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Inhibitive effect of diphenytriazol on rat cytochrome P450 enzyme *in vitro*

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The inhibiting effect of diphenytriazol, a non-hormonal early pregnancy-terminating agent, towards cytochrome P450 (CYP) enzymes in rat liver microsomes was studied *in vitro*. The inhibiting effect of diphenytriazol on CYP was investigated by coincubating diphenytriazol with the specific CYP1A substrates, ethoxyresorufin and phenacetin, in microsome induced by β-naphthoflavone, with the specific CYP2B substrates, pentoxyresorufin and aminopyrine, in the microsome induced by phenobarbital, and with the specific CYP3A substrates, diazepam, testosterone, nifedipine and quinine sulfate in microsome induced by dexamethasone. The results showed that diphenytriazol inhibited the metabolism of ethoxyresorufin and phenacetin significantly, and its inhibition potential on CYP1A was higher than the typical inhibitor fluvoxamine. Diphenytriazol also inhibited the metabolism of diazepam, testosterone, nifedipine and quinine sulfate to different degrees, but its inhibition potential was relatively weaker than that of the typical inhibitor, ketoconazole. No inhibiting effect of diphenytriazol was seen on the metabolism of pentoxyresorufin and aminopyrine. The ability of diphenytriazol to inhibit rat liver CYP1A and CYP3A suggests that in human patients complex interactions may result from co-adiministration of diphenytriazol with other agents which are also substrates for CYP1A or CYP3A enzymes.

1. Introduction

Cytochrome P450 (CYP) enzymes are well known for their ability to metabolize a broad range of chemical entities, many of which are foreign to the host organism. Due to the prominent role of P450 enzymes in the metabolism of many pharmaceutical agents and in the activation or deactivation of potential carcinogens, the inhibition or induction of cytochrome P450 enzymes by xenobiotics has received considerable attention. Inhibition of cytochrome P450 enzymes is of clinical concern because drug interactions resulting in impaired drug metabolism can be detrimental and even fatal. Inhibition of drug metabolism may cause the rapid onset of symptoms of drug overdose. A case in point is terfenadine, which produces potentially fatal ventricular arrhythmias when the metabolism of this antihistamine is blocked by the CYP3A inhibitors ketoconazole and erythromycin. The potential for such drug interactions can now be predicted on the basis of *in vitro* studies of the P450 enzymes that either metabolize drugs or are inhibited by them (Biglin et al. 1994).

Diphenytriazol [3-(2-ethyl phenyl)-5-(3-methoxy phenyl)-1*H*-1,2,4-triazol], a new chemical entity (NCE), has high contragestational activity in rodents, dogs, and primates. The *in vitro* metabolism of diphenytriazol is mainly catalyzed by CYP1A in rat hepatic microsomes, and diphenytriazol has been shown to be a novel inducer of CYP1A, the induction being isoenzyme specific (Hu and Yao 2004). In the present study, we conducted a detailed, systematic investigation to evaluate the inhibitory effect of diphenytriazol on rat liver cytochrome P450, and to provide some useful pharmacokinetics information for avoiding metabolic drug interactions.

2. Investigations and results

2.1. Effect of diphenytriazol on rat hepatic CYP1A

Microsome induced with BNF was used as an enzyme source. The inhibiting effect of diphenytriazol on rat hepatic CYP1A was tested. The result was compared with that of fluvoxamine, a typical inhibitor of CYP1A (Yao et al. 2001). Ethoxyresorufin and phenacetin were used as selective markers for CYP1A. In the EROD activity inhibition studies, the final concentrations of fluvoxamine were 3.84, 19.2 and 38.4 μM, and those of diphenytriazol were 4.48, 8.96 and 26.9 μM. In phenacetin inhibition studies, the final concentrations of fluvoxamine were 2.3, 11.5, 34.5 and 115.0 μM, and those of diphenytriazol were 3.58, 17.9, 53.7, 89.5 and 179.0 μM.

