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Toluene Metabolism by cDNA-Expressed Human Hepatic Cytochrome P450

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ABSTRACT. The metabolism of toluene in human liver microsomes and by cDNA-expressed human cytochrome P450s (CYPs) was investigated. Toluene was metabolized mainly to benzyl alcohol and slightly to *o*- and *p*-cresol by human liver microsomes. Formation of *o*-cresol was elevated in microsomes from human livers derived from cigarette smokers, but the induced CYP isoforms were not clear. Of the eleven human CYP forms studied, CYP2E1 was the most active in forming benzyl alcohol, followed by CYP2B6, CYP2C8, CYP1A2, and CYP1A1, in that order. The activities of CYP2A6, CYP2C9, CYP2D6, CYP3A3, CYP3A4, and CYP3A5 were negligible. In addition, CYP2B6 and CYP2E1 catalyzed the formation of *p*-cresol (11–12% of total metabolites), and CYP1A2 catalyzed the formation of both *o*-(22%) and *p*-cresol (35%). The relationship between the amino acid sequence of rat CYP2B1 cDNA and the activity for toluene metabolism was investigated using variants, because of great differences in the forming of toluene ring products between CYP2B1 and CYP2B6. These results suggest that the structure of CYP2B1 at the site of Leu 58 rather than Ile-114 and Glu-282 plays an important role in the formation of toluene ring products, whereas in CYP2B1 Ile-114 plays an important role in the formation of benzyl alcohol. These results may explain, in part, the lower activity of CYP2B6, which has Phe at position 58 of the protein, for toluene ring oxidations than that of CYP2B1. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:271–277, 1997.

KEY WORDS. human cytochrome P450; rat cytochrome P450; mouse cytochrome P450; toluene metabolism; human liver microsomes; smoking

Since toluene is the most common organic solvent used in the production of benzene, as a paint solvent or coating in the leather industry, and in inks and adhesives[1] a large number of workers are exposed to toluene. The National Institute of Occupational Safety and Health estimated that 1,278,000 workers were potentially exposed to toluene in the U.S.A. in 1981–1983.

Toluene is metabolized by CYP¶ mainly to benzyl alcohol, which is easily converted to benzoic acid via benzaldehyde, and is excreted into urine as hippuric acid [3, 4]. *o*-Cresol and *p*-cresol are formed by the catalytic action of CYP as minor metabolites from toluene. Studies on the formation of benzyl alcohol and *o*-cresol are of importance in the biological monitoring of toluene-exposed workers [5, 6].

In rats, CYP2B1/2 and CYP2C11/6 contribute to the formation of benzyl alcohol and *o*- and *p*-cresol with different K_m and V_{max} values; CYP1A1/2 contributes to the formation of *o*- and *p*-cresol; CYP2E1 contributes to the formation of benzyl alcohol and *p*-cresol [7]. We found no documentation on the contribution of human CYP forms to the metabolism of toluene.

In the present study, the contribution of human hepatic CYPs to toluene side-chain oxidation and ring oxidation is described. Using cDNA-expressed human CYP forms [8], we compared results from the metabolic activity in human liver microsomes, and investigated the effect of the structure of CYP2B1 cDNA on the metabolism of toluene, in relation to the activity of CYP2B6 for toluene ring oxidation.

MATERIALS AND METHODS

Human Liver Specimens

Liver samples were obtained at laparotomy from 35 patients (23 men and 12 women) who were undergoing surgery for either primary liver tumors or hepatic metastases at the

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¶ *Abbreviations:* CYP, cytochrome P450; Hep G2, hepatoma G2; and DMEM, Dulbecco's modified Eagle's medium.

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Fourth Hospital of Hebei Medical College in Shijiazhuang, China. The use of liver tissues was approved by the Ethical Committee of the College. Tobacco use and/or drinking habits of the patients were investigated during personal interviews prior to surgery. The patients were separated into the following groups: non-smokers and smokers, and those who consumed alcoholic beverages regularly and those who did not. There were no ex-smokers among these patients.

Human liver microsomes were prepared by standard differential centrifugation as reported elsewhere [9].

