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Induction of diphenytriazol on cytochrome CYP1A[1]

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KEY WORDS cytochrome P-450 CYP1A; diphenytriazol; phenacetin; liver microsomes; metabolism

ABSTRACT

AIM: To study the effects of diphenytriazol on cytochrome P-450 (CYP) enzymes. **METHODS:** SD rats were pretreated with diphenytriazol. The catalytic activities of rat liver microsomes were determined by assaying ethoxyresorufin-*O*-deethylase (EROD) and pentoxyresorufin-*O*-dealkylase. Phenacetin and aminopyrine were selected as the substrate of CYP1A and CYP2B, respectively. The concentration of remaining substrate in microsomal incubates was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). The inhibition of fluvoxamine or α-naphthoflavone on phenacetin metabolism was measured. **RESULTS:** Phenacetin was significantly metabolized in the diphenytriazol-treated microsomes and the metabolic degree increased according to the diphenytriazol-treatment days. There existed a significant correlation between the metabolic degree of phenacetin and EROD in the microsomes pretreated with diphenytriazol. Both fluvoxamine and α-naphthoflavone inhibited the metabolism of phenacetin significantly, and the inhibition constants (K_i) were (5.4±1.0) μmol/L and (10.4±0.5) μmol/L, respectively. The activity of microsomes pretreated with diphenytriazol for 4 d was similar to that in β-naphthoflavone group, but was significantly different from those in control group and phenobarbital group. **CONCLUSION:** These results reveal that diphenytriazol is a novel inducer of CYP1A.

INTRODUCTION

Diphenytriazol [3-(2-ethyl phenyl)-5-(3-methoxy phenyl)-1*H*-1,2,4 triazol] (Fig 1), a new chemical entity (NCE), has high contragestational activity in rodents, dogs, and primates. The *in vitro* metabolism of diphenytriazol was mainly catalyzed by β-naphthoflavone-induced rat hepatic microsomes, and also catalyzed by itself-pretreated microsomes[1]. These observations revealed that diphenytriazol not only was the substrate of cytochrome P-450 (CYP)1A, but also might be an inducer of CYP1A. The inductive or inhibitive effect of drugs on enzymes may change the pharmacological and toxicological effects of drug itself or other drugs, which is one of the reasons resulting in drug interaction in pharmacokinetics[2,3], so acquiring the information about the effect of drugs on CYP enzymes is essential in instructing clinical secure and efficient medication,

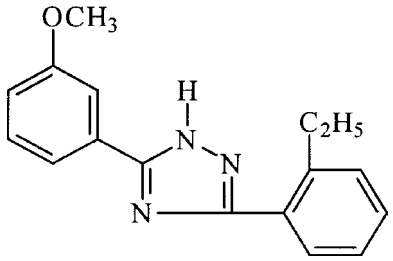

Fig 1. Chemical structure of diphenytriazol.

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especially in new drugs investigation. This paper was designed to find some evidences to prove above mentioned possibility, such as whether diphenytriazol is an inducer of CYP1A. In this study, we designed two approaches: firstly, identificating catalytic activity on diphenytriazol-pretreated rat liver microsomes through assaying the metabolic degree of marker substrates of CYP; secondly, using specific inhibitors of CYP1A to study metabolic inhibition of the substrates. Thereby to provide some useful information for clinical medicate.

MATERIALS AND METHODS

Chemicals Diphenytriazol was kindly donated by Xianju Pharmaceutical Factory (Zhejiang, China). Phenobarbital (PB), α-naphthoflavone (α-NF), β-naphthoflavone (β-NF), trinatric isocitric acid, isocitric dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), ethoxyresorufin, and pentoxyresorufin were purchased from Sigma (St Louis, Mo, USA). Phenacetin, aminopyrine, acetanilide, and fluvoxamine were offered by Department of Pharmaceutical Chemistry (Zhejiang University, Hangzhou, China). All other chemicals and solvents were analytical reagent or chromatographic grade.

Experimental animals and pretreatment Female Sprague-Dawley (SD) rats were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Grade II, Certificate No 003). The rats (180-200 g) were divided into 6 groups (every group including 3 batches, 2-4 rats per batch). The 6 groups rats were treated as follow: diphenytriazol (30 $mg \cdot kg^{-1} \cdot d^{-1}$ in tea oil) was given to 3 groups by intraperitoneal (ip) for 1 d, 2 d, 4 d, respectively; β-NF (80 $mg \cdot kg^{-1} \cdot d^{-1}$ in tea oil) and PB (80 $mg \cdot kg^{-1} \cdot d^{-1}$ in saline) were given to the other 2 groups by ip for 3 d; to the last group none of drug was given (control group). Eighteen batches of rat liver microsomes were obtained as the enzyme sources for metabolism investigation.

