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Metabolism of Terephthalic Acid and Its Effects on CYP4B1 Induction[1]

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Objective To investgate the metabolism of terephthalic acid (TPA) in rats and its mechanism. **Methods** Metabolism was evaluated by incubating sodium terephthalate (NaTPA) with rat normal liver microsomes, or with microsomes pretreated by phenobarbital sodium, or with 3-methycholanthrene, or with diet control following a NADPH-generating system. The determination was performed by high performance liquid chromatography (HPLC), and the mutagenic activation was analyzed by *umu* tester strain *Salmonella typhimurium* NM2009. Expression of CYP4B1 mRNA was detected by RT-PCR. **Results** The amount of NaTPA (12.5-200 μmol·L^{-1}) detected by HPLC did not decrease in microsomes induced by NADPH-generating system. Incubation of TPA (0.025-0.1 mmol·L^{-1}) with induced or noninduced liver microsomes in an NM2009 *umu* response system did not show any mutagenic activation. TPA exposure increased the expression of CYP4B1 mRNA in rat liver, kidney, and bladder. **Conclusion** Lack of metabolism of TPA in liver and negative genotoxic data from NM2009 study are consistent with other previous short-term tests, suggesting that the carcinogenesis in TPA feeding animals is not directly interfered with TPA itself and/or its metabolites.

Key words: Terephthalic acid; Metabolism; Microsomes; High performance liquid chromatography; *umu* gene expression; CYP4B1

INTRODUCTION

Numerous chemical compounds are oxidized by multiple forms of cytochrome P450 (CYP) in laboratory animals and human bodies[1]. CYPs capable of oxygenating or metabolizing a variety of drugs, chemical carcinogens and other foreign compounds, are versatile catalysts[1-4]. CYPs detoxify and/or toxify these compounds to more polar, and sometimes more reactive metabolites that may contribute to carcinogenesis[5]. In mammals, the main chemical-metabolizing families of CYP are primarily expressed in the liver, and some specific isoforms are present in extrahepatic organs and tissues[6]. Imaoka *et al.*[6-7] have found that CYP4B1 could contribute to the initiation of carcinogenesis in rat and mouse bladders by activating aromatic amines such as 2-naphthylamine (2-NA), 3, 2'-dimethyl-4-aminobiphenyl (DMAB). Activation of carcinogenic aromatic amines in human bodies also increases the risk of bladder tumor with a high expression of CYP4B1.

Terephthalic acid (TPA) is an important chemical intermediate in the plastic industry. It has been extensively used for the production of polyesters, fiber, dye and plastic films[8-9], and also for training smoke by U. S. Army[10]. In 2001, more than 2 million tons of TPA was produced in China. Laboratory experiments demonstrated[11-13] that rats exposured to 3%-5% TPA in the diet for two weeks result in formation of bladder calculus, then bladder hyperplasia, and finally, transitional epithelial carcinomas. Since TPA is apparently a non-genotoxic chemical compound with LD_{50}>1500 mg/kg[14], we hypothesize that TPA might be biotransformed to more active metabolites involved in carcinogenesis.

To evaluate the role of TPA in the induction of bladder carcinomas, we measured the residues of sodium terephthalate (NaTPA) from incubated liver microsomes in rats treated or untreated with P450 inducers. In addition, we also studied the effects of TPA on CYP4B1 expression in the tissues of liver, kidney, and bladder of 90-day TPA exposed rats.

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MATERIALS AND METHODS

Chemicals

TPA, white powder with its purity ⩾99.99%, was obtained from Yizheng Chemical Fiber Co. (Jiangsu, China). NaTPA was synthesized by dissolving TPA with 1 mmol·L^{-1} NaOH. Methanol was added to the solution, and NaTPA was harvested, washed with methanol and dried for 12 h at 100℃. *Salmonella typhimurium* NM2009 for the *umu* test was a gift from Dr. Oda, Osaka Prefectural Insititute of Public Health. 3-methycholanthrene (3-MC, Fluka), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, dihydronicotinamide adenine dinucleotide phosphate (NADP) and dimethyl sulphoxide (DMSO) were obtained from Sigma Co. All other chemicals used were of the highest purity.