2.1.1. Inhibiting effect of diphenytriazol on ethoxyresorufin-O-deethylation activity

Ethoxyresorufin-*O*-deethylation (EROD) was used to indicate the activity of CYP1A (Cawley et al. 2001). The metabolic reaction was performed in 1.0 ml of incubation mixture containing microsome protein 0.3 mg, isocitric acid trisodium salt 0.011 mmol, isocitric dehydrogenase 0.35 units, $MgCl_2$ 0.015 mmol, Tris-HCl buffer 0.1 mol/L

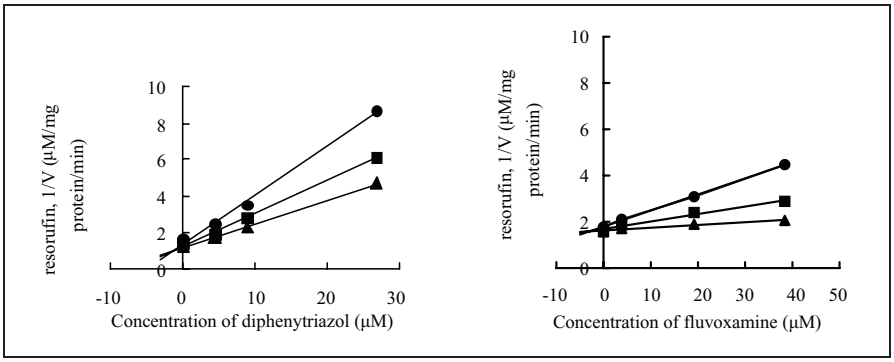

Fig. 1:
Inhibition by diphenytriazol or fluvoxamine of ethoxyresorufin-*O*-deethylation metabolism in microsomes treated with BNF. Each point was the average of three experiments in triplicate determination with each inhibitor. Qualitative assessment of competitive inhibition was demonstrated by the intersection of the regression lines above the x-axis. Inhibition constant (K_i) of diphenytriazol 1.222 ± 0.230 μM, that of fluvoxamine 3.094 ± 0.154 μM. ● 3.25 μM ethoxyresorufin, ■ 6.50 μM ethoxyresorufin, ▲ 13.0 μM ethoxyresorufin

(pH 7.4), and ethoxyresorufin 3.25, 6.5 or 13.0 μM. Oxygen was bubbled though the incubation mixture for 2 min before use. The reaction was started by adding NADP and NADPH (final concentration 0.25 mM and 0.1 mM) after pre-incubation at 37 °C for 5 min, and was terminated by adding 2.0 ml ice-cold methanol after metabolizing for 5 min. The sample was vortex-mixed and centrifuged at $10000 \times g$ for 10 min. The supernatant was directly analyzed for the metabolite, resorufin, with an F-4000 fluorescence spectrophotometer at an excitation wavelength of 565 nm (5 nm slit width) and an emission wavelength of 587 nm (5 nm slit width). Calibration curves were constructed with authentic resorufin and linear regression analysis was used to calculate the amount of resorufin formed in each incubation sample.

EROD activity was assayed by the fluorometric method mentioned above. Because of the substrate, the inhibitor diphenytriazol or the microsomes may have fluorescent properties, so in order to prove that there was no interference in the determination of resorufin concentration, we assayed a series of samples which might interfere with the determination at an excitation wavelength of 565 nm and an emission wavelength of 587 nm. The results showed that the fluorescence intensities of control microsome, 5 μM ethoxyresorufin, 17.9 μM diphenytriazol and 19.2 μM fluvoxamine were all less than 0.15, while that of resorufin was 347. This indicated the specificity of this method.

The inhibition by diphenytriazol or fluvoxamine of ethoxyresorufin-*O*-deethylation metabolism in microsomes treated with BNF was assayed using the method described above. Diphenytriazol significantly inhibited EROD activity. K_i values were determined from Dixon plots. Qualitative assessment of competitive inhibition was demonstrated by the intersection of the regression lines above the x-axis (concentration of inhibitor). The inhibition constant (K_i) of diphenytriazol was 1.222 ± 0.230 μM, compared with fluvoxamine, a specific inhibitor of CYP1A, of which the inhibition constant (Ki) was 3.094 ± 0.154 μM (Fig 1.).