Preparation of liver microsomes Liver microsomes were prepared by calcium precipitation method[4]. The microsomal preparations were stored at -20 ºC until used. Protein concentrations were determined by the Lowry method[5], with bovine serum albumin as the standard.

Equipment and chromatographic conditions The DU 640 nucleic acid protein analyzer (Beckman coulter); the high-performance liquid chromatography (HPLC) system (Agilent 1100) consisted of a series G1311A pump, mode G1314A UV, G1313A auto-injector, and ChemStations software. A C_{18} column (Lichrospher, 25 cm×4.6 mm ID, 5 mm) was used for the HPLC separations. The mobile phases and UV detection wavelength varied according to substrates : methanol-water (7:3) and 254 nm for phenacetin; methanol-acetonitrile-pH 7.2 phosphate buffer solution (1:2:7) and 235 nm for aminopyrine.

Marker activity determination Ethoxyresorufin-*O*-deethylation (EROD) and 7-pentoxyresorufin-*O*-dependentylation (PROD) were used to indicate the activities of CYP1A and CYP2B, respectively. Metabolic reaction was performed in a 3.0 mL of incubation mixture containing microsome protein 0.9 mg, isocitric acid trisodium salt 0.033 mmol, isocitric dehydrogenase 1.05 units, $MgCl_2$ 0.045 mmol, Tris-HCl buffer 0.1 mol/L (pH 7.4), ethoxyresorufin 42.0 mmol or pentoxy-resorufin 18.0 mmol. The mixture was bubbled with oxygen for 2 min before use. After pre-incubation at 37 ºC for 5 min, reaction was started by adding NADP and NADPH (final concentration was 0.9 mol/L and 0.2 mol/L). The A-T curves were scanned under double wavelength (λs=572 nm, λ_R=700 nm) model for 15 min. The maximum absorbance of the metabolite was recorded.

Incubation of typical substrates of CYP with rat liver microsomes Phenacetin and aminopyrine were chosen as the typical substrates for CYP1A and CYP2B. The incubation was performed in 1.0 mL of incubation mixture containing 111.6 nmol phenacetin or 86.5 nmol aminopyrine as described under marker activity determination but 1.5 g/L of microsomes protein. The catalytic reaction was stopped by adding 3.0 mL of chloroform to the incubation mixture at 0, 10, 20, 30 and 60 min, respectively. Internal standard acetanilide (for phenacetin) or phenacetin (for aminopyrine) was added to the mixture. The contents were vortex-mixed for 3 min, and centrifuged at 3500×*g* for 20 min. The organic layer was separated and evaporated to dryness under a stream of air at ambient temperature. Residue was reconstituted in 1.0 mL of mobile phase. An aliquot of 10 μL supernatant was injected into the RP-HPLC system.

Metabolism inhibition of phenacetin The inhibition experiments were carried out at three different concentrations of phenacetin (27.9 μmol/L, 55.8 μmol/L, or 111.6 μmol/L) with fluvoxamine (11.5 μmol/L and 23.0 μmol/L) or α-naphthoflavone (18.4 μmol/L and 36.8 μmol/L). The reaction was stopped by adding 3.0 mL of chloroform to the incubation mixture at 3 min.

RESULTS

Validation of phenacetin assay method Phenacetin, internal standard acetanilide and metabolites were separated at base line under the chromatographic condition developed. There was no interfering peak found at the same retention time of substrate and internal standard in the chromatogram of blank microsomal incubate (Fig 2).

The calibration curve of phenacetin was constructed by analyzing the blank microsomal incubates spiked with a series concentrations of phenacetin. The results exhibited excellent linearity with a correlation coefficient of 0.9997 (n=7) at the concentration range from 11.1 to 558.0 μmol/L. The regression equation of the calibration curve, which was based on the peak-area ratio (y) of phenacetin and internal standard against the concentration (x) of phenacetin spiked in the blank microsomal incubates, was y=0.01332x+0.03139. The limit of detection and of quantitation for method was 0.112 μmol/L (S/N=3) and 0.558 μmol/L (RSD 12.3 %, n=3), respectively. The method was reproducible and accurate. The average recovery for the method was 97.3 %±1.3 % and the relative standard deviations of intra-day and inter-day were less than 4 %.