Preparation of Rat Liver Microsomes

Male Sprague-Dawley (SD) rats (5-6 weeks old, weighing about 200 g) obtained from Shanghai B & K Animal Center were treated with phenobarbital sodium (75 mg·kg^{-1}, daily for 3 days), 3-MC (30 mg·kg^{-1}, daily for 3 days), or diet control for 16 h before being killed[15]. Liver microsomes from rat liver were prepared by differential centrifugation of homogenates as described elsewhere[16]. The livers were excised, washed in isotonic saline, and then blotted to dryness. All subsequent steps were carried out at 4℃. The liver was minced with scissors and then diluted at 1:4 (w/v) with 0.05 mol·L^{-1} Tris buffer (pH 7.4) containing 0.25 mol·L^{-1} sucrose and 5.5 mmol·L^{-1} EDTA. The pulp was then homogenized with a Potter (Haimen, China) homogenizer. The homogenate was centrifuged at 13 500×g for 20 min. After a further centrifugation at 105 000×g for 1 h, the pellets were resuspended in 0.05 mol·L^{-1} Tris buffer (pH 7.4). Prior to storage at -80℃, protein concentration of the microsomal fractions was measured using the method of Coomassie brilliant blue G 250 with bovine serum albumin as standard.

Mutagenicity Assay

Both TPA and 3-MC were dissolved in DMSO respectively. P450-dependent activation of procarcinogens to reactive products that cause induction *umu* gene expression in tester strain *S. typhimurium* NM2009 was determined as described[16]. In brief, the standard reaction mixtures contained rat microsomes (40 μg of protein/mL) in a final volume of 1.0 mL of 50 mmol·L^{-1} potassium phosphate buffer (pH 7.4) including TPA (10 μL, 0.025-0.1 mmol·L^{-1}) and NADPH-generating system with 0.5 mmol·L^{-1} $NADP^+$, 5 mmol·L^{-1} glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, 1.5 mmol·L^{-1} $MgCl_2$ and 0.75 mL of bacterial suspension. A portion of 0.2 mL reaction mixture was assayed for β-galactosidase activity using *o*-nitropheny-β-D-galactopyranoside as a substrate, and *umu* gene expression was measured as the specific-galactosidase activity per unit per protein.

HPLC Analysis

P450-dependent metabolism of NaTPA was determined as follows. Standard incubation was conducted at a final volume of 1.0 mL of 50 mmol·L^{-1} potassium phosphate buffer (pH 7.4) containing rat liver microsomes (1 mg of protein) and NaTPA (12.5-200 μmol·L^{-1}). The incubation mixture was incubated at 37℃ by addition of the NADPH generating system (final concentrations: 10 nmol·L^{-1} glucose-6-phosphate, 1.0 mmol·L^{-1} NADP, and 1.0 units of glucose-6-phosphate dehydrogenase and 5 mmol·L^{-1} magnesium chloride). Aliquots of the supernatant solution were taken for determination by HPLC (Waters Model 2010 chromatographic system equipped with a Model 996 detector) with a Lichrospher 7-C_{18} analytical column (5 μm, 4.6×250 mm) at 35℃. Elution was conducted by methanol-tetrapropylammonium solution containing 5 mmol·L^{-1} tetrapropylammonium and 20% (v/v) methanol (pH 7.00) at a flow rate of 1.3 mL/min, and the elution was monitored at 238 nm. TPA residue was quantified by comparing the HPLC peak heights to the standard (NaTPA, 6.25-200 μmol·L^{-1}). Fifty μL samplers mixed with 450 μL pure water was treated in boiling water for 10 min, and then homogenized at 12 000×g for 10 min to precipitate protein. Aliquots of diluted samples (10 μL) were injected into the HPLC. NaTPA emerged as a sharp peak at approximately 6.5 min post-injection. Standard solutions were prepared from a stock aqueous solution of NaTPA.