2.1.2. Inhibiting effect of diphenytriazol on phenacetin metabolism

Phenacetin was chosen as the typical substrate for CYP1A (Murray et al. 2001; Yun et al. 2000). Incubation mixtures consisted of rat hepatic microsomes induced by BNF and 27.9, 55.8 or 111.6 μM phenacetin respectively, and the microsome protein was 1.0 mg in a total volume of 1.0 ml. The reaction was started by adding NADP and NADPH (final concentrations 0.25 mM and 0.10 mM respectively). Chloroform (3.0 ml) was added to the incubation mixture to end the incubation reaction after 5 min at 37 °C in a shaking water bath. Acetanilide was added as an internal standard. The contents were vortex-mixed for 3 min, and centrifuged at $10000 \times g$ for 10 min. The organic layer was separated and evaporated to dryness under a stream of air. The residue was reconstituted in 1.0 ml of mobile phase, and the contents were vortex-mixed for 3 min, and centrifuged at $10000 \times g$ for 10 min. An aliquot of 10 μl supernatant was injected into the RP-HPLC system.

Diphenytriazol significantly inhibited the metabolism of phenacetin in microsomes treated with BNF with an inhibition constant (K_i) of 6.836 ± 0.10 μM, while the inhibition constant (K_i) of fluvoxamine was 7.301 ± 0.192 μM (Fig. 2).

2.1.3. Time-dependent inhibition of CYP1A by diphenytriazol

Rat liver microsomes induced by BNF with various concentrations of protein were preincubated with various concentrations of diphenytriazol at 37 °C in the presence or absence of an NADPH system, the total volume of incuba-

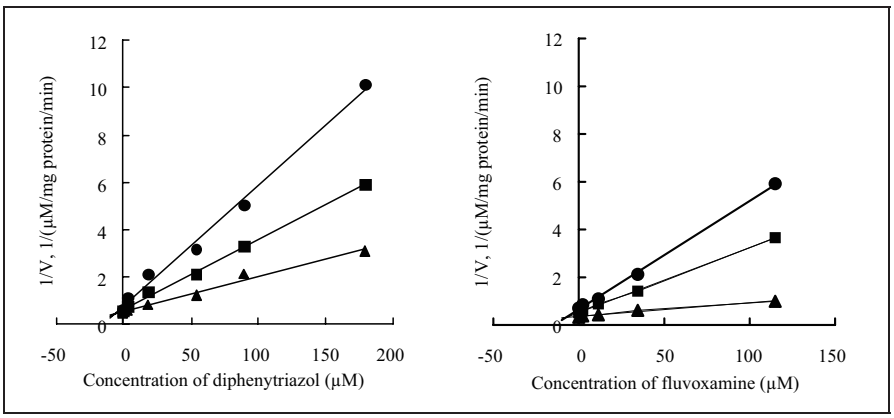

Fig. 2:
Inhibition by diphenytriazol or fluvoxamine of phenacetin metabolism in microsomes treated with BNF. Inhibition constant (K_i) of diphenyltriazol 6.836 ± 0.084 μM, that of fluvoxamine 7.301 ± 0.192 μM. ● 27.9 μM phenacetin, ■ 55.8 μM phenacetin, ▲ 111.6 μM phenacetin