Validation of aminopyrine assay method Aminopyrine, internal standard phenacetin and metabolites were separated at base line under the chromatographic condition developed (Fig 3). The calibration curve of aminopyrine was constructed by analyzing the blank microsomal incubates spiked with a series concentrations of aminopyrine. The results showed an excellent linearity with a correlation coefficient 0.9999 (n=7) at the concentration range from 8.85 to 432.3 μmol/L. The regression equation was 0.007101x-0.0157. The limit of detection and of quantitation for method was 0.432 μmol/L (S/N=3) and 2.162 μmol/L (RSD 11.95 %, n=3). The average recovery for the method was 99.0 %±1.6 %. The relative standard deviations of within-day and between-day were less than 3 %.

Effect of diphenytriazol on hepatic CYP enzymes in rats

The metabolism profile of phenacetin Phenacetin was incubated in the rat liver microsomes pretreated with diphenytriazol (for 1 d, 2 d, and 4 d), β-naphthoflavone (for 3 d), and control microsomes. The incubate reaction was terminated at 10, 20, 30, and 60 min, respectively. Phenacetin was notably metabolized in the diphenytriazol-treated microsomes, and there was a significant correlation between the metabolic degree of phenacetin and the treatment day of diphenytriazol, with the correlation coefficient *of r*> 0.95. The activity of microsomes pretreated with diphenytriazol for 4 d was in agreement with that induced by β-naphtho-flavone, but was significantly different from control microsomes (P<0.01) (Fig 4A). A significant correlation existed between the metabolic degree of phenacetin and EROD activity in the microsomes pretreated with dipheny-triazol, with the correlation coefficient of r>0.99.

Fluvoxamine and α-naphthoflavone remarkably inhibited the metabolism level of phenacetin in the diphenytriazol-treated microsomal incubates, which was in a dose-dependent manner. The inhibition constants (K_i) for fluvoxamine and α-naphthoflavone were (5.4±1.0) μmol/L and (10.4±0.5) μmol/L, respectively (Fig 5).

Metabolism profile of aminopyrine Aminopyrine incubation was carried as the same procedure of

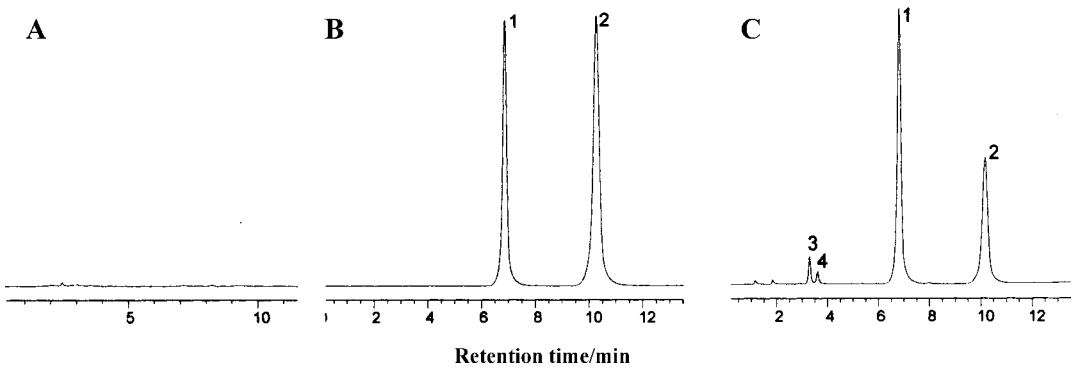

Fig 2. HPLC chromatograms of phenacetin in rat liver microsomal incubate. A: blank microsome; B: blank microsome spiked with phenacetin and internal standard; C: incubated at 37 ºC for 60 min. Peaks: 1, acetanilide (IS); 2, phenacetin; 3, metabolite 1; 4, metabolite 2.

