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Inhibition of Benzo(a)pyrene Hydroxylation by Lignans Isolated from *Justicia procumbens*

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ABSTRACT

Five lignans, neojusticin A (neo A), neojusticin B (neo B), justicidin A (jus A), justicidin B (jus B), and chinensinaphthol methyl ether (CME) were isolated from the ethanol extract of *Justicia procumbens* (*J. p.*), which has been used as a herbal remedy in traditional Chinese medicine. The *in vitro* effects of lignans on rat hepatic cytochrome P450-catalyzed oxidations were studied. Addition of 10 μM lignans caused 21% to 57% decreases of microsomal benzo(a)pyrene hydroxylation (AHH) activity. Among these lignans, neo B had the strongest inhibitory effect on AHH activity with an IC_{50} of 6 ± 1 μM. Lignans at 10 μM decreased 7-ethoxyresorufin O-deethylation activities by 20% to 48%. Neo B at 10 μM caused a 70% decrease of 7-methoxyresorufin O-demethylation activity whereas this oxidation activity was relatively less affected by other lignans. Lignans (10 μM) also decreased testosterone 6β-hydroxylation activity by 19% to 74% and neo B had the least inhibitory effect on this activity. Kinetic analysis of AHH activity revealed that neo B was a mixed type inhibitor of competitive and noncompetitive characteristics with K_i and K_i' of 2 μM and 11 μM, respectively. With NADPH as the variable cofactor, neo B showed a uncompetitive type of inhibition of AHH activity. These *in vitro* results suggested that lignans from *J. p.* inhibited monooxygenase activities differentially and neo B might have a role in diminishing the oxidative activation of benzo(a)pyrene.

Key words: *Justicia procumbens*, lignan, benzo(a)pyrene hydroxylation

INTRODUCTION

The cytochrome P450 (CYP)-dependent monooxygenase system consists of CYP, NADPH-CYP reductase, and phospholipids. This monooxygenase plays a major role in the detoxification and bioactivation of xenobiotics including drugs, environmental pollutants, and chemical carcinogens [1,2]. Benzo(a)pyrene (B(a)P), a polycyclic aromatic hydrocarbon (PAH), is released into the atmosphere from incomplete combustion processes. Humans ingest B(a)P and other PAHs from charbroiled meat, cigarette smoke, and automobile emissions[3]. CYP-mediated metabolic activation of B(a)P is necessary for its conversion to form the reactive metabolite, B(a)P 7,8-diol-9,10-epoxide, which binds to DNA and initiates tumor formation[4]. The importance of the metabolic activation of B(a)P has led to the suggestion that inhibitors of B(a)P hydroxylation (AHH) activity could diminish the mutagenicity and carcinogenicity of B(a)P[5,6]. Therefore, we examined the inhibition of AHH activity by traditional Chinese medicine.

Justicia procumbens (*J. p.*) has been used as a traditional herbal remedy for the treatment of fever and pain from pharyngolaryngeal swelling. Recently, it has been used as a constituent of a herbal tea in Taiwan. Arylnaphthalene lignans have been reported as the major active components in *J. p.*[7,8]. These lignans also showed antiviral and antitumor activities[9]. Five lignans (Scheme 1), neojusticin A (neo A), neojusticin B (neo B), justicidin A (jus A), justicidin B (jus B), and chinensinaphthol methyl ether (CME) have been isolated and purified from the ethanol extract of *J. p.* by monitoring the anti-platelet activity[10]. To investigate the beneficial effects of these lignans against B(a)P, the effects of lignans on microsomal AHH and related monooxygenase activities were studied *in vitro*.

MATERIALS AND METHODS

I. *Lignans and Chemicals*

Lignans were isolated from air-dried *J. p* by the method described previously[10]. The purity of these lignans were > 95% as analyzed by NMR and HPLC analyses. NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytochrome *c*, B(a)P, 7-ethoxyresorufin, 7-methoxyresorufin, testosterone and 6β-hydroxytestosterone were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methanol, acetone, *n*-hexane, and dichloromethane were purchased from Merck Chemical Co. (Darmstadt, Germany).