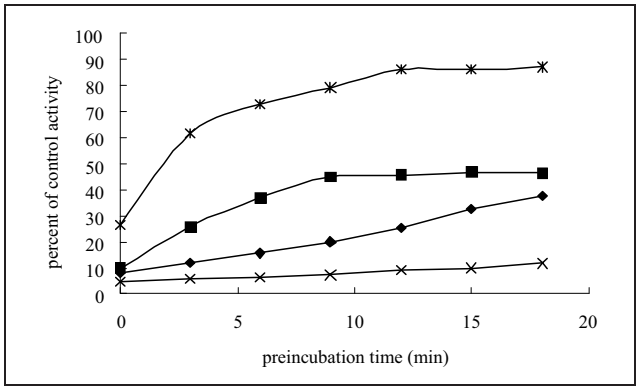

Fig. 3: Time-dependent inhibition of ethoxyresorufin-*O*-deethylation activity by diphenytriazol. Pooled rat liver microsomes with various concentrations of protein (0.1 mg/ml or 0.5 mg/ml) induced by BNF were preincubated with various concentrations of diphenytriazol (8.95 μM or 17.9 μM) at 37 °C for 0, 3, 6, 9, 12, 15, or 18 min in the presence of an NADP-generating system. Final concentration of ethoxyresorufin 5.0 μM. Metabolic time of ethoxyresorufin 3 min. —✳— 0.5 mg/ml protein + 8.95 μM diphenytriazol, —■— 0.5 mg/ml protein + 17.9 μM diphenytriazol, —◆— 0.1 mg/ml protein + 8.95 μM diphenytriazol, —×— 0.1 mg/ml protein + 17.9 μ M diphenytriazol

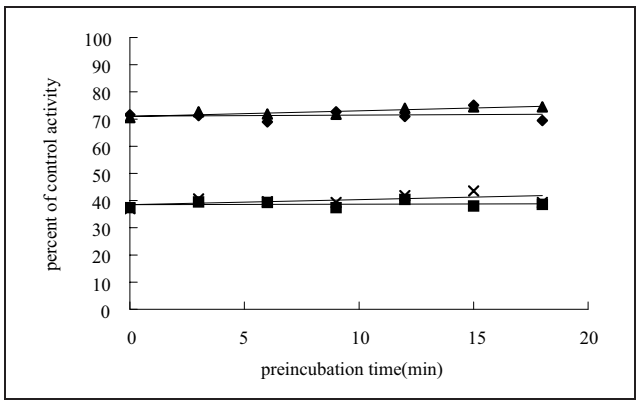

Fig. 4: Inhibition of ethoxyresorufin-*O*-deethylation activity by fluvoxamine. Pooled rat liver microsomes with various concentrations of protein (0.1 mg/ml or 0.5 mg/ml) induced by BNF were preincubated with various concentrations of fluvoxamine (5.76 μM or 28.8 μM) at 37 °C for 0, 3, 6, 9, 12, 15, or 18 min in the present of an NADP-generating system. Final concentration of ethoxyresorufin 5.0 μM. Metabolic time of ethoxyresorufin 3min. ◆ 0.5 mg/ml protein + 5.76 μM fluvoxamine, ■ 0.5 mg/ml protein + 28.8 μM fluvoxamine, ▲ 0.5 mg/ml protein + 5.76 μM fluvoxamine, × 0.1 mg/ml protein + 28.8 μM fluvoxamine

tion mixture being 2.0 ml. After 0, 3, 6, 9, 12, 15, and 18 min, respectively, an aliquot (0.2 ml) of the preincubation mixture was transferred to a 0.8 ml enzyme activity assay mixture prewarmed to 37 °C containing the buffer, 5 μM ethoxyresorufin, and the NADPH system. Ethoxyresorufin-*O*-deethylation activity was determined as described above.

The preincubation of rat liver microsomes with diphenytriazol alone did not result in an alteration in EROD activity. In the present of NADPH, as seen in Fig. 3, we found that the inhibiting effect of diphenytriazol gradually weakened according to the preincubation time. This result showed that the preincubation of microsomes with diphenytriazol and NADPH did not result in a decrease in EROD activity, but on the contrary, an increase in EROD activity. Therefore, diphenytriazol had not produced the mechanism-based inactivation of rat CYP1A enzyme. In this test, EROD activity was also determined in rat liver microsomes preincubated with fluvoxamine and NADPH. The results showed no obvious alteration in EROD activity accompanying the preincubation of microsomes and fluvoxamine (Fig. 4). From these results, we concluded that the inhibition mechanisms of diphenytriazol and fluvoxamine were different.

2.2. Effect of diphenytriazol on rat hepatic CYP2B

7-Pentoxyresorufin-*O*-dependentylation (PROD) was used to indicate CYP2B activity. The final concentrations of pentoxyresorufin were 1.0, 3.0 and 5.0 μM. The incubation was carried out according to the method described in section 2.1.1.