Preparation of Total RNA and RT-PCR of CYP4B1 mRNA

SD male rats were quarantined for 7 days before being used in the experiments. The room temperature and the relative humidity were controlled at 22℃± 3℃ and 60%±10%, respectively. Fluorescent lighting was provided in a 12 h light/dark cycle. The animals were divided into five groups including 0%, 0.04%, 0.2%, 1%, and 5% TPA, and the treatment schedule was continued for 90 days. Rat liver, kidney, and bladder were immediately excised, quick-frozen in liquid nitrogen and stored at -80℃. Total RNA was isolated by TriPure^{TM} method (Roche, USA), and

quantified spectophotometrically at 260 nm. cDNA was synthesized from total RNA (2 μg) by reverse transcriptase reaction (total 30 μL) using 1 μg oligod (T)18 primer (Pharmacia No. 27-7858, Piscatway, NJ) and 10 units of AMV reverse transcriptase (Promega No. M510, Madison, WI). The mixture was preincubated for 5 min at 70℃, incubated for 90 min at 42℃ for the mRNA conversion to cDNA, and then the enzyme was inactivated for 5 min at 70℃. PCR was conducted in a 30 μL final volume in the presence of 1 μL RT product, 1.5 mmol·L^{-1} $MgCl_2$, 0.1 mmol·L^{-1} dNTPs, and Taq DNA polyerase (Promega, USA) 2U and 5 pmol of the appropriate oligonucleotide primers. The primers for rat CYP4B1 and GAPDH were designed according to previous reports[6,17-18]. The forward and reverse primers for CYP4B1 were 5′-CCAGTACCATAATGACTTCA-3′ and 5′-TAGAGGCGGAAGCACTCCTT-3′, respectively, and were predicted to produce 444-bp fragments. The forward and reverse primers for GAPDH were 5′-GGTGCTGAGTATGTCGTGGA-3′ and 5′-GCCATGCCAGTGAGCTTCCC-3′, respectively, and were predicted to produce 425-bp fragments. The reaction condition was at 94℃ for 2 min, followed by 35 cycles at 94℃ for 30 s, at 56.5℃ for 45 s, and at 72℃ for 1 min (for CYP4B1) and 28 cycles at 94℃ for 30 s, at 67℃ for 45 s, and at 72℃ for 1 min (for GAPDH), a final template extension at 72℃ for 7 min. PCR products (10 μL) were separated by electrophoresis on 2% agarose gel containing ethidium bromide. The density of the bands of the PCR products was analyzed with JD801-gel imaging system (Nanjing, China) and the CYP4B1 to GAPDH ratio was calculated.

Statistical Analysis

Student *t*-test was employed to calculate the significance of difference between the control and experimental values. $P<0.05$ was considered statistically significant.

RESULTS

Mutagenic Activation of TPA by Rat Liver Microsomes

The concentration of chemical could increase to near 0.1 mmol·L^{-1} when weak mutagens were tested with *umu* test strain. Thus, we performed the *umu* test for TPA (0.025-0.1 mmol·L^{-1}) and 3-MC (positive control, 0.025 mmol·L^{-1}) bioactivation using chemical-induced rat microsomes (Table 1). All forms of rat microsomes had high activity toward 3-MC, but their activity toward TPA was similar to that of DMSO (Table 1). These results suggested that TPA did not have mutagenic activity.

TABLE 1

Induction of *umu* Gene Expression After Metabolism of TPA by Liver Microsomes (40 μg Protein/mL) From Chemical-treated or Untreated Rats ($\bar{x} \pm s$, n=4)

Treatment of Rats	*umu* Gene Expression (Units/mg Protein)				
	Control	3-MC (mmol·L^{-1})	TPA (mmol·L^{-1})		
	(DMSO)	0.025	0.025	0.05	0.1
Untreated	189±23	592±38**	210±30	159±90	176±36
Phenobarbital	226±20	530±47**	187±32	211±40	187±10
3-MC	192±29	510±27	237±22**	241±30	197±17
Diet Control	234±20	594±24**	248±25**	247±80	247±80

Note. $^{**}P<0.01$, compared with control.