II. *Animal Treatments*

Male Sprague Dawley rats (four weeks, weighing 200 ~ 250 g) were purchased from the Animal Center of National Yang-Ming University, Taipei, Taiwan. Microsomes were isolated from rat livers by differential centrifugation[11]. Washed microsomal pellets were covered with 0.1 M potassium phosphate buffer, pH 7.4 and stored at –75˚C. Monooxygenase activities were determined within two weeks.

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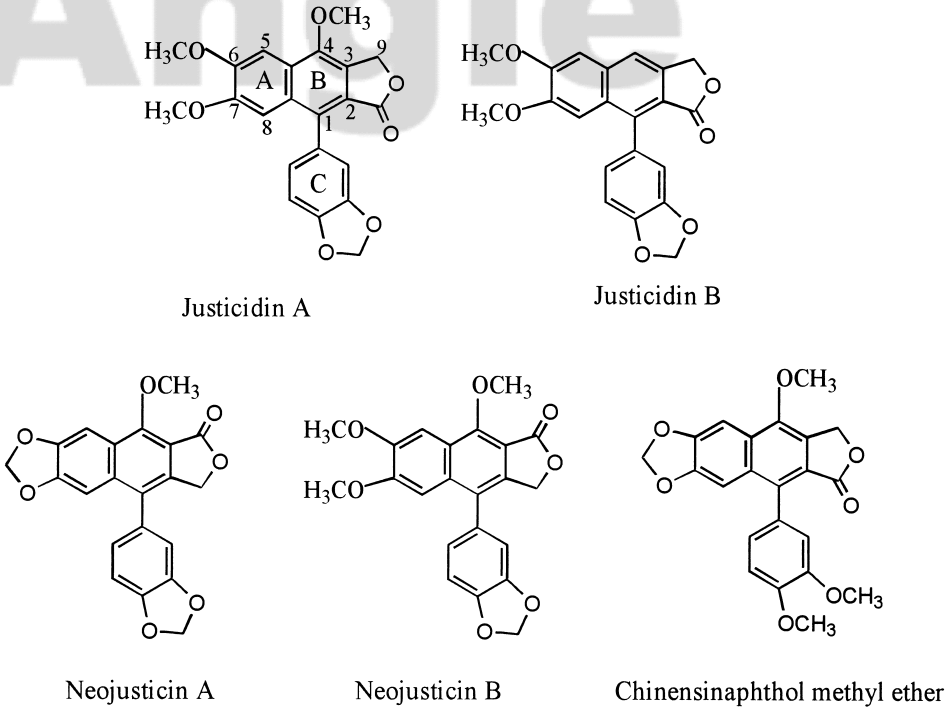

Scheme 1. Structures of arylnaphthalene lignans isolated from *Justicia procumbens*[10].

III. *Monooxygenase Assays*

The CYP contents were determined by CO-difference spectral analysis as described by Omura and Sato[12]. NADPH-CYP reductase activity was determined following the method of Phillips and Langdon[13] using cytochrome *c* as a substrate. In the AHH assay, formation of 3-hydroxybenzo(a)pyrene was detected by fluorometry[14]. Activities of 7-ethoxyresorufin O-deethylation (EROD) and 7-methoxyresorufin O-demethylation (MROD) were determined by measuring fluorescence of resorufin[15]. For testosterone 6β-hydroxylation (TESH), the formation of 6β-hydroxytestosterone was analyzed by HPLC using a C18 column (100 Å, 4.6 × 250 mm, Varian Analytical Instrument, CA, U.S.A.) and detected by A_{254nm}[16]. Microsomal protein concentration was determined by the method described by Lowry *et al.*[17].