Preparation of Microsomes from Human CYP-Containing Cell Lysate

Recombinant vaccinia viruses containing the cDNAs encoding human CYP1A2 [10], CYP2A6 [11], CYP2B6 [12], CYP2C8, CYP2C9 [13], CYP2D6, CYP2E1 [14], CYP3A3 [15], CYP3A4 and CYP3A5 [16], mouse CYP1A1 [17] and CYP1A2 [10], and rat CYP2B1 [18] and CYP2B2 [19], respectively, were constructed and characterized as described elsewhere [20]. Microsomes containing recombinant human CYP1A1 were purchased from Daiichi Pure Chemicals (Tokyo, Japan), Three variants of CYP2B1, cDNA, CYP2B1-2 cDNA, which substituted Phe for Ile-114 of CYP2B1 cDNA, CYP2B1-1,2 cDNA, which substituted Phe for Leu-58 and Ile-114, respectively, and CYP2B1-1,2,3 cDNA, which substituted Phe, Phe, and Val for Leu-58, Ile-114, and Glu-282, respectively, (see Fig. 1) were also constructed, as described elsewhere [19]. Human hepatoma G2 (Hep G2 cells), from the American Type Culture Collection (Rockville, MD, U.S.A.) were cultured in DMEM, containing 10% fetal bovine serum and were infected with recombinant viruses. The cells were scraped from the plates 20 hr after infection and washed once with 10 mM sodium phosphate buffer, pH 7.5, containing 0.14 M NaCl. Cell lysates were prepared by sonication, and microsomal fractions were prepared by the method reported elsewhere [9]. Protein content was measured by the method of Lowry *et al.* [21].

The level of expression of each CYP was determined as described previously [16]. Cell lysate (100 mg protein) was solubilized directly in 0.1 mM phosphate buffer (pH 7.4) containing 20% glycerol and 0.2% Emulgen 913. The solution was centrifuged at 4000 *g* for 5 min, and spectra were measured on the supernatant. The soluble lysate was then divided into two cuvets, designated sample and reference cuvets, carbon monoxide gas was bubbled gently into the sample cuvet, and then a few crystals of sodium hydrosulfite were dissolved in both cuvets. Spectra were measured using an Hitachi U-3210 spectrophotometer. CYP content was calculated as described [22].

Metabolism of Toluene by Microsomes

Toluene metabolism in liver microsomes and in microsomes from the cell lysate was assessed by measuring the rate of formation of benzyl alcohol and *o*- and *p*-cresol in a reaction system that contained 50 mM potassium-potassium phosphate buffer, NADPH-generating system (1 mM NADP, 20 mM glucose-6-phosphate, 2 IU glucose-6-phosphate dehydrogenase, and 50 mM magnesium chloride), 200 μg protein of liver microsomes and cell lysate microsomes, and 0.2 or 5.0 mM toluene in water, to a final volume of 0.5 mL. The reaction was initiated by adding the substrate, and the reaction vials were placed in a thermoregulated, shaking water bath (37°). After 10 min (liver samples) and 20 min (cell lysate sample) of incubation, 0.1 mL of a 15% zinc sulfate and saturated barium hydroxide solution was added to stop the reaction, followed by measurement of toluene metabolites, which was reported earlier [9].

In Situ *Metabolism with Culture Cells*

Twenty hours after recombinant virus infection as described above, the medium bathing the Hep G2 cells was

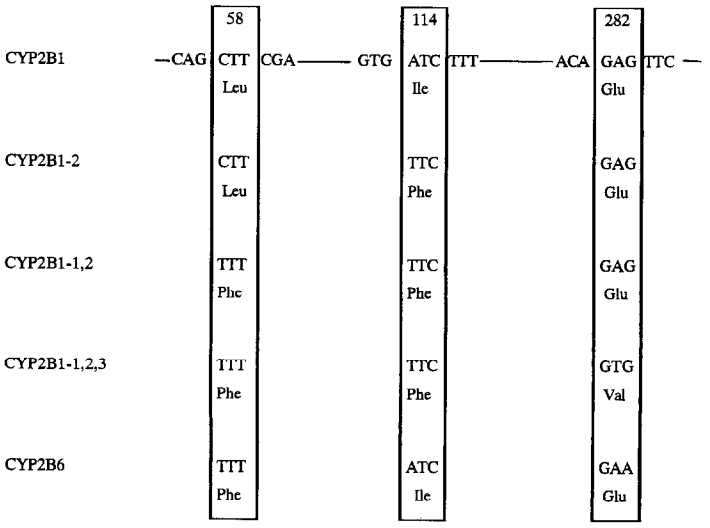

Fig. 1. Diagram of amino acids at positions 58, 114, and 282 of cDNA of CYP2B6, CYP2B1, and the variants.

replaced with DMEM without phenol red, and 50 μL of toluene in methanol at a final concentration of 5 mM was added as described elsewhere [20]. The dish was incubated at 37° in a CO_2 incubator. Two hours after the addition of substrate, an aliquot of 1 mL of the medium was taken, and 0.2 mL of 15% zinc sulfate and saturated barium hydroxide solution was added. The mixture was centrifuged at 1800 *g* for 15 min, and 10 μL of the supernatant was taken for measurement of metabolites of toluene, by a high-performance liquid chromatograph with an UV-vis detector and a Chromatointegrator. The analytical conditions for measuring benzyl alcohol and *o*- and *p*-cresol were described elsewhere [7]. The amount of benzyl alcohol and/or *o*- and *p*-cresol formed from toluene increased with linear kinetics for at least 4 h, in all dishes. Therefore, the incubation was stopped after 2 h. Under these conditions, benzaldehyde, benzoic acid, and hippuric acid were not detected in the incubation medium.

