethylation for CYP2D6 activity (Dayer et al., 1989), chlorozoxazone 6-hydroxylation for CYP2E1 activity (Peter et al., 1990), and dextromethorphan N-demethylation for CYP3A4 activity (Jacqz-Aigrain et al., 1993). Each assay was carried out with incubations that were conducted as described above for CYP2A6 inactivation by menthofuran.

**Results**

**Time-Dependent Inhibition of Human CYP2A6 by Menthofuran.** Inhibition of CYP2A6 activity by menthofuran was found to be time- and concentration-dependent in both human microsomal and reconstituted CYP2A6 incubations (figs. 2a and 3a). The K_i values for menthofuran were determined from nonlinear regression analysis of the data to be 2.5 and 0.84 μM for CYP2A6 in human liver microsomes and for the reconstituted purified enzyme, respectively (figs. 2b and 3b). The k_{max} values were determined to be 0.22 and 0.25 min⁻¹ for the microsomal and purified enzymes, respectively.

**NADPH Dependence and Effects of Trapping Agents.** In our studies, the concentration of menthofuran (5 μM) and the inactivation time (20 min) were chosen so that approximately 10% of the enzyme activity remained (table 1). Inactivation of CYP2A6 by menthofuran was demonstrated to be NADPH dependent. The protective effects of various trapping agents on the inactivation were also determined. The nucleophilic trapping agents glutathione and N-acetylcycteine failed to protect CYP2A6 from inactivation. Sodium cyanide protected 78% of the CYP2A6 activity. The free iron scavenger deferoxamine failed to protect CYP2A6 from inactivation. Furthermore, superoxide dismutase and catalase, scavengers of reactive oxygen species, did not protect the enzyme.

**CYP1A2, CYP2D6, CYP2E1, and CYP3A4 Inactivation.** Little inactivation of CYP1A2, CYP2D6, CYP2E1, or CYP3A4 by menthofuran was observed at concentrations greater than those required to completely inactivate CYP2A6 (table 2). Complete inactivation of CYP2A6 occurred under those conditions at 10 μM (fig. 2n).

**Covalent Binding of Menthofuran Metabolites to CYP2A6.** Immunochemical methods were used to detect CYP2A6 adducts with reactive metabolites of menthofuran. Western blots of proteins incubated with menthofuran in the presence of NADPH showed a band corresponding in molecular mass to CYP2A6 (~52 kDa) and a band corresponding in molecular mass to NADPH-P450 reductase (~76 kDa) that were absent when NADPH was not present in the incubation mixture (fig. 4, lanes 1 and 2). Various trapping agents did not protect CYP2A6 from adduction. However, glutathione, methoxyamine, and semicarbazide prevented adduction to NADPH-P450 reductase, as shown by the intensities of bands at ~76 kDa (fig. 4). N-Acetyllysine, on the other hand, did not change the intensities of the bands observed, compared with control.

**Partition Ratio of Menthofuran with CYP2A6.** The menthofuran metabolite formation/CYP2A6 inactivation partition ratio was deter-
mined using an incubation time of 60 min, to ensure complete inactivation of CYP2A6. The ratio was 3.5 ± 0.6 nmol/nmol of P450.

Discussion

Menthofuran was previously shown to be metabolically activated to chemically reactive intermediates that are capable of covalent binding to cellular proteins (Thomassen et al., 1992). Investigations with inhibitors and inducers of hepatic P450 demonstrated an association between hepatocellular damage caused by menthofuran and its metabolomic activation and covalent binding to target organ proteins.

In this study we observed inactivation of CYP2A6 by menthofuran. The inactivation of CYP2A6 by menthofuran was determined to be mechanism-based, by considering the following observations. 1) Menthofuran inactivated CYP2A6 in a time- and concentration-dependent manner (figs. 2 and 3), 2) The loss of activity was NADPH dependent (table 1). 3) The loss of activity was irreversible and was not recoverable by extensive dialysis. 4) Inactivation was not protected against by exogenous nucleophiles. However, sodium cyanide was not recovered by extensive dialysis. 5) Inactivation was not protected by trapping agents (table 1).

The bands at ~52 and ~76 kDa correspond to proteins with the molecular masses of CYP2A6 and NADPH-P450 reductase, respectively.

![FIG. 3](image)

**Fig. 3.** Menthofuran-mediated inactivation of purified expressed CYP2A6 in the presence of a NADPH-generating system (a) and double-reciprocal plot of the relationship between the inactivation rate and the menthofuran concentration (b).

In α, results are averages of duplicate values. The concentrations of menthofuran in the inactivation assay were 0 µM (●), 0.1 µM (●), 0.5 µM (○), 1 µM (■), and 5 µM (○).