IV. *Data Analysis*

The IC_{50} value of neo B for AHH activity was calculated by graph fitting (Grafit, Erithacus Software Ltd., Staines, UK). Kinetic analysis of AHH activity was done following the Michaelis-Menten kinetic property. Values of velocity (v) at various substrate concentrations (s) were fitted by nonlinear least-squares regression without weight due to the equation, v = Vm(s/(Km + s) (Sigma plot, Jandel Scientific, San Rafael, CA). The mixed type inhibitor constants for enzyme (K_i) and enzyme-substrate complex (K_I) were obtained by linear regression of the plots of I *vs* 1/Vm(i) and I *vs* Km(i)/Vm(i), where I was the concentrations of neo B and Vm(i) and Km(i) were Vm and Km in the presence of neo B, respectively. Values of velocity at various NADPH concentrations were fitted by nonlinear least-squares regression without weight due to the equation, consistent with uncompetitive inhibition according to the Michaelis-Menten equation: v = Vm·S/{Km+S·[1+(I/Ki)]}. Estimates of variances (denoted by ±) are presented from analysis of individual sets of data.

RESULTS AND DISCUSSION

To study the effects of lignans on CYP-catalyzed oxidations, monooxygenase activities were determined in the presence of increasing concentrations of lignans (Fig. 1). The lignans at 10 μM inhibited rat microsomal AHH activity by 21% to 57% (Fig. 1A). Among these lignans, neo A and neo B showed greater inhibitory effects on AHH activity and neo B had the most potent inhibitory effect on AHH activity with an IC_{50} of 6 ± 1 μM. Many studies have been done on rat hepatic CYP enzymes and have suggested that CYPs including CYP1A, CYP2C, and CYP3A were involved in the oxidation of B(a)P[18]. CYP1A has the highest AHH activity and CYP3A is the most abundant CYP in mammalian livers [18,19,20]. CYP1A1 and CYP1A2 are two major members of the CYP1A subfamily. CYP1A2 is the main hepatic CYP1A member and CYP1A1 is essentially an extrahepatic CYP1A in rodents and humans[20]. EROD and MROD were preferentially catalyzed by CYP1A1 and CYP1A2, respectively[21,22]. In control rats, EROD was mainly catalyzed by CYP2C and CYP2B[22]. Our data showed that 10 μM lignans caused 20% to 48% decreases of EROD activity (Fig. 1B). The inhibitory effects of lignans were jus B > CME $\simeq$ neo B > neo A $\simeq$ jus A. The presence of neo B inhibited MROD

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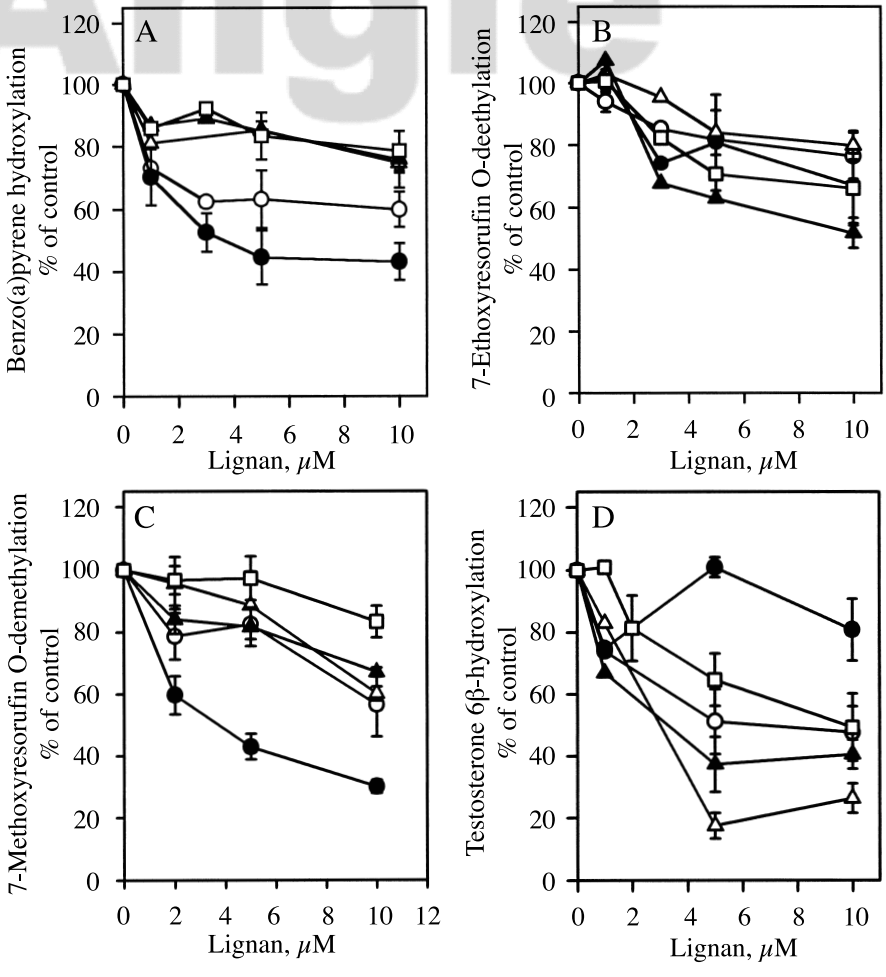