Statistics

Analysis of variance was used, and when there was any significant difference among groups, Student's *t*-test was used.

RESULTS

Metabolism of Toluene by Human Liver Microsomes

The effect of alcohol consumption on the formation of benzyl alcohol and *o*- and *p*-cresol from toluene in liver microsomes from 35 patients is shown in Table 1. At both concentrations (0.2 and 5.0 mM), benzyl alcohol and *o*- and *p*-cresol were formed in all microsomes from the patients: the average formation of benzyl alcohol was 92%, that of *o*-cresol 3%, and that of *p*-cresol 5% of the total metabolites. The lowest and the highest activities for the formation of benzyl alcohol at a high substrate concentration were 0.67 and 2.54 nmol/mg protein/min, respectively, suggesting that the interindividual variation was only 3.8 times. Similarly, the lowest and the highest activities for the formation of *p*-cresol were 0.045 and 0.143 nmol/mg protein/min; the highest activity for the formation of *o*-cresol was 0.069 nmol/mg protein/min, a value 3-fold higher than that of the lowest one, 0.023 nmol/min protein/min.

Regular consumption of alcohol did not seem to influence the formation of benzyl alcohol or of *o*- and *p*-cresol from toluene in human liver microsomes.

Tobacco use also did not influence activities in the formation of benzyl alcohol and *p*-cresol in human liver microsomes, but *o*-cresol formation was enhanced (Table 2). Although the majority of smokers were also alcohol drinkers, smokers not using alcohol had a higher activity (data not shown), suggesting that enhancement of *o*-cresol formation may be due to tobacco but not alcohol consumption.

Metabolism of Toluene by cDNA-Expressed Human CYPs

The amounts of metabolites from toluene by eleven human CYPs and two rat and mouse CYP forms are given in Tables 3 and 4. Of the human CYPs, five catalyzed the formation of benzyl alcohol, the major metabolite of toluene by these CYPs: CYP2E1 was the most active for the formation, followed by CYP2B6; the activity of CYP2C8 was one-third that of CYP2E1, and the activity of CYP1A2 was one-seventh; CYP1A1 showed a low and non-significant activity; the activities of CYP2A6, CYP2C9, CYP2D6, CYP3A3, CYP3A4, and CYP3A5 were not detectable. CYP1A2 also formed *o*- and *p*-cresol. CYP2B6 and CYP2E1 also catalyzed the formation of *p*-cresol (11–12% of the total metabolites), but not *o*-cresol.

When comparing the formation of toluene metabolites between low (0.2 mM) and high (5.0 mM) substrate concentrations, the differences were only 3- to 5-fold by CYP2E1 and CYP1A2, whereas differences were 9-fold by CYP2B6 and CYP2C8.

TABLE 1. Effect of alcohol consumption on the metabolism of toluene in human liver microsomes

Source of tissues	N	Benzyl alcohol	o-Cresol	p-Cresol
			(nmol/mg protein/min)	
			Toluene 0.20 mM	
Non-drinker	28 (8)	0.53 ± 0.19	0.015 ± 0.009	0.030 ± 0.008
Drinker	7 (7)	0.57 ± 0.34	0.020 ± 0.007	0.030 ± 0.008
			Toluene 5.0 mM	
Non-drinker	28 (8)	1.50 ± 0.46	0.044 ± 0.008	0.084 ± 0.022
Drinker	7 (7)	1.27 ± 0.38	0.050 ± 0.009	0.083 ± 0.024

The number of tobacco users in the group is given in parentheses. Pack years of smokers were 27.6 ± 9.9. Alcohol consumption was 77 ± 53 g/day calculated as pure ethanol, and 32 ± 8 years. Values are means ± SD.

TABLE 2. Effect of tobacco use on the metabolism of toluene in human liver microsomes

Source of tissues	N	Benzyl alcohol	*o*-Cresol	*p*-Cresol
		(nmol/mg protein/min)		
			Toluene 0.20 mM	
Non-smoker*	21	0.56 ± 0.19	0.013 ± 0.004	0.030 ± 0.008
Smoker†	14	0.52 ± 0.27	0.020 ± 0.009‡	0.030 ± 0.010
			Toluene 5.0 mM	
Non-smoker*	21	1.51 ± 0.47	0.040 ± 0.007	0.082 ± 0.020
Smoker†	14	1.38 ± 0.42	0.050 ± 0.008‡	0.082 ± 0.021

Values are means ± SD.