Incubation mixtures consisted of rat hepatic microsomes induced with phenobarbital and 21.6, 43.2 or 86.4 μM aminopyrine respectively, with 1.0 mg of microsome protein in a total volume of 1.0 ml. Adding 0.2 ml 6% perchloric acid was added to the incubation mixture to end the incubation reaction after 5 min at 37 °C in a shaking water bath. Phenacetin was added as an internal standard. The contents were vortex-mixed for 3 min, and centrifuged at 10000 × g for 10 min. An aliquot of 10 μl supernatant was injected into the RP-HPLC system.

Diphenytriazol was coincubated with pentoxyresorufin or aminopyrine in microsomes induced with phenobarbital. In PROD activity inhibition studies, the final concentrations of diphenytriazol were 4.48, 8.96 and 26.9 μM. In aminopyrine inhibition studies, the final concentrations of diphenytriazol were 17.9, 89.5 and 179.0 μM.

There was no inhibiting effect of diphenytriazol on PROD activity or aminopyrine metabolism in microsomes induced with phenobarbital. The data are not given in detail here.

2.3. Effect of diphenytriazol on rat hepatic CYP3A

The inhibiting effect of diphenytriazol on rat hepatic CYP3A was determined. In this test, diphenytriazol was

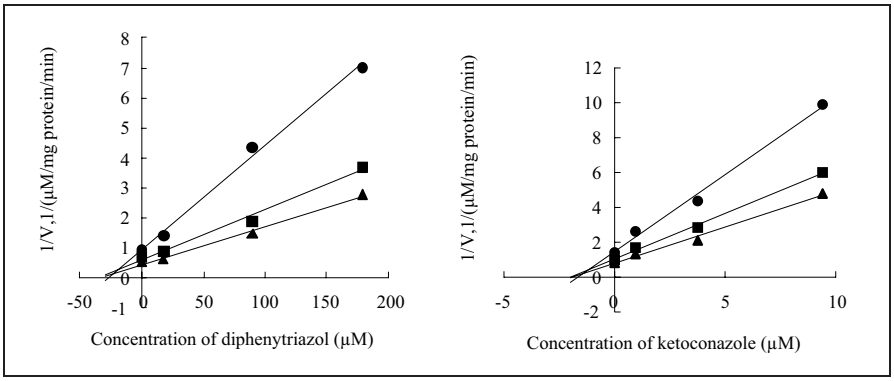

Fig. 5:
Inhibition by diphenytriazol or ketoconazole of diazepam metabolism in microsomes treated with dexamethasone. Inhibition constant (K_i) of diphenytriazol 21.48 ± 2.65 μM, that of ketoconazole 1.634 ± 0.603 μM. ● 17.6 μM diazepam, ■ 35.2 μM diazepam, ▲ 70.4 μM diazepam

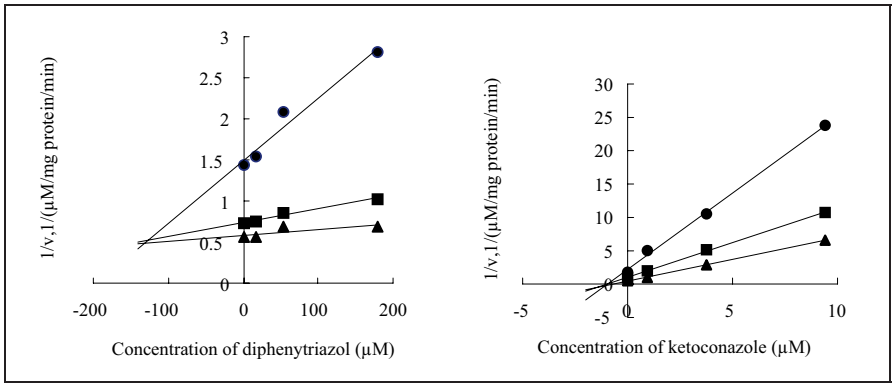

Fig. 6:
Inhibition by diphenytriazol or ketoconazole of testosterone metabolism in microsomes treated with dexamethasone. Inhibition constant (K_i) of diphenytriazol 137.5 ± 13.6 μM, that of ketoconazole 1.206 ± 0.271 μM. ● 17.3 μM testosterone, ■ 51.9 μM testosterone, ▲ 103.8 μM testosterone