Metabolism of NaTPA by Rat Liver Microsomes

Incubation of NaTPA with rat liver microsomes in the presence of an NADPH-generating system resulted in a single peak of parent NaTPA. Fig. 1. shows a typical chromatogram obtained from incubation containing 50 μmol·L^{-1} NaTPA, NADPH-generating system, and rat liver microsomes at 2 h. The amount of NaTPA did not decrease with increasing incubation time, various microsomes induced by P450, and substrate concentration (Figs. 2 and 3).

Expression of CYP4B1 mRNA in Male Rats Exposed to TPA

Expression of CYP4B1 mRNA was detected in all six samples of rat bladders exposed to 0.04% TPA (Fig. 4), however its expression in other groups was detectable in fewer samples or even undetectable. In

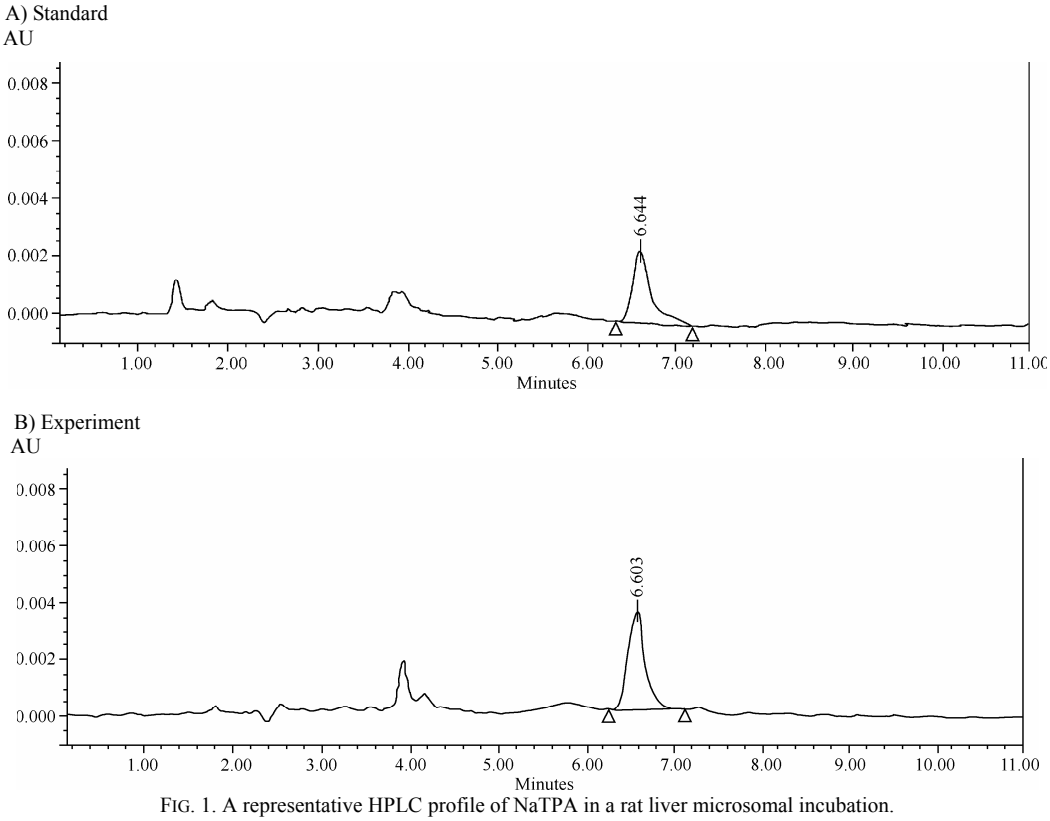

FIG. 1. A representative HPLC profile of NaTPA in a rat liver microsomal incubation.

