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EFFECTS OF CINNAMOPHILIN ON MOUSE HEPATIC DRUG-METABOLIZING ENZYMES *IN VITRO* AND *IN VIVO*

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Cinnamophilin (CIN) showed protective effect against oxidative damage. Cytochrome P450-dependent monooxygenase (P450) and glutathione S-transferase (GST) are important drug-metabolizing enzymes and P450 plays an important role in drug-drug interactions. To assess the possible drug interactions, effects of CIN on mouse P450 and GST were studied. *In vitro*, CIN inhibited liver microsomal nifedipine oxidation activity with an IC_{50} of 17.3 ± 1.8 μM. However, CIN had no effect on 7-ethoxyresorufin O-deethylation and coumarin hydroxylation activities. *In vivo*, oral administration of CIN had no effects on liver microsomal P450 content and NADPH-P450 reductase activity. Hepatic P450-catalyzed oxidations of 7-ethoxyresorufin, 7-methoxyres-orufin, coumarin, tolbutamide, N-nitrosodimethylamine, and nifedipine were not affected by CIN. These results revealed that CIN was a mouse CYP3A inhibitor *in vitro* without affecting P450 and GST activities *in vivo*.

**Key words**: Cinnamophilin, Cytochrome P450, Glutathione S-transferase.

INTRODUCTION

Cytochrome P450 (P450)-dependent monooxygenase is the primary enzyme system involved in the metabolism of many endogenous substrates such as fatty acids and steroid hormones, as well as many xenobiotics including drugs, carcinogens and environmental pollutants[1]. The microsomal monooxygenase system consists of a family of P450 hemoproteins, NADPH-P450 reductase and phospholipids. Endogenous and exogenous factors, such as steroids, drugs and natural products can modulate P450 enzymes. Cytosolic glutathione S-transferase (GST)is a main enzyme responsible for xenobiotic conjugative metabolism[2]. GST plays a primary role in the detoxication of drugs, procarcinogens, and toxins. GST-catalyzed conjugation reduced the toxicities of the active metabolite of and the metabolites of benzo(a)pyene and aflatoxin B_1. However, GST may also play a bioactivation role in the toxic

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reaction of pro-mutagens, such as ethylene dibromide. GST is responsive to the induction and inhibition by xenobiotics. Alterations in the pool of P450 and GST enzymes can influence the biological fate of foreign chemicals in experimental animals and cause drug interactions and toxicity[2-4].

Due to the broad substrate specificity of P450, specific substrates can be used as chemical probes for P450 enzymes. CYP1A1 is essentially an extrahepatic P450 and CYP1A2 is the CYP1A member in human and untreated rodent liver. 7-Ethoxyresorufin O-deethylation (EROD) and 7-methoxyresorufin O-demethylation (MROD) were catalyzed by both CYP1A1 and CYP1A2. Although there was controversy about the selectivity of 7-ethoxyresorufin O-deethylation (EROD) and 7-methoxyresorufin O-demethylation (MROD), the induction and inhibition studies suggested that EROD and MROD were preferentially metabolized by CYP1A1 and CYP1A2, respectively[5,6]. Coumarin was recognized as a model representative substrate of human CYP2A6[7]. Tolbutamide hydroxylation is the marker reaction for human CYP2C9[7]. Nitroso-compounds occur in many foodstuffs special in sea-foods and have carcinogenic effects in experimental animals. The demethylation of N-nitrosodimethylamine (NDM) is mainly performed by CYP2E1, which is an ethanol inducible form in rodents[8]. Nifedipine, a calcium channel blocker is a marker substrate for human CYP3A4[9]. Therefore, these substrates can be used as specific substrates for respective P450 forms.

