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Inhibition of human cytochrome P450 enzymes by the natural hepatotoxin safrole

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Abstract

The hepatotoxin, safrole is a methylenedioxy phenyl compound, found in sassafras oil and certain other essential oils. Recombinant cytochrome P450 (CYP, P450) and human liver microsomes were studied to investigate the selective inhibitory effects of safrole on human P450 enzymes and the mechanisms of action. Using *Escherichia coli*-expressed human P450, our results demonstrated that safrole was a non-selective inhibitor of CYP1A2, CYP2A6, CYP2D6, CYP2E1, and CYP3A4 in the IC_{50} order CYP2E1 < CYP1A2 < CYP2A6 < CYP3A4 < CYP2D6. Safrole strongly inhibited CYP1A2, CYP2A6, and CYP2E1 activities with IC_{50} values less than 20 μM. Safrole caused competitive, non-competitive, and non-competitive inhibition of CYP1A2, CYP2A6 and CYP2E1 activities, respectively. The inhibitor constants were in the order CYP1A2 < CYP2E1 < CYP2A6. In human liver microsomes, 50 μM safrole strongly inhibited 7-ethoxyresorufin *O*-deethylation, coumarin hydroxylation, and chlorzoxazone hydroxylation activities. These results revealed that safrole was a potent inhibitor of human CYP1A2, CYP2A6, and CYP2E1. With relatively less potency, CYP2D6 and CYP3A4 were also inhibited.

Keywords: Safrole; Human; CYP1A2; CYP2A6; CYP2E1

1. Introduction

Cytochrome P450 (CYP, P450)-dependent monooxygenase is the primary enzyme system catalyzing the oxidations of a variety of endogenous and exogenous chemicals including steroids, drugs, and chemical carcinogens (Guengerich, 1995). Oxidations catalyzed by this monooxygenase require a hemoprotein P450, a flavoprotein NADPH-P450 reductase, and phospholipids. In human liver, CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 are the major forms, which constitute about 13%, 4%, 18%, 2%, 7%, and 29% of total P450 contents, respectively (Shimada et al., 1994). These P450 forms have broad substrate specificities and are responsive to the inductive and inhibitory effects of xenobiotics including plant constituents (Ioannides, 2002; Ueng and Chen, 2004).

Methylenedioxybenzenes are the substrates, inducers, and inhibitors of P450 (Murray, 2000). These compounds have differential modulatory effects and selectivities on P450 forms. Safrole (4-allyl-1,2-methylenedioxybenzene) is a natural derivative of this group and constitutes 70–80% of sassafras oil. Some flavouring spices such as star anise, cumin, black pepper, and ginger also had about 9, 3, 1, and 0.5 mg/g safrole, respectively (Farag and Abo-Zeid, 1997). Safrole was a hepatotoxin and metabolized by human CYP2C9 and CYP2E1 (Ueng et al., 2004). Safrole inhibited 7-methoxyresorufin *O*-demethylation, 7-ethoxyresorufin *O*-deethylation, and *p*-nitrophenol

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hydroxylation activities of untreated, 3-methylcholanthrene-, and acetone-treated mouse liver microsomes, respectively (Zhao and O'Brien, 1996). This result suggested that safrole was an inhibitor of mouse CYP1A and CYP2E1. However, the effects of safrole on human P450 forms were not reported. Thus, the in vitro effects of safrole on human P450 enzymes were studied using *Escherichia coli*-expressed bicistronic human P450 enzymes and human liver microsomes. In this report, our results revealed that safrole was a non-selective inhibitor of human P450s. Safrole was a competitive, non-competitive, and non-competitive inhibitor of CYP1A2, CYP2A6, and CYP2E1, respectively.

2. Materials and methods

2.1. Chemicals

Chlorzoxazone, coumarin, dextromethorphan, dextrorphan, 7-ethoxyresorufin, β-NADP, safrole, and tolbutamide were purchased from Sigma-Chemico Inc. (St Louis, MO, USA). 6-Hydroxychlorzoxazone and 4-hydroxytolbutamide were purchased from Sigma/RBI (Natick, MA). Safrole was distilled and the purity was 98% as analyzed by HPLC.