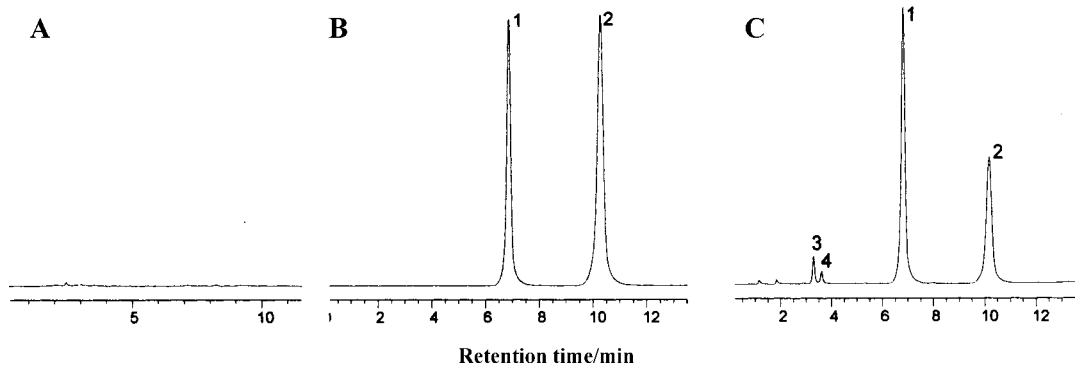

Fig 3. HPLC chromatograms of aminopyrine. A: Blank microsome spiked with aminopyrine and internal standard; B: Incubated at 37 ºC for 60 min in microsomes pretreated with diphenytriazol; C: Incubated at 37 ºC for 60 min in microsomes pretreated with phenobarbital. Peaks: 1, aminopyrine; 2, phenacetin (IS); 3, 4, metabolites.

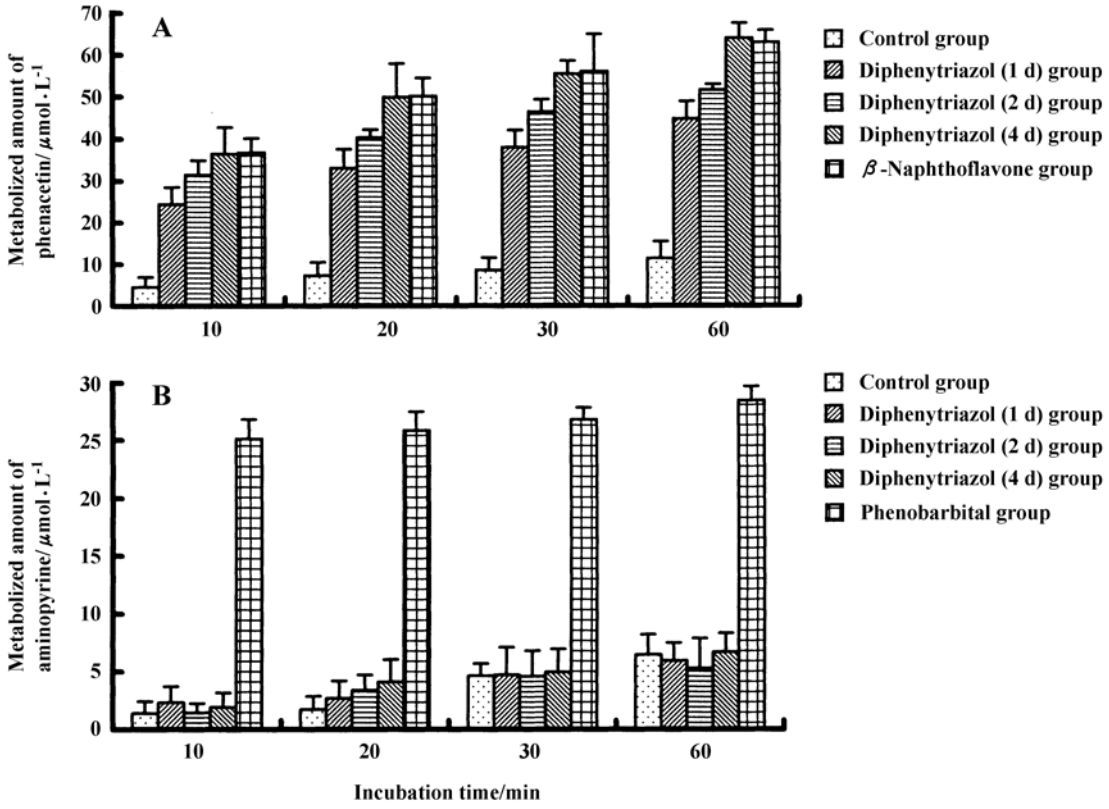

Fig 4. A: Metabolism profile of phenacetin in the microsomal incubates pretreated with diphenytriazol, β-naphthoflavone and control group. B: The metabolism profile of aminopyrine in the microsomal incubates pretreated with diphenytriazol, phenobarbital and control group. The each metabolized amount of phenacetin or aminopyrine in the figure was the average value of three batches of rat liver microsome.

phenacetin. The metabolic ability of aminopyrine in microsomes pretreated with diphenytriazol had no significant difference compared with control group ($P>0.05$), and there was no correlation between the metabolic degree of aminopyrine and the treatment day of diphenytriazol. But aminopyrine was obviously metabolized in the microsomal incubate induced by phenobarbital (Fig 4B), and fluvoxamine could not inhibit the metabolism of aminopyrine.