Many edible plants and herbs have been reported to cause drug interactions with pharmaceutical drugs[4]. One glass of grapefruit juice increases the serum concentration of felodipine through the inhibition of intestinal CYP3A. Administration of the anti-depressant, St. John's wort, resulted in pregnane X receptor-mediated CYP3A4 induction and caused drug interactions with CYP3A4 substrates in human[10]. These reports demonstrated the crucial role of P450 in drug-drug interaction. Cinnamophilin (CIN) (scheme 1) was isolated from *Cinnamomum philippinense* and found to be a novel antioxidant and a free radical scavenging agent[11]. CIN inhibited the function of thromboxane synthase and reduced the activation of thromboxane A2 receptor[12,13]. Modulation of drug-metabolism is a crucial factor of drug-drug interaction. During drug development, examination of the drug metabolizing enzymes affected by target compound is an important criterion. To assess the possible drug-drug interaction, we have studied the *in vitro* effect of CIN on P450 activities and the *in vivo* effects of CIN on P450 and GST activities in mouse liver.
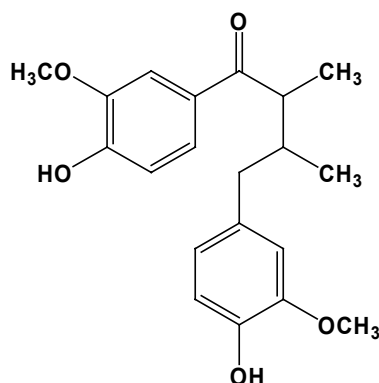
Scheme 1. Structure of cinnamophilin.

MATERIALS AND METHODS

Materials

CIN, cytochrome c, 7-methoxyresorufin, 7-ethoxyresorufin, NADH, NADPH, N-nitrosodimethylamine, and nifedipine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U. S. A.). CIN was isolated and purified from the root of *Cinnamomum philippinense* as described before[13]. The structure of CIN was identified by NMR analysis. The purity of CIN was > 99% when analyzed by HPLC. Pyridine metabolite of nifedipine was a gift from Dr. F. Peter Guengerich, Vanderbilt University, Nashville, TN, U. S. A..

Animal Treatment and Microsomal and Cytosolic Preparation

Male C57BL/6J mice (5 weeks old, weighing 10~15 g) were purchased from the National Animal Center in Taiwan. Before experimentation, mice were allowed a one-week acclimation period at the animal quarters with air conditioning and an automatically controlled photoperiod of 12 hr light daily. For the *in vitro* studies, CIN was dissolved in DMSO and the final concentration of DMSO was less than 0.5%. For the *in vivo* studies, CIN was dissolved in corn oil. Control group was treated with the same dose of corn oil without CIN. Livers were removed 20 hours after the last treatment. Liver cytosol and washed microsomes were prepared using differential centrifugation at 4 °C[14]. Enzyme activities were determined within two weeks.

Enzyme Assays

P450 content was determined by the spectrophotometric method of Omura and Sato[15]. NADPH-P450 reductase activity was determined following the method of Phillips and Langdon[16] using cytochrome c as a substrate. The O-dealkylations of 7-ethoxyresorufin and 7-methoxyresorufin were determined by measuring fluore-scence of resorufin[17]. Coumarin hydroxylation activity was determined by HPLC with a fluorescence detector[18]. Tolbutamide hydroxylation was determined by HPLC[19]. N-Demethylation of N-nitrosodimethylamine was determined by measuring the formation of formaldehyde using Nash's reagent[20]. Nifedipine oxidation was determined following the method of Guengerich et al.[21]. Cytosolic GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate in the presence of glutathione[22]. Microsomal and cytosolic protein concentrations were determined by the method of Lowry *et al.*[23].

Data and Statistical Analysis

The IC_{50} of CIN for the inhibition of nifedipine oxidation activity was calculated by graph fitting (Grafit, Erithacus Software Ltd., Staines, UK). Estimates of variances (denoted by±) are presented from analysis of individual sets of data. In the *in vivo* studies, the statistical significance of differences between control and treated animals was evaluated by the Student's t-test. For multiple comparisons, data were analyzed by one-way (ANOVA)

followed by *post-hoc* Dunnett's *t*-test. A *p* < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

CYP3A is the most abundant hepatic P450 form in rodents and humans[7]. In the *in vitro* study, our results demonstrated that CIN inhibited liver microsomal nifedipine oxidation, which is the model reaction catalyzed by CYP3A（Fig. 1）. The IC_{50} value of CIN for this inhibition was 17.3 ± 1.8 μM. However, simultaneous addition of CIN with substrate to the enzyme reaction mixture had no effects on 7-ethoxyresorufin O-deethylation and coumarin hydroxylation activities. This result indicated that CIN was an inhibitor of CYP3A *in vitro*. Lee *et al.*[24] reported that mouse brain infraction was reduced by intraperitoneal treatment of mice with a single injection of 80 mg/kg CIN 2 hour after transient middle cerebral artery occlusion. The plasma concentration of CIN was 130 μM. It is higher than the IC_{50} value for inhibition of nifedipine oxidation activity in our mouse study. Thus, mice were treated with CIN at the dose up to 100 mg/kg to elucidate the *in vivo* effect of CIN. For medical application, oral ingestion is the most common way for the treatment of medicines. Therefore, mice were treated with CIN by gastrogavage and CIN was given daily for 5 days in the following *in vivo* study.