2.2. Enzyme preparations

The constructed plasmids of bicistronic human P450s were kindly provided by Dr. F. Peter Guengerich (Nashville, TN, USA). Bicistronic human CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 constructs consisting of the coding sequence of a P450 followed by that of NADPH-P450 reductase were transformed to *E. coli* DH5α by electroporation (Gene pulser II, Bio-Rad, Hercules, CA, USA). Bacterial membrane fractions were prepared by differential centrifugation following the method of Parikh et al. (1997). Bacterial membrane fractions were stored at −75 °C until use. The non-tumor human liver samples were obtained from patients who underwent liver resection in Taipei Veteran General Hospital, Taipei. All liver samples were kept at −70 °C immediately after surgery according to a protocol approved by the medical committee for conducting human research at the hospital. Experiments using human liver samples were under the agreement of Department of Surgery, Taipei Veteran General Hospital, Taipei. Human liver microsomes were prepared following the method of Guengerich (1994) and stored at −75 °C.

2.3. Enzyme assays

P450 content was determined using the spectrophotometric method of Omura and Sato (1964). 7-Ethoxyresorufin *O*-deethylation activity was determined by measuring the fluorescence of resorufin (Pohl and Fouts, 1980). The hydroxylations of chlorzoxazone and tolbutamide and the oxidation of nifedipine were determined by HPLC with an UV detector (Guengerich et al., 1986; Peter et al., 1990; Yamazaki et al., 1998). Coumarin hydroxylation and dextromethorphan *O*-demethylation activities were determined by HPLC with a fluorescence detector (Souček, 1999; Koenigs et al., 1997). The substrate concentrations were 0.5 mM chlorzoxazone, 20 μM coumarin, 200 μM dextromethorphan, 2 μM 7-ethoxyresorufin, 0.2 mM nifedipine, and 2.5 mM tolbutamide. Microsomal protein concentration was determined by the method of Lowry et al. (1951). Oxidations were initiated by the addition of a NADPH-generating system containing NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. Duplicated reactions were carried out at 37 °C with shaking for 10–20 min. Stock solution of safrole was prepared using methanol and the final content of methanol was less than 1% in the reaction volume of 0.2–1.0 ml. The same amount of methanol was added in the control reactions.

2.4. Data and kinetic analyses

The concentration of safrole required for 50% inhibition of catalytic activities (IC_{50}) was calculated by curve fitting (Grafit, Erithacus Software Ltd., Staines, UK). Kinetic analyses of P450 activities were done following Michaelis–Menten kinetic property. Values of velocities (v) at various substrate concentrations (S) were fitted by non-linear least-squares regression without weight due to the equation consistent with respective inhibitory type according to the Michaelis–Menten equation: competitive inhibition, $v = V_{max} \times S/\{S + K_m \times [1 + (I/K_i)]\}$ and non-competitive inhibition, $v = V_{max} \times S/(K_m + S)[1 + (I/K_i)]$ (Sigma Plot, Jandel Scientific, San Rafael, CA, USA). V_{max}, K_i, and I are the maximal velocity, inhibitor constant, and safrole concentration, respectively. The initial estimates of K_i values were obtained from the Lineweaver–Burk and Dixon Plots. For the spectral binding of safrole with P450, values of absorbance differences ($\Delta A_{389-420\ nm}$) at various concentrations of safrole were fitted using non-linear least-squares regression without weight due to the equation: $\Delta A = (\Delta A_{max} \times I)/(K_s + I)$, where K_s is the spectral dissociation constant.

3. Results

P450 activities of the membrane fractions from *E. coli*-expressing human P450 forms were determined. 7-Ethoxyresorufin was used as a common substrate of CYP1A1, CYP1A2, and CYP1B1. Coumarin, tolbutamide, dextromethorphan, chlorzoxazone, and nifedipine were used as the model substrates for CYP2A6,

CYP2C9, CYP2D6, CYP2E1, and CYP3A4, respectively. The 7-ethoxyresorufin *O*-deethylation activities of CYP1A1, CYP1A2, and CYP1B1 were 74.8, 4.9, and 8.7 nmol/min/nmol P450, respectively. The coumarin hydroxylation, tolbutamide hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone hydroxylation, and nifedipine oxidation activities of each form were 2.0, 11.6, 3.2, 5.7, 20.3 nmol/min/nmol P450, respectively. Safrole inhibited human CYP1A2, CYP2A6, CYP2D6, CYP2E1, and CYP3A4 activities. The IC_{50} values for CYP1A2, CYP2A6, CYP2D6, and CYP3A4 were 3-, 7-, 65-, and 26-fold higher than that for CYP2E1, respectively (Table 1). In contrast, the addition of safrole at the concentration up to 100 μM had no effect on CYP1A1, CYP1B1, and CYP2C9 catalytic activities. To examine the metabolism-dependent P450 inhibition, P450 was preincubated with 5–100 μM safrole in the absence or presence of a NADPH-generating system at 37 °C for 10 min. However, the inhibition with preincubation in the presence of NADPH was not different from that in the absence of NADPH (results not shown).