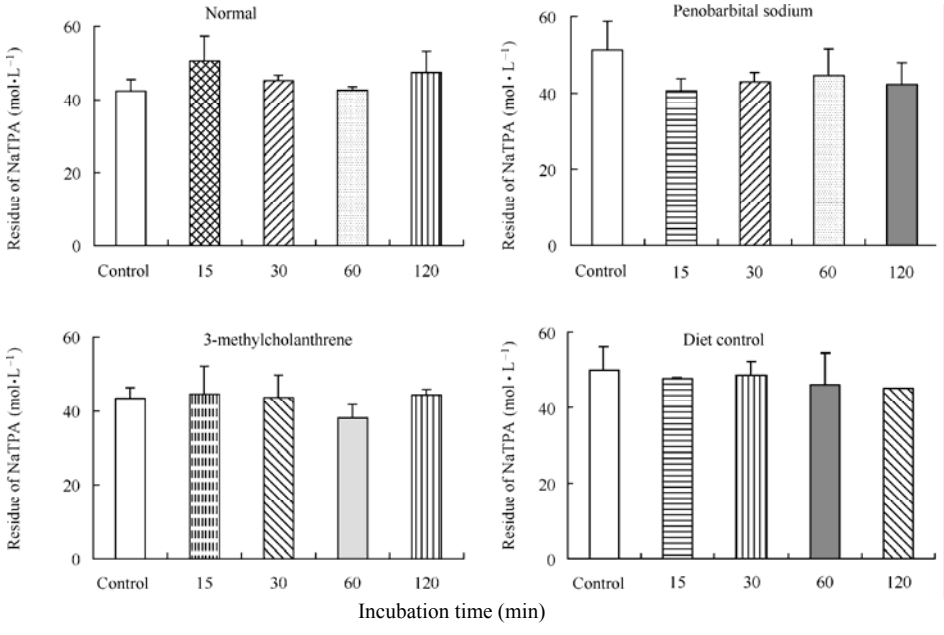

FIG. 2. Effect of incubation time on the residue of NaTPA (50 μmol•L^{-1}) in chemical-induced rat microsomes.

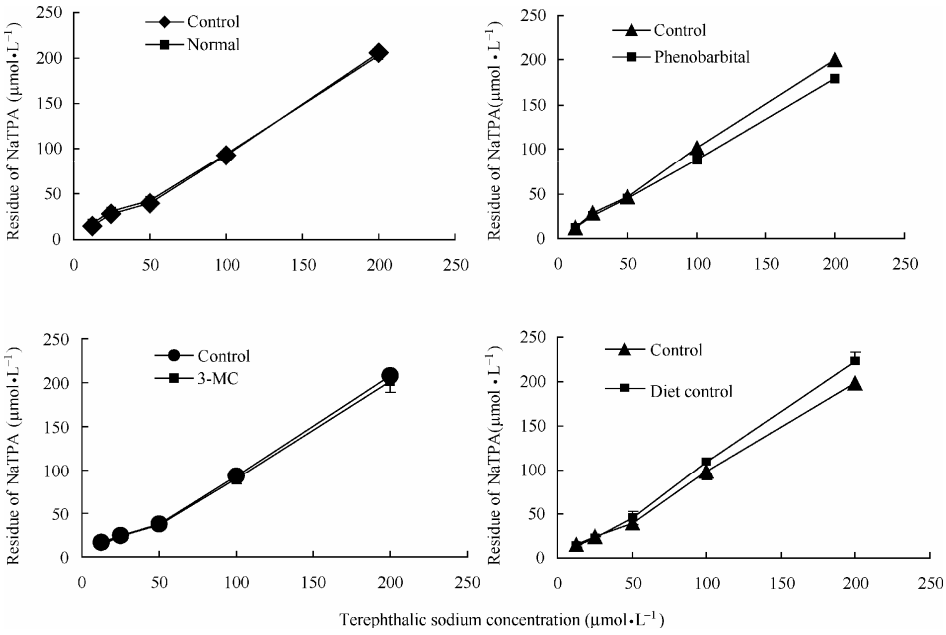

FIG. 3. Effect of CYP450 selective inducers on metabolism of NaTPA (12.5-200 μmol•L^{-1}) in rat liver microsomes.