In the *in vivo* studies, oral administration of CIN at the dose range between 25 and 100 mg/kg/day for 5 days had no effect on hepatic activities of 7-ethoxyresorufin O-deethylation, coumarin hydroxylation, and nifedipine oxidation (Fig. 2). Treatment of mice with 100 mg/kg CIN for 5 days had no effects on mouse body and liver weights (Table 1). Liver microsomal P450 content and NADPH-P450 reductase activity were not affected by CIN at 100 mg/kg. Besides of the P450 activities described above, this treatment had no effect on other P450 activities including 7-methoxyresorufin O-demethylation, tolbutamide hydroxylation, and N-nitrosodimethylamine N-demethylation activities (Table 2). These results suggested that CIN had no effects on CYP1A-, CYP2A-, CYP2C-,
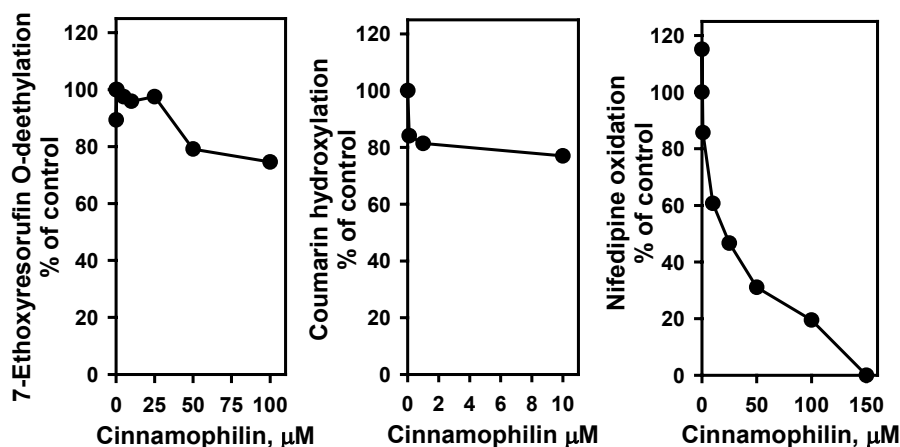

Fig. 1.   *In vitro* **effects of cinnamophilin on 7-ethoxyresorufin O-deethylation, coumarin hydroxylation, and nifedipine oxidation activities of mouse liver microsomes. Results represent the mean of duplicated determination.**
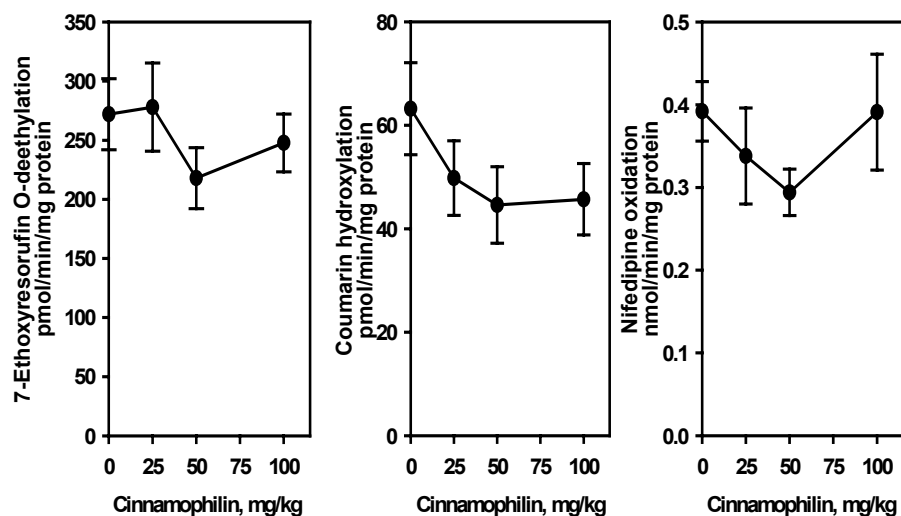
Fig. 2. **Dietary effects of cinnamophilin on 7-ethoxyresorufin O-deethylation, coumarin hydroxylation, and nifedipine oxidation activities. Cinnamophilin was dissolved in corn oil. Mice were treated with 25, 50, and 100 mg/kg cinnamophilin for five days. Control group receive the same dose of corn oil without cinnamophilin.**