Inhibitory kinetic parameters of safrole were determined by non-linear regression following the Michaelis–Menten equation using bacterial membranes expressing CYP1A2, CYP2A6, and CYP2E1 (Fig. 1). For CYP1A2, safrole was a competitive inhibitor with a K_i value of 1.5 ± 0.1 μM. For CYP2A6, safrole was a non-competitive inhibitor with a K_i value of 30.9 ± 3.6 μM. For CYP2E1, safrole was a non-competitive inhibitor with a K_i value of 2.8 ± 0.1 μM. Safrole caused a type I binding spectrum of CYP1A2 and CYP2E1 using 0.25 nmol P450/ml (results not shown). However, no obvious binding spectrum was formed by safrole and CYP2A6 at 0.25 nmol P450/ml. The apparent spectral dissociation constant K_s of safrole for CYP1A2 and CYP2E1 were 13.6 ± 1.1 and 20.5 ± 1.8 μM, respectively. The maximal absorbance changes (ΔA_{max}) caused by CYP1A2 and CYP2E1 were 0.017 ± 0.000 and 0.022 ± 0.001, respectively.

Table 1
IC_{50} values of the inhibition of cytochrome P450 activities by safrole

CYP	Assay	IC_{50}, μM
1A1	7-Ethoxyresorufin *O*-deethylation	>100
1A2	7-Ethoxyresorufin *O*-deethylation	5.7 ± 0.9
1B1	7-Ethoxyresorufin *O*-deethylation	>100
2A6	Coumarin hydroxylation	12.0 ± 0.5
2C9	Tolbutamide hydroxylation	>100
2D6	Dextromethorphan *O*-demethylation	110 ± 12
2E1	Chlorzoxazone hydroxylation	1.7 ± 0.8
3A4	Nifedipine oxidation	43.5 ± 8.5

Bacterial membrane fractions of *E. coli* expressing human P450s were prepared and activities were determined as described in Section 2. P450 concentration in the assays was 20 pmol/ml.

To investigate the effects of safrole on activities of P450 forms in human liver microsomes, microsomes were prepared from three human liver samples. The addition of 50 μM safrole caused 71%, 86%, 36%, and 87% decreases of 7-ethoxyresorufin *O*-deethylation, coumarin hydroxylation, dextromethorphan *O*-demethylation, and chlorzoxazone hydroxylation activities, respectively (Table 2). In contrast, the influence on tolbutamide hydroxylation, dextromethorphan *O*-demethylation, and nifedipine oxidation were less than 15%. In the presence of 20 μM safrole, human liver microsomal NADPH-P450 reductase activity was 92% of the control value (Fig. 2). Addition of 50–150 μM safrole caused about 30% decreases of the cytochrome *c* reduction activity of NADPH-P450 reductase in human liver microsomes.

4. Discussion

The methylenedioxyphenyl compound, safrole is an aromatic constituent of many plant oils and spices. In mouse liver microsomes, safrole inhibited CYP1A and CYP2E1 catalytic activities (Zhao and O'Brien, 1996). Our results of human P450s demonstrated that safrole was an inhibitor not only for CYP1A2 and CYP2E1 but also for CYP2A6, CYP2D6, and CYP3A4 (Table 1). Among CYP1 family, safrole selectively inhibited CYP1A2 catalytic activity. The IC_{50} values of safrole for P450 forms were in the order CYP2E1 < CYP1A2 < CYP2A6 < CYP3A4 < CYP2D6. Safrole was a competitive, non-competitive, and non-competitive inhibitor of CYP1A2, CYP2A6, and CYP2E1, respectively (Fig. 1). The inhibitor constant K_i values were in the order of CYP1A2 < CYP2E1 < CYP2A6. These results suggested that safrole bound either to P450 or both P450 and P450-substrate complex depending on P450 forms. Safrole was oxidized by P450 to form epoxides, catechols, and hydroxyl metabolites (Ioannides et al., 1981). Human CYP2E1 and maybe CYP2A6 catalyzed the 1′-hydroxylation of safrole (Ueng et al., 2004; Jeurissen et al., 2004). However, the results of different heterologous expressing systems of CYP2A6 were inconsistent and the correlation of coumarin hydroxylation and safrole 1′-hydroxylation activities was consistently not significant in human liver microsomes. Our previous report showed that *E. coli* expressed human CYP1A2 also catalyzed this reaction but its activity was only 3% of that of CYP2E1. Ioannides et al. (1985) reported that safrole caused a type I binding spectrum with the K_s values of 22 and 98 μM of phenobarbital- and 3-methylcholanthrene-treated rat liver microsomes, respectively. Our results of binding spectra using bacterial membranes showed that the K_s values of safrole for CYP1A2 (13.6 μM) and CYP2E1 (20.5 μM) appeared to be similar. This result suggested that CYP1A2 and CYP2E1 had similar binding affinity to