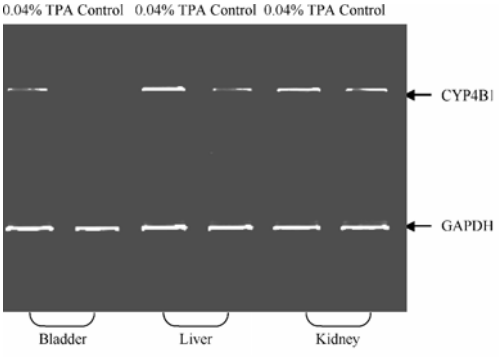

FIG. 4. RT-PCR of rat bladder, liver, and kidney RNA.

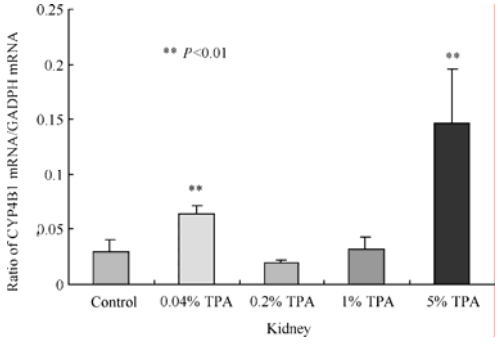

FIG. 6. Regulation of CYP4B1 by terephthalic acid in rat kidney.

the kidney and liver of 90-day subchronic feeding rats, expression of CYP4B1 increased in groups treated with 0.04% and/or 5% TPA, respectively (Figs. 5 and 6).

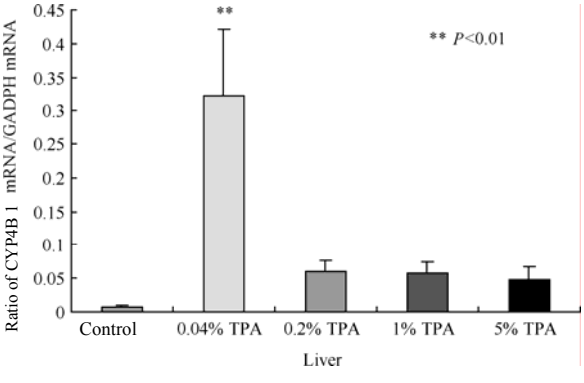

FIG. 5. Expression of CYP4B1 in rat liver.

DISCUSSION

With increasing TPA production, there is a great concern for the health of occupational workers and contamination of TPA derived from its workshop effluent. The manifestation of toxicity to baby *ctenopharyngodon idellus*[19] and the diagnosed bladder carcinoma in rats with 5% TPA in diet lead to increasing concern regarding TPA as being devoid of any metabolites, although the formation of TPA-induced calculi may be a primary factor for bladder hyperplasis and carcinogenesis.

TPA-containing wastewater usually can be biodegraded by aerobic or anaerobic system[20-22], suggesting that the similar mechanism may exist in

mammalian species. However, whether TPA is biotransformed by mammalian liver remains unclear.

In the present study, the metabolism of NaTPA was examined *in vitro* using rat liver microsomal preparations. The results showed that concentration of NaTPA was not influenced by chemical-induced microsomes, even the treatment time was prolonged for 120 min. As was reported previously, absorbed TPA excreted in urine does not change, and no metabolite is found with[14] C labelled TPA[23]. Apparently, the present in *vitro* results agree well with these *in vivo* studies.