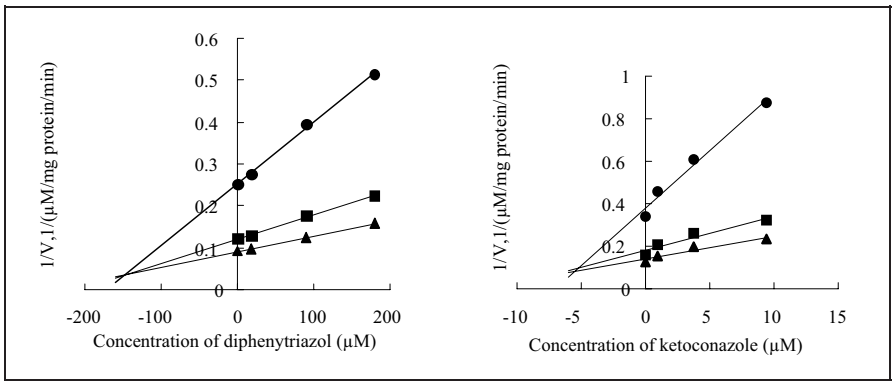

Fig. 7:
Inhibition by diphenytriazol or ketoconazole of nifedipine metabolism in microsomes treated with dexamethasone. Inhibition constant (K_i) of diphenytriazol 146.3 ± 7.4 μM, that of ketoconazole 6.120 ± 1.342 μM. ● 14.4 μM nifedipine, ■ 43.2 μM nifedipine, ▲ 86.4 μM nifedipine

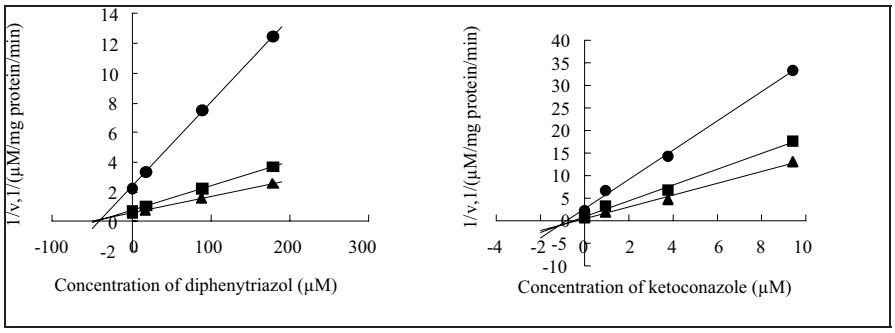

Fig. 8:
Inhibition by diphenytriazol or ketoconazole of quinine sulfate metabolism in microsomes treated with dexamethasone. Inhibition constant (K_i) of diphenytriazol 37.03 ± 1.71 μM, that of ketoconazole 1.136 ± 0.100 μM. ● 6.40 μM quinine sulfate, ■ 32.0 μM quinine sulfate, ▲ 64.0 μM quinine sulfate

coincubated with diazepam, testosterone, nifedipine or quinine sulfate in microsomes induced by dexamethasone. The results were compared with those of ketoconazole, a typical inhibitor of CYP3A (Fabre et al. 1993). The final concentrations of diazepam were 17.6, 35.2 or 70.4 μM, those of testosterone 17.3, 51.9 or 103.8 μM, those of nifedipine 14.4, 43.2 or 86.4 μM, and those of quinine sulfate 6.40, 32.0 or 64.0 μM. In each substrate of CYP3A, the final concentrations of diphenytriazol were 17.9, 89.5 and 179 μM, while those of ketoconazole were 0.94, 3.76 and 9.41 μM.