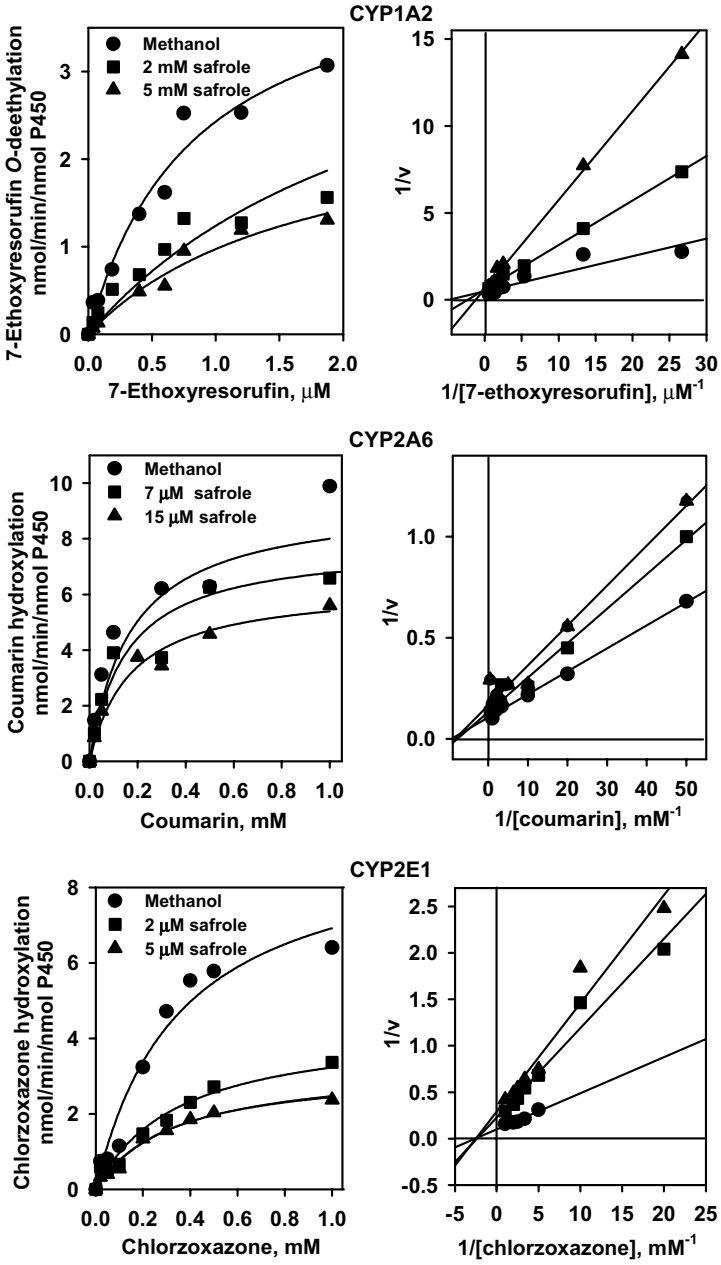

Fig. 1. Inhibition kinetics of safrole on CYP1A2, CYP2A6, and CYP2E1 activities. Bacterial membranes expressing CYP1A2, CYP2A6, and CYP2E1 containing 5, 20, and 20 pmol/ml P450 were used in the assays, respectively. Right panels: the plots of velocity (v) versus substrate concentration. Left panels: The Lineweaver–Burk plots. The solid lines show the best fit determined as described in Section 2. Data are the means of duplicate determinations.

safrole to cause the iron spin state change of P450. Thus, the roles of CYP1A2 and CYP2A6 in other oxidative pathways of safrole are of interest.