**TABLE 1**

<table>
<thead>
<tr>
<th>Components</th>
<th>Activity</th>
<th>Amount Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>20 min</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Menthofuran (5 µM) - NADPH</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td>Menthofuran (5 µM) + Glutathione</td>
<td>94</td>
<td>11</td>
</tr>
<tr>
<td>+ N-Acetylcycteine</td>
<td>91</td>
<td>11</td>
</tr>
<tr>
<td>+ Sodium cyanide</td>
<td>57</td>
<td>34</td>
</tr>
<tr>
<td>+ Deferoxamine</td>
<td>96</td>
<td>10</td>
</tr>
<tr>
<td>+ Catalase</td>
<td>92</td>
<td>11</td>
</tr>
<tr>
<td>+ Superoxide dismutase</td>
<td>79</td>
<td>8</td>
</tr>
<tr>
<td>Menthofuran + NADPH (dialyzed)²</td>
<td>95</td>
<td>8</td>
</tr>
<tr>
<td>Control (dialyzed)²</td>
<td>99</td>
<td>92</td>
</tr>
</tbody>
</table>

*Human liver microsomes (HL109) were incubated at 30°C with the components. Results are averages of duplicate runs, in which the difference in values was <5%.

* 100% is 5.21 nmol of 7-hydroxycoumarin formed/nmol of P450/min.

* Glutathione, N-acetylcycteine, sodium cyanide, and deferoxamine were used at 2 mM concentrations. Catalase was used at 2000 units and superoxide dismutase at 500 units.

* Human liver microsomes, menthofuran (5 µM), and NADPH were incubated for 20 min. The reaction mixtures were dialyzed against 1 liter of 25 mM potassium phosphate, pH 7.4, three times for 4 hr at 4°C.

* Reactions were incubated as described for d, except menthofuran was absent.

to cellular proteins (Thomassen et al., 1992). Investigations with inhibitors and inducers of hepatic P450 demonstrated an association between hepatocellular damage caused by menthofuran and its metabolomic activation and covalent binding to target organ proteins.

In this study we observed inactivation of CYP2A6 by menthofuran. The inactivation of CYP2A6 by menthofuran was determined to be mechanism-based, by considering the following observations. 1) Menthofuran inactivated CYP2A6 in a time- and concentration-dependent manner (figs. 2 and 3), 2) The loss of activity was NADPH dependent (table 1). 3) The loss of activity was irreversible and was not recovered by extensive dialysis. 4) Inactivation was not protected against by exogenous nucleophiles. However, sodium cyanide was able to protect CYP2A6 from inactivation, at least in part because of inhibition of CYP2A6 activity (table 1). 5) Inactivation was not a consequence of the generation of reactive oxygen species, because catalase and superoxide dismutase did not protect CYP2A6 from inactivation, at least in part because of inhibition of CYP2A6 activity (table 1).

Menthofuran was found to be a relatively efficient inactivator of CYP2A6, with a $k_i$ of ~1 µM, a $k_{inact}$ of ~0.2 min⁻¹ (figs. 2 and 3), and a partition ratio of ~3.5 nmol/mm. It was highly selective for CYP2A6, because several other P450 isoforms were not inactivated at concentrations of menthofuran significantly greater than those required for inactivation of CYP2A6 (table 2).

Interestingly, oxidative metabolites of menthofuran formed adducts with CYP2A6 and with NADPH-P450 reductase (fig. 4). The intensities of the CYP2A6 bands were unchanged when various trapping
agents were used in the incubations. The intensities of the bands corresponding to NADPH-P450 reductase were affected by various trapping agents. The decreases in the intensities of the bands demonstrate that the reactive electrophilic metabolite that leaks out of the active site of CYP2A6 is a soft electrophile (trapped with glutathione and methoxylamine) and contains an aldehyde group (trapped with semicarbazide). It is unclear whether these adducts are those that lead to inactivation of CYP2A6; addition of the reductase does not apparently affect its ability to reduce other P450 isoforms, inasmuch as CYP2E1 activity in human liver microsomes is unaffected under conditions in which inactivation of CYP2A6 occurs (table 2).

Acknowledgments. We are grateful to Stella J. Thompson and Allan E. Rettie (Department of Medicinal Chemistry, University of Washington) for providing us with the recombinant baculovirus for human CYP2A6. The cDNA for human CYP2A6 was kindly provided by Dr. Frank J. Gonzalez (National Institutes of Health).

References


Erratum

In the article entitled “Metabolism of β-Arteether to Dihydroqinghaosu by Human Liver Microsomes and Recombinant Cytochrome P-450” (Drug Metab Dispos 26:313–317, 1998), the name of a secondary author was inadvertently omitted. The author list, with the name originally omitted shown in bold, should read as follows: James M. Grace, Antonio J. Aguilar, Kimberly M. Trotman, James O. Peggins, and Thomas G. Brewer.