Diphenytriazol partially inhibited the metabolism of diazepam, testosterone, nifedipine and quinine sulfate in the microsomes treated with dexamethasone. But its inhibitory power was much weaker than that of ketoconazole, a typical inhibitor of CYP3A. The inhibitory potential of diphenytriazol and ketoconazole are shown in Table and Fig. 5–Fig. 8.

Table: Inhibition constants (K_i) of diphenytriazol and ketoconazole for different substrates of CYP3A

Substrate of CYP3A	Diphenytriazol	Ketoconazole
Diazepam	21.48 ± 2.65 μM	1.634 ± 0.603 μM
Testosterone	137.5 ± 13.6 μM	1.206 ± 0.271 μM
Nifedipine	146.3 ± 7.4 μM	6.120 ± 1.342 μM
Quinine sulfate	37.03 ± 1.71μM	1.136 ± 0.100 μM

3. Discussion

The majority of drug-drug interactions have a metabolic-basis; that is, two or more drugs are competing for metabolism by the same enzyme, and the majority of these drug-drug interactions involve cytochromes P450 (CYPs). If the compound is a potent cytochrome P450 inhibitor, it might inhibit the metabolism of a coadministered medication and cause a pharmacokinetic drug-drug interaction. New chemical entities (NCE) that cause drug-drug interactions could be more costly to develop, and might suffer from decreased market acceptance or fail to survive the drug registration process.

Prediction of drug-drug interactions is based on the paradigm of individual enzyme inhibition. Basically, if an NCE inhibits the metabolism of one substrate for a cytochrome P450 then it inhibits the metabolism of all substrates for that cytochrome P450. This paradigm provides the basis for screening for drug-drug interactions. A single, easily assayed probe substrate is selected and the abil-

ity of an NCE to inhibit the metabolism of the probe substrate is measured. The potential for drug-drug interactions is then estimated in relation to the potency for enzyme inhibition (IC_{50} or K_i) of the inhibitor.

For competitive inhibitors, the concentration of the chemical required to inhibit a reaction by 50% (IC_{50}) is dependent on the concentration of the substrate. When multiple substrates for the same enzyme are considered, the IC_{50} value will depend not only on the concentration of the substrate, but on its relative K_m. Likewise, when multiple enzymes are inhibited by the same chemical, the degree of inhibition will depend not only on the concentration of the inhibitor, but on its relative K_i. For competitive inhibitors, therefore, the specificity of the inhibition depends on the K_i of the inhibitor, the K_m of the substrate and both concentrations. Consequently, the idea that a certain concentration of an inhibitor can be added to a microsomal incubation to achieve selective inhibition of one particular P450 enzyme is untenable, unless the chemical functions as a specific noncompetitive inhibitor. Each individual P450 enzyme may interact with chemical inhibitors with a characteristic affinity, as measured by the inhibitory constant, Ki. In contrast to IC_{50} values, which are extrinsic constants, Ki values are intrinsic constants that should not vary from one laboratory to the next.

In addition, we should be aware that for CYP3A4 (unlike CYP1A2, CYP2C9, CYP2C19, and CYP2D6), the extent of inhibition is commonly substrate-dependent. For some inhibitors, qualitative differences and quantitative differences of up to 300-fold in IC_{50} values can be obtained depending on the substrate being used in the assay (Kenworthy et al. 1999). This property is not fully understood, but may be related to the unique ability of CYP3A enzymes to often bind multiple molecules in their active site (Korzekwa et al. 1998). As a practical matter, it is prudent to use multiple probe substrates in any test for CYP3A4 inhibition, and some level of uncertainty will remain regarding the potential to inhibit CYP3A4.