Besides the competitive effects, formation of methylenedioxyphenyl metabolite intermediate (MI)–P450 complex was suggested to be another important inhibitory mechanism of methylenedioxyphenyl compounds in vitro and in vivo (Ioannides et al., 1981; Murray, 2000). However, our results showed that the inhibitory effects of safrole were not enhanced by preincubation of P450 with safrole up to 100 μM in the presence of a NADPH-generating system (results not shown). The K_m value of human CYP2E1 for safrole oxidation was 1.2 mM and was relatively high as compared to the concentration required for inhibition of CYP1A2, CYP2E1, and CYP2A6 activities (Ueng et al., 2004). Since the oxidative metabolism is the crucial step of complex formation, the influence of NADPH-dependent reaction on the inhibition might require high concentration of safrole. In addition, safrole decreased NADPH-P450 reductase activity at concentra-

Table 2
Effects of safrole on model reactions of P450 forms in human liver microsomes

Model reactions	Percent of control
7-Ethoxyresorufin *O*-deethylation (CYP1A2)	29 ± 16
Coumarin hydroxylation (CYP2A6)	14 ± 4
Tolbutamide hydroxylation (CYP2C9)	92 ± 5
Dextromethorphan *O*-demethylation (CYP2D6)	64 ± 12
Chlorzoxazone hydroxylation (CYP2E1)	13 ± 3
Nifedipine oxidation (CYP3A4)	85 ± 9

Safrole was dissolved in methanol. Activities were determined in the absence or presence of 50 μM safrole. The same concentration of methanol was added in the control incubation. The methanol concentration was less than 0.2%. The representative control activities of 7-ethoxyresorufin *O*-deethylation, coumarin hydroxylation, tolbutamide hydroxylation, chlorzoxazone hydroxylation, and nifedipine oxidation were in the range of 27.0–33.2 pmol/min/mg protein, 0.19–0.30 nmol/min/mg protein, 0.06–0.12 nmol/min/mg protein, 0.02–0.15 nmol/min/mg protein, 0.25–0.53 nmol/min/mg protein, and 0.78–1.43 nmol/min/mg protein, respectively. Results represent mean ± SEM of three human liver samples. The main P450 forms involved in the oxidations are shown in parentheses.

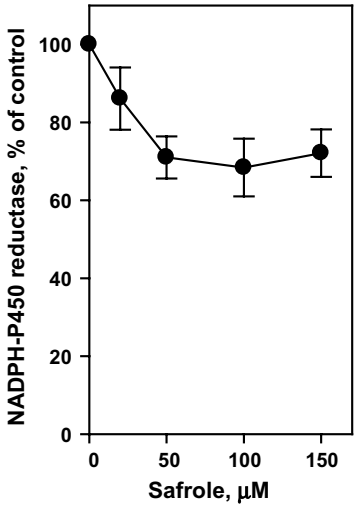

Fig. 2. Effect of safrole on NADPH-cytochrome P450 reductase activity in human liver microsomes. Results represent the mean ± SEM of three human liver samples. The cytochrome *c* reduction activities of microsomal reductase were 235.0, 201.3, and 228.0 nmol/min/mg protein.

tion higher than 50 μM. Thus, reversible competitive inhibition plays a major role in the inhibition at low concentration, <50 μM. At higher concentration of safrole, both MI-complex formation and reductase inhibition may be involved in the P450 inhibition.

In Taiwan, areca quid was commonly chewed together with *Piper betel inflorescence*, which has high safrole content (15 mg/g). In human orally treated with 1.66 mg safrole, there was about 12 μM safrole in plasma after 30 min (Benedetti et al., 1977). The hepatic concentration of safrole may be higher in areca quid-chewing persons. In human liver microsomes, safrole at 50 μM caused more than 70% loss of 7-ethoxyresorufin *O*-deethylation, coumarin hydroxylation and chlorzoxazone hydroxylation activities (Table 2). In mice, treatment with safrole for 1 h before carbon tetrachloride treatment decreased the hepatic toxicity of carbon tetrachloride, which was mainly activated by CYP2E1 (Zhao and O'Brien, 1996). These reports suggested that the P450 inhibitory effect of safrole might occur in vivo. In addition to the expression of P450s in the liver, the mRNA of safrole affected P450s, CYP1A2 and CYP2E1, were detectable by reverse transcription-polymerase chain reaction in human buccal mucosa (Vondracek et al., 2001). In areca quid-chewing persons, the saliva concentration of safrole could be as high as 420 μM (Wang and Hwang, 1993). This concentration was higher than the IC_{50} values for human P450 inhibition. Safrole was a hepatocarcinogen in experimental rodents and possibly in human (Ioannides et al., 1981; Lin et al., 2002). Besides the carcinogenicity of safrole, the detoxication and activation of food-born toxicants might be changed by the exposure to safrole.

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