EROD and PROD activities The EROD activity in the microsomal incubates pretreated with diphenytriazol was remarkably increased contrast to the

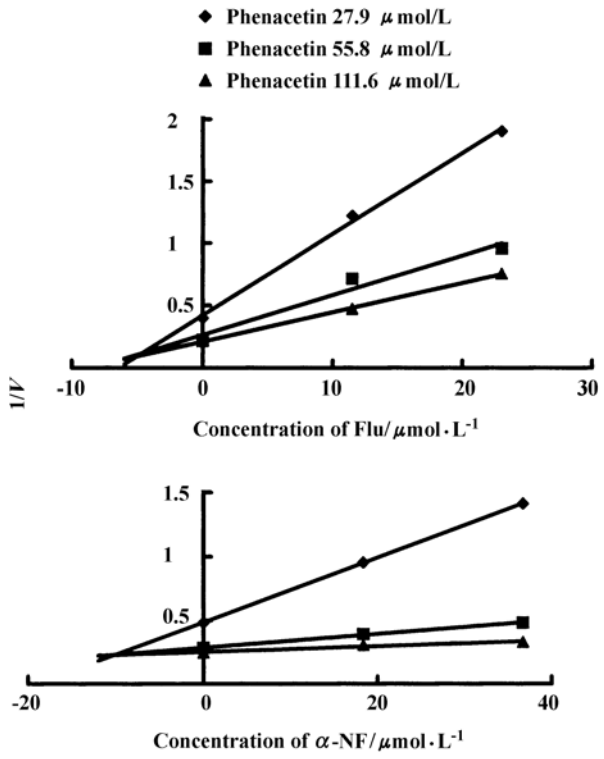

Fig 5. Inhibition by fluvoxamine or α-naphthoflavone on phenacetin metabolism in the microsomes treated by diphenytriazol. Each point was the average of three experiments in triplicate determination with each inhibitor. Qualitative assessment of competitive inhibition was demonstrated by the intersection of the regression lines above the x-axis. The inhibition constant (K_i) of fluvoxamine was (5.4±1.0) μmol/L, which of α-naphthoflavone was (10.4±0.5) μmol/L. V: μmol·L^{-1}·min^{-1}·mg^{-1} protein.

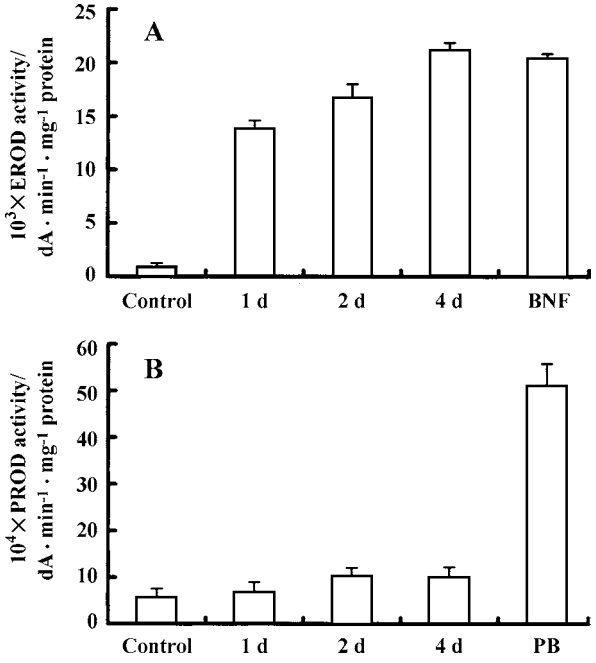

Fig 6. A: EROD activity in rat hepatic microsomes pretreated with diphenytriazol, β-naphthoflavone (BNF) and control group. B: PROD activity in rat hepatic microsomes pretreated with diphenytriazol, phenobarbital (PB) and control group. The EROD and PROD activity in the figure were the average value of three batches of rat liver microsome.

control group, with a trend of treatment day-dependent increase (Fig 6A). The EROD activity of microsomes pretreated with diphenytriazol for 4 d was in agreement with that induced by β-naphthoflavone for 3 d. The EROD level of diphenytriazol-pretreated for 1-4 d was 15-22 fold of control values. However, PROD activity in the microsomal incubates pretreated with diphenytriazol was not notably increased, only 1.8-fold of control value. But the PROD activity was distinctly increased in the microsomes induced by phenobarbital, being approximately 9-fold of control value (Fig 6B).