Figure 1. Inhibitory effects of arylnaphthalene lignans on monooxygenase activities of rat liver microsomes. Increasing concentrations of lignans (❍, neo A; ●, neo B; Δ, jus A; ▲, jus B; ❑, CME) were added to the NADPH-supported microsomal reaction mixtures and monooxygenase activities were determined as described in the section of Materials and Methods.

activity in a concentration-dependent manner, whereas there were relatively less inhibitory effects from the other lignans (Fig. 1C). Neo B at 10 μM caused a 70% decrease of MROD activity. TESH was selectively catalyzed by CYP3A[23]. Lignans (10 μM) decreased TESH by 19% to 74%. The inhibitory effects of lignans were jus A > jus B $\simeq$ neo A $\simeq$ CME > neo B (Fig. 1D). Neo B had the least inhibitory effect on TESH activity. Our results suggest that these lignans could affect CYP activities with differential potency and neo B had stronger inhibitory effects on both AHH and MROD activities. The relationship between structures and the inhibitory effects of lignans is not clear. However, neo B showed the strongest inhibition on MROD activity, but the least inhibition on TESH activity. Jus A and jus B had a stronger inhibition effect on TESH activity, but less inhibition on AHH and MROD activities. These results suggest that the relative position of the C ring and the keto group in the γ-lactone moiety and the presence of 4-, 6- and 7-methoxy groups might play roles in the determination of inhibition selectivity of CYP1A and CYP3A.

To understand the inhibitory property of neo B on AHH activity, kinetic analysis of AHH activity in the presence of various concentrations of neo B were performed. Fig. 2A shows the velocity (v) *vs* substrate concentration (s) plot. Nonlinear regression analysis generated the V_m and K_m values in the presence of increasing concentrations of neo B (Table 1). In the presence of 10 μM neo B, the V_m value was decreased to 48% of the value in the absence of neo B (Table 1). In the presence of 1, 5, and 10 μM neo B, the K_m values were 9 ± 2, 12 ± 4, and 12 ± 6 μM, respectively (Table 1). Kinetic analysis of the Lineweaver-Burk plots suggested that neo B was a mixed type of competitive and noncompetitive

Table 1. Kinetic analyses of AHH activity in the presence of neo B

Neo B, μM	V_m	K_m	r^2*
0	415 ± 13	11 ± 4	0.95
1	316 ± 13	9 ± 2	0.99
5	251 ± 16	12 ± 4	0.97
10	200 ± 19	12 ± 6	0.94

Hepatic AHH activity and kinetic parameters were determined as described in the section of Materials and Methods. * r^2 represents coefficient of the nonlinear regression determination.