Table 1. **Effects of cinnamophilin on body and liver weights and P450 content and NADPH-P450 reductase activity in mouse liver**

	Control	Cinnamophilin
Body weight, g	16.2 ± 0.53	17.2 ± 0.39
Liver weight, g	0.90 ± 0.03	0.96 ± 0.05
Liver weight/body weight, g/g	0.06 ± 0.00	0.06 ± 0.00
Cytochrome P450 nmol/mg protein	0.68 ± 0.02	0.62 ± 0.05
NADPH-Cytochrome P450 reductase nmol/min/mg protein	245.5 ± 24.3	304.5 ± 35.8

Mice were treated with 100 mg/kg/day cinnamophilin dissolved in corn oil by gastrogavage for 5 days. Results represent means ± SEM of 6 and 5 mice in control and cinnamophilin-treated groups, respectively.

Table 2. **Effects of cinnamophilin on mouse hepatic monooxygenase and glutathione S-transferase activities *in vivo***

Assay	Control	Cinnamophilin
7-Methoxyresorufin O-demethylase nmol/min/mg protein	0.781 ± 0.068	0.775 ± 0.105
7-Ethoxyresorufin O-deethylase nmol/min/mg protein	0.272 ± 0.03	0.247 ± 0.024
Coumarin hydroxylation pmol/min/mg protein	63.2 ± 8.9	45.7 ± 6.9
N-Nitrosodimethylamine N-demethylase nmol/min/mg protein	2.92 ± 0.24	2.97 ± 0.28
Tolbutamide hydroxylation pmol/min/mg protein	30.2 ± 4.0	22.2 ± 2.2
Nifedipine oxidase n mol/min/mg protein	0.39 ± 0.04	0.39 ± 0.07
Glutathione S-transferase µmol/min/mg protein	6.78 ± 0.81	5.39 ± 0.64

Mice were treated with control or 100 mg/kg cinnamophilin for 5 days. Data are presented as mean ± SEM of 6 and 5 animals for control and cinnamophilin-treated group, respectively.

CYP2E1-, and CYP3A-catalyzed oxidations toward their respective model substrates in mouse liver. CIN had no effect on cytosolic GST activity in mouse liver, either.

It is clear that *in vitro* results should be extrapolated to *in vivo* with caution. First, P450 modulation shows tissue specificity. Grapefruit juice strongly inhibited CYP3A-catalyzed oxidations *in vitro*[25]. However, the administration of grapefruit juice had no effect on hepatic CYP3A but inhibited intestinal CYP3A4 expression and

activities *in vivo*. The inhibition of intestinal CYP3A caused herb-drug interactions in clinics. Other possible causes of lose of inhibitory effect of CIN *in vivo* includes age of mice, oral bioavailability of CIN, and the time-dependence of modulatory effect. To illustrate the causes described above, further investigation will be performed.

ACKNOWLEDGEMENTS

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Cinnamophilin 對 C57BL/6J 鼷鼠肝藥物代謝之體外及體內之作用

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　　Cinnamophilin（CIN）具有抗氧化傷害之保護作用。細胞色素 P450（P450）及 glutathione 硫-轉移酶（GST）為重要之藥物代謝酶，藥物代謝酶之調控影響是產生藥物交互作用之重要因素。為評估 CIN 因影響藥物代謝酶而產生藥物交互作用之可能性，因而研究 CIN 對鼷鼠 C57BL/6J 肝 P450 及 GST 之影響，體外實驗之結果顯示 CIN 對微粒體 nifedipine oxidation 活性有抑制作用，其 IC_{50} 值為 17.3 ± 1.8 μM。以餵管給藥方式，將鼷鼠處理 CIN，結果顯示以 100 毫克/公斤/天 CIN 連續處理鼠 5 天後，對鼠肝微粒體 P450 含量及 NADPH-P450 還原酶活性無影響。這處理不影響鼠肝微粒體 P450 氧化代謝 7-ethoxyresorufin，7-methoxyresorufin，coumarin，tolbutamide，N-nitrosodimethylamine 及 nifedipine 等的活性，CIN 處理對鼠肝細胞質液 GST 活性亦無影響。這些結果顯示 CIN 在體外抑制 nifedipine oxidation 活性，但以口服處理鼠，CIN 對鼠肝 P450 及 GST 活性均無影響。

關鍵詞：cinnamophilin，細胞色素 P450，glutathione 硫-轉移酶。

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