The genotoxicity of TPA has been evaluated in short-term tests, but no positive results are obtained in Ames assay, with or without metabolic activation[24-26]. Data about rates of micronuclei and sister chromatid exchange do not reflect genotoxic effects differing between groups[14]. The *umu* test is a simple and sensitive procedure for the detection of chemical carcinogens and mutagens[16,27-28]. It is applicable to kinetic analysis of enzymatic activation of chemical carcinogens by P450 enzyme, and only one tester strain is required in a standard assay condition. The *S. typhimurium* NM2009 is a highly sensitive *umu* tester strain for studies on the mechanisms of activation of chemical carcinogens by P450s. We evaluated TPA with NM2009 test system, but obtained no positive results at all incubation conditions.

Although the content of P450s in extrahepatic tissues is low, it may be sufficient to initiate tumors upon long exposure to carcinogens. CYP4B1 is a specific isoforms of P450s that bioactivates procarcinogens such as DMAB which induce bladder cancer in experimental animals. The expression of CYP4B1 is also significantly higher in transurethral samples from patients with bladder tumors than that from non-bladder tumor patients. Hence, a high expression of CYP4B1 contributes to the initiation of bladder carcinoma by activating carcinogenic aromatic amines[7]. Our study illustrated that expressions of CYP4B1 mRNA in rat liver, kidney and bladder did not increase with TPA exposure. Although expression of CYP4B1 could increase with different doses of TPA, its functions in TPA-induced bladder lesion are still unclear.

In conclusion TPA is not a mutagenic chemical and cannot be metabolized by rat microsomes, thus not supporting our hypothesis that TPA might be biotransformed into active derivatives.

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REFFERENCES

1. Hasler J A, Estabrook R, Murray M, *et al*. (1999). Human cytochromes P450. *Mol Asp Med* **20**, 1-137
2. Bestervelt L L, Vaz A N D, and Coon M J (1995). Inactivation of enthanol-inducible cytochrome P450 and other microsomal P450 isozymes by trans-4-hydroxy-2-nonenal, a major product of membrane lipid peroxidation. *Proc Natl Acad Sci* **92**, 3764-3768.
3. Yu L J, Matias J, Scudiero D A, *et al*. (2001). P450 enzyme expression patterns in the NCI human tumor cell line panel *Drug Met Disp* **29**, 304-312.
4. Yamazaki H, Komatsu T, Takemoto K, *et al*. (2001). Decreases in phenytoin hydroxylation acitivaties catalyzed by liver microsomal cytochrome P450 enzymes in phenytoin- treated rats. *Drug Met Disp* **29**, 427-434.
5. Guengerich F P (2001). Forging the links between metabolism and carcinogenesis. *Mut Res* **488**, 195-209.
6. Imaoka S, Yoneda Y, Matsuda T, *et al*. (1997). Mutagenic activation of urinary bladder carcinogens by CYP4B1 and the presence of CYP4B1 in bladder mucosa. *Biochem Pharmacol* **54**, 677-683.
7. Imaoka S, Yoneda Y, Sugimoto T, *et al*. (2000). CYP4B1 is a possible risk factor for bladder cancer in humans. *Biochem Biophys Res Commun* **277**, 776-780.
8. Wang L, Zhou L, Zeng C (2003). Production of pure terephthalic acid and the technology development. *Jiangsu Chem Ind* **31**, 21-25.
9. Ding M, Li X (2003). About the construction of PTA. *Chem Tech Econ* **21**, 13-15.
10. Muse W T, Anthony J S, Bergmann J D, *et al*. (1997). Chemical and toxicological evaluation of pyrotechnically disseminated terephthalic acid. *Drug Chem Toxicol* **20**, 293- 302.
11. Chin T Y, Tyl R, W, Popp J A, *et al*. (1981). Chemical urolithiasis. I. Characteristics of bladder stone induction by terephthalic acid and dimethyl terephthalate in weanling Fischer-344 rats. *Toxicol Appl Pharmacol* **58**, 307-321.
12. Wolkowki-Tyl R, Chin T Y, Popp J A, *et al*. (1982). Chemically induced urolithiasis in weanling rats. *Am J Pathol* **107**, 419-421.
13. Heck H D, Tyl R W (1985). The induction of bladder stones by terephthalic acid, dimethyl terephthalate, and melamine (2, 4, 6-triamino-s-triazine) and its relevance to risk assessment. *Regul Toxicol Pharmacol* **5**, 294-313.
14. Gong N, Xu X K, Wang X R (1999). Study on the acute toxicity and genotoxicity of terephthalic acid. *Chin. Pub. Health* **18**, 203-204.
15. Yuan Z X, Sikka H C, Munir S, *et al*. (2003). Metabolism of the polynuclear sulfur heterocycle benzo[b] phenanthro [2,3-d] thiophene by rodent liver microsomes: evidence for multiple pathways in the bioactivation of benzo [b] phenanthro [2,3-d] thiophene. *Chem Res Toxicol* **16**, 1581-1588.
16. Shimada T, Oda T, Yamazaki H, *et al*. (1994). SOS function tests for studies of chemical carcinogenesis using *Salmonella typhimurium* TA1535/Psk 1002, NM2009, and NM3009. In Adolph K W (ed) Methods in Molecular Genetics, Vol. 5, Gene and Chromosome Analysis, Part C. Academic Press, San Diego, CA, pp. 342-355.
17. Imaoka S, Yoneda Y, Sugimoto T, *et al*. (2001). Androgen regulation of CYP4B1 responsible for mutagenic activation of bladder carcinogens in the rat bladder: detection of CYP4B1