DISCUSSION

The commercial success of a new chemical entity (NCE) depends on its pharmacological activity and several absorption, distribution, metabolism, and excretion properties. An important property of which is the ability of the NCE to cause metabolism-based pharmacokinetic drug-drug interactions. CYP superfamily plays a key role in the metabolism of numerous exogenous compounds[6]. Many of the individual isoenzyme forms of cytochrome P450 exhibiting distinct substrate specificities can be inhibited or induced by these compounds. Precious studies had found that diphenytriazol metabolism was not only catalyzed by β-naphthoflavone-induced rat hepatic microsomes, but also catalyzed by itself-pretreated microsomes. These observations revealed that diphenytriazol might be an inducer of CYP1A.

The induction and inhibition experiments were carried out in order to identify whether diphenytriazol is an inducer of CYP1A, which was based on the metabolism activities for special substrates of CYP isoenzyme in the microsomal incubates pretreated with different chemicals. The results indicated that: (1) Phenacetin[7,8] and ethoxyresorufin[9], the typical substrates of CYP1A, were well metabolized in diphenytriazol-treated rat liver microsomes. The metabolic degree increased 5-8 fold and 15-22 fold respectively versus the control group (P<0.01). The microsomes pretreated with diphenytriazol could function as that induced by β-naphthoflavone, and the metabolic level for phenace-

tin and EROD activity showed no difference between both microsomes by *t*-test ($P>0.05$). (2) The characterization of microsomes pretreated with diphenytriazol was different from that pretreated with phenobarbital. Aminopyrine[10,11] and pentoxyresorufin[9], the typical substrates of CYP2B, showed low metabolic activities in diphenytriazol-treated rat liver microsomes. The PROD activity and the metabolic degree of aminopyrine in the diphenytriazol-pretreated rat liver microsomes were completely different from that induced by phenobarbital, which is a special inducer of CYP2B and CYP3A ($P<0.01$). Above experiments indicated that diphenytriazol had the same function as the special inducer of CYP1A, β-naphthoflavone, in inducing rat liver microsomes.

The inhibition experiments were carried out using the special inhibitors of CYP1A fluvoxamine and α-naphthoflavone[12,13]. Both inhibited significantly the metabolic activities of phenacetin in the microsomes treated by diphenytriazol. But the metabolic rate of aminopyrine has not been affected by the addition of fluvoxamine or α-naphthoflavone. These results further supported that induction of phenacetin metabolic activity by diphenytriazol was equivalent to that of β-naphthoflavone.

In order to make clear that diphenytriazol activated CYP1A enzymes, not CYP2B or CYP3A, we also analyzed the catalytic activities of the special substrate of CYP3A in rat hepatic microsomal pretreated with diphenytriazol, and observed no significant metabolism. Meantime the metabolic activities of the other substrates of CYP, eg ipriflavone, zolmitriptan and so on, were determined and compared in the microsomes pretreated with diphenytriazol or special inducers of CYP subfamilies, respectively. These substrates showed the similar metabolism profile between microsomes pretreated with diphenytriazol and β-naphthoflavone.

All these results proved that diphenytriazol was a novel inducer of CYP1A, and the induction was isoenzyme specific.

Rat liver microsomal incubation *in vitro* system is an economical and simple method for preliminary evaluating and screening new molecular entities whether an inducer or inhibitor of CYP enzymes. The experimental results suggest that diphenytriazol worth further investigation, such as obtaining the information about the induction potential of diphenytriazol toward CYP1A1 or CYP1A2 by cell and molecular biology methods, evaluating the ability on metabolic drug-drug interaction *in vivo* by the inhibitory effects of diphenytriazol toward CYP *in vitro* and so on. All these will help to successfully develop diphenytriazol to a drug in the market and to instruct clinical secure and efficient medication.

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