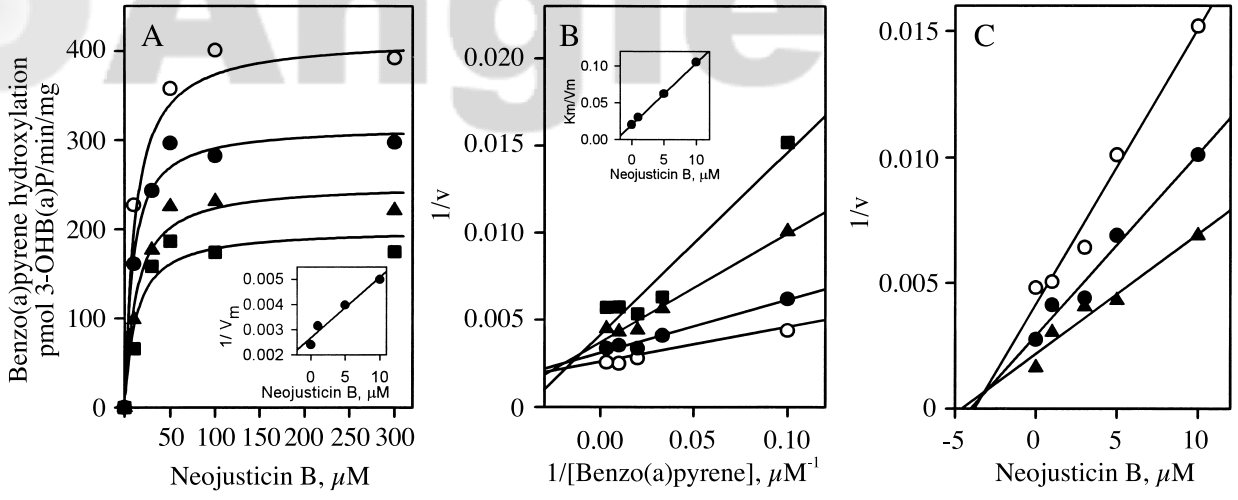

Figure 2. Steady-state kinetics of AHH activity and the effects of neo B. Plots of v, expressed in pmol/ min/ mg protein, *vs* S, benzo(a)pyrene concentration (A) and Lineweaver-Burk plots (B) at increasing concentrations of neo B (○, 0 μM; ●, 1 μM; ▲, 5 μM; ■, 10 μM) are presented, with lines drawing according to the fitting results using Sigma-Plot program as described in the section of Materials and Methods. Plots of 1/v *vs* neo B concentration (C) at various concentrations of benzo(a)pyrene (○, 10 μM; ●, 30 μM; ▲, 100 μM).

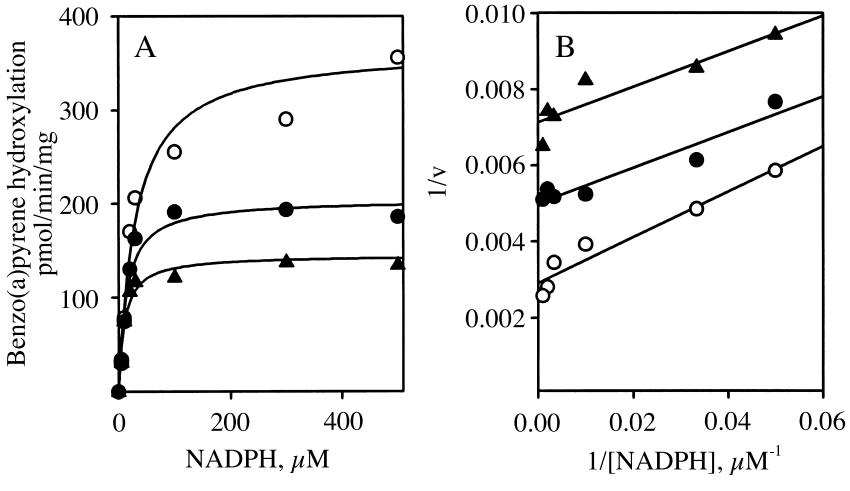

Figure 3. Kinetic analysis of the inhibition of AHH by neo B with respect to the cofactor NADPH. .Plots of v, expressed in pmol/ min/ mg protein, *vs* NADPH concentrations (A) and Lineweaver-Burk plots (B) at increasing concentrations of neo B (○, 0 μM; ●, 5 μM; ▲, 10 μM).