So in the present study, in order to determine the inhibiting effect of diphenytriazol on rat cytochrome P450, we chose two typical substrates of CYP1A – ethoxyresorufin and phenacetin, two typical substrates of CYP2B – pentoxyresorufin (Cawley et al. 2001) and aminopyrine (Tsyrlov et al. 1991), and four typical substrates of CYP3A – diazepam (Reilly et al. 1999), testosterone (Maenpaa et al. 1993), nifedipine (Yamazaki et al. 1996) and quinine sulfate (Zhao et al. 1996). The results showed that diphenytriazol significantly inhibited EROD activity and phenacetin metabolism. Based on the inhibition constant (K_i), diphenytriazol was a more potent inhibitor of CYP1A than fluvoxamine. However, preincubation of microsomes with diphenytriazol and NADPH resulted in a time-dependent increase in EROD activity, not mediated by mechanism-based inactivation. The ratio between the K_i values of diphenytriazol and fluvoxamine was 0.40 and 0.94 corresponding to the substrates ethoxyresorufin and phenacetin. Also, diphenytriazol inhibited the metabolism of diazepam, testosterone, nifedipine and quinine sulfate to different degrees, but compared with the inhibition potential of the typical inhibitor ketoconazole, it was relatively weaker. The ratio between the K_i values of diphenytriazol and ketoconazole was from 13.1 to 114.0 according to the substrate. No inhibitory effect of diphenytriazol was seen on PROD activity or aminopyrine metabolism.

CYP1A and CYP3A are well known to be responsible for the metabolism of many drugs. The ability of diphenytriazol to inhibit rat liver CYP1A and CYP3A suggests that complex interactions may result in human patients from co-administration of diphenytriazol with other agents which are also substrates for CYP1A or CYP3A enzymes.

4. Experimental

4.1. Chemicals and reagents

Diphenytriazol was kindly donated by Xianju Pharmaceutical Factory (Zhejiang, China). Diazepam was obtained from Changshu Pharmaceutical Factory (Jiangsu, China). Phenacetin, aminopyrine, acetanilide and fluvoxamine were supplied by the department of pharmaceutical chemistry (Zhejiang University, Hangzhou, China). Dexamethasone (Dex), phenobarbital (PB), ethoxyresorufin, pentoxyresorufin, β-naphthoflavone (BNF), nifedipine, testosterone, ketoconazole, quinine sulfate, trinatric isocitric acid, isocitric dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADP) and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were purchased from Sigma (St. Louis, Mo, USA). All other chemicals and solvents were analytical reagent or chromatographic grade and were obtained from usual commercial sources.

4.2. Experimental animals and enzyme induction

Female Sprague-Dawley (S.D.) rats (Grade II) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (SLAC.CAS). Rats of 180–200 g were used. They were divided into 4 groups (every group including three batches, with two to four rats per batch). These four groups of rats were treated as following: Dex ($100\ mg \cdot kg^{-1} \cdot d^{-1}$ in water) was given to one group by ig for 3 days; BNF ($80\ mg \cdot kg^{-1} \cdot d^{-1}$ in tea oil) and PB ($80\ mg \cdot kg^{-1} \cdot d^{-1}$ in saline) were given to the other two groups by ip for 3 days; no drug was given to the remaining group (control group). The 12 batches of rat liver microsomes obtained following the above pretreatment were used as the enzyme sources for metabolism investigation.

4.3. Preparation of liver microsomes

Liver microsomes were prepared by calcium precipitation method (Gibson and Skett, 1994). The microsomal preparations were stored at −20 °C until used. Protein concentrations were determined by the Lowry method (Lowry et al., 1951), with bovine serum albumin as the standard.

4.4. Equipment and chromatographic conditions

A DU 640 nucleic acid protein analyzer (Beckman Coulter) was used; the 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 I.D., 5 μm) was used for the reversed-phase high-performance liquid chromatography (RP-HPLC) separations. The mobile phases and UV detection wavelengths for the different substrates were respectively: 1) substrate of CYP1A: methanol-water (5 : 5) and 254 nm for phenacetin; 2) substrate of CYP2B: methanol-acetonitrile-pH 7.2 phosphate buffer solution (1 : 2 : 7) and 235 nm for aminopyrine; 3) substrates of CYP3A: methanol-water (7 : 3) and 235 nm for diazepam, methanol-water (65 : 35) and 240 nm for testosterone, methanol-water (6 : 4) and 235 nm for nifedipine, acetonitrile-pH 7.5 phosphate buffer solution (3 : 7) and 235 nm for quinine sulfate. The flow rate was $1.0\ ml \cdot min^{-1}$ for all determinations.

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