mRNA by competitive reverse transcription-polymerase chain reaction. *Cancer Lett* **166**, 119-123.
18. Swafford D S, Middleton S K, Palmisano W A, *et al*. (1997). Frequent aberrant methylation of $p16^{INK4a}$ in primary rat lung tumors. *Mol Cell Biol* **17**, 1336-1374.
19. Gao X (1999). Acute toxicity of PTA of ctenoph-aryngodon idellus. *Environ Monit Chin* **15**, 57-58.
20. Guan B H, Wu Z B, Wu Z C, *et al*. (2003). Biodegradability of terephthalic acid in terylene artificial silk printing and dyeing wastewater. *J Environ Sci* **15**, 296-301.
21. Li X M, Zeng G M, Liu J J, *et al*. (2002). A two-stage anaerobic system for biograding wastewater containing terephthalic acid and high strength easily degradable pollutants. *J Environ Sci* **14**, 478-481.
22. Li X M, Zeng G M, Liu J J, *et al*. (2002). A novel technology for quick acclimation of an anaerobic microbial consortia used for biograding terephthalic acid (TA). *J Environ Sci* **14**, 204-209.
23. Hoshi A, Kuretani K (1967). Metabolism of terephthalic acid. Absorption of terephthalic acid from gastriointestinal tract and detection of its metabolites. *Chem Pharm Bull* **15**, 1979-1984.
24. Zeiger E, Haworth S, Speck W, *et al*. (1982). Phthalate ester testing in the national toxicology program's environmtental mugagenesis test development program. *Environ Health Perspect* **45**, 99-101.
25. Kozumbo W J, Kroll R, Rubin R J (1982). Assessment of the mutagenicity of phthalate esters. *Environ Health Perspect* **45**, 103-109.
26. Brooks A L, Seiler F A, Hanson R L, *et al*. (1989). In vitro genotoxicity of dyes present in colored smoke munitions. *Environ Mor Mutagen* **13**, 304-313.
27. Reifferscheid G, Heil J (1996). Validation of the SOS/*umu* test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. *Mut Res* **369**, 129-145.
28. Oda Y, Yamazaki H, Their R, *et al*. (1996). New *Salmonella typhimurium* NM5004 strain expressing rat glutathione S-transferase 5-5: use in detection of genotoxicity of dihaloalkanes using an SOS/*umu* test system. *Carcinogenesis* **17**, 297-302.

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