inhibitors (Fig. 2B). The inhibitor constants of neo B, K_i and K_I for the enzyme and enzyme-substrate complex were obtained by linear regression (the inserted figures in Fig. 2A and B; Fig. 2C). The K_I value was larger than the K_i value (Table 2). This result also suggested that neo B was a mixed type inhibitor. This mixed type inhibitor suggests that neo B may bind to both CYP and CYP-substrate complex and interfere with the substrate binding and product formation. In supporting CYP-catalyzed oxidations, NADPH serves as an electron donor to NADPH-CYP reductase. Kinetic analysis of AHH activity was performed in conditions with varying concentrations of NADPH. With NADPH as the variable substrate, neo B showed an uncompetitive type of inhibition in AHH (Fig. 3). The inhibition constant K_i calculated by nonlinear regression was 7 ± 1 μM (Table 2).

Several possibilities may contribute to the *in vitro* inhibition of neo B. First, neo B may have caused protein destruction and resulted in the inhibition of catalytic activity. To reveal this possibility, microsomes were incubated with or without 10 μM neo B for 10 min at room temperature and the CO-difference spectra were then determined. The microsomal CO-difference spectra were not affected by the treatment of lignans. This result suggested that lignans did not cause the destruction of the active heme moiety of CYP. Second,

Table 2. The inhibitor constants of neo B for AHH activity with respect to varying concentrations of benzo(a)pyrene and NADPH

Varying factor	Constant	μM	r2*
Benzo(a)pyrene	K_i	2	0.99
	K_I	11	0.96
NADPH	K_i	7 ± 1	0.96

Inhibitor constants were determined as described in the section of Materials and Methods. *r^2 represents the coefficient of regression determination.

neoB may interfere with the electron transfer from NADPH through NADPH-CYP reductase by reducing NADPH-CYP reductase activity. To examine this possibility, NADPH-CYP reductase activity toward cytochrome *c* was determined. However, the addition of neo B in the reaction mixture had no effect on NADPH-CYP reductase activity (data not shown). Together with the results of kinetic analysis, this datum suggested that neo B interfered with the electron transfer from NADPH to CYP-substrate complex without directly affecting NADPH-CYP reductase reduction activity. In summary, our results indicated that lignans isolated from *J. p*. had the ability to affect CYP-catalyzed oxidations *in vitro*. To better understand the pharmacological implications and pharmaceutical applications, further studies should be done on the *in vivo* effects of *J. p.* extracts and lignans.

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爵床木質素對benzo(a)pyrene氫氧化活性之抑制作用

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摘 要

爵床在傳統中藥中被用為治療發燒及喉嚨痛之處方用藥。由爵床乙醇萃取物中分離純化得neojusticin A（neo A），neojusticin B（neo B），justicidin A（jus A），justicidin B（jus B）及chinensinaphthol methyl ether（CME）五種木質素（lignan）。分析木質對大白鼠肝微粒體單氧酵素催化benzo(a)pyrene羥化（AHH）活性之影響，結果顯示10 μM木質素對此活性具21%至57%之抑制作用，其中以neo B具最強之抑制作用，其IC_{50}為6 ± 1 μM。這些木質素（10 μM）對鼠肝微粒體7-ethoxyresorufin O-脫乙基作用活性造成20%至48%之抑制作用。對7-methoxyresorufin O-脫甲基作用活性，neo B造成較其他木質素強之抑制作用，10 μM neo B使7-methoxyresorufin O-脫甲基作用活性下降70%。對睪酮6β-羥化活性造成19%至74%抑制作用，而neo B之抑制作用最小。酵素動力學分析neo B抑制AHH活性之作用，結果顯示neo B為一競爭性（competitive）及非競爭性（noncompetitive）之混合型抑制物，其K_i及K_I分別為2 μM及11 μM。分析neo B對NADPH濃度與AHH活性相關性之結果顯示neo B非競爭性（uncompetitive）抑制NADPH支持之AHH活性。這些結果顯示木質素可對鼠肝單氧酵素催化活性造成不同程度之抑制影響，其中neo B對AHH活性具較強抑制作用，neo B可能可降低benzo(a)pyrene之氧化活化作用。

關鍵詞：爵床，木質素，benzo(a)pyrene羥化作用