* Non-smokers mean never having been smokers.

† Pack years of smokers were 27.6 ± 9.9.

‡ Significantly different ($P < 0.002$) from non-smokers.

Mouse CYP1A1 catalyzed the formation of benzyl alcohol more than did mouse CYP1A2, but not *o*- and *p*-cresol. Formation of benzyl alcohol by mouse CYP1A2 was similar to that by human CYP1A2.

Rat CYP2B1 catalyzed the formation of benzyl alcohol and *o*- and *p*-cresol. The percentages of benzyl alcohol and *o*- and *p*-cresol of the total metabolites were 70, 12, and 18%, respectively, differing greatly from those by human CYP2B6. Rat CYP2B2 catalyzed only the formation of benzyl alcohol.

The rate of formation of toluene metabolites by mouse CYP1A2 between low and high substrate concentration was 6-fold, and that of rat CYP2B1 was 9-fold, values very similar to that of the respective human CYP isoform.

TABLE 3. Formation of benzyl alcohol and *o*- and *p*-cresol from toluene in microsomes from cell lysate containing vaccinia virus-expressed human P450s

Recombinant virus	P450 content (pmol/mg protein)	Benzyl alcohol	*o*-Cresol	*p*-Cresol	Total
		(nmol/mg protein/min)			
Wild-type	<0.2	ND[a]	ND	ND	ND
		ND[b]	ND	ND	ND
CYP1A1*	36.3*	ND	ND	ND	ND
		0.006 (100)	ND	ND	0.006
CYP1A2	80.5	0.004 (50)	0.002 (25)	0.002 (25)	0.008
		0.020 (43)	0.010 (22)	0.016 (35)	0.046
CYP2A6	74.1	ND	ND	ND	ND
		ND	ND	ND	ND
CYP2B6	82.8	0.011 (100)	ND	ND	0.011
		0.096 (89)	ND	0.012 (11)	0.108
CYP2C8	72.4	0.006 (100)	ND	ND	0.006
		0.054 (100)	ND	ND	0.054
CYP2C9	70.3	ND	ND	ND	ND
		ND	ND	ND	ND
CYP2D6	55.9	ND	ND	ND	ND
		ND	ND	ND	ND
CYP2E1	68.3	0.059 (91)	ND	0.006 (9)	0.065
		0.140 (88)	ND	0.019 (12)	0.159
CYP3A3	86.6	ND	ND	ND	ND
		ND	ND	ND	ND
CYP3A4	73.0	ND	ND	ND	ND
		ND	ND	ND	ND
CYP3A5	88.5	ND	ND	ND	ND
		ND	ND	ND	ND

HepG2 cells expressing P450 isozyme were disrupted by sonication, and the microsomal fraction was used as a source of enzyme. Each value in the first row ([a]) for each recombinant represents the mean formation rate of duplicate determinations at 0.2 mM toluene, and that in the second row ([b]) represents the mean formation rate of duplicate determinations at 5.0 mM. The numbers in parentheses represent percentage of the metabolite. ND, not detected.

* Microsomes containing CYP1A1 were purchased from Daiichi Pure Chemicals. CYP1A1 content was 36.3 pmol/mg protein, according to the manufacturer's measurements.

TABLE 4. Formation of benzyl alcohol and *o*- and *p*-cresol from toluene in microsomes from cell lysate containing vaccinia virus-expressed mouse and rat P450s

Recombinant virus	P450 content (pmol/mg protein)	Benzyl alcohol	*o*-Cresol	*p*-Cresol	Total
		(nmol/mg protein/min)			
Wild-type	<0.2	ND[a]	ND	ND	ND
		ND[b]	ND	ND	ND
Mouse					
CYP1A1	95.3	0.005 (100)	ND	ND	0.005
		0.040 (100)	ND	ND	0.040
CYP1A2	86.7	0.004 (100)	ND	ND	0.004
		0.024 (100)	ND	ND	0.024
Rat					
CYP2B1	103.0	0.029 (67)	0.006 (14)	0.008 (19)	0.043
		0.252 (70)	0.044 (12)	0.064 (18)	0.360
CYP2B2	81.9	0.004 (100)	ND	ND	0.004
		0.024 (100)	ND	ND	0.024
Rat CYP2B1 variant					
CYP2B1-2	77.4	0.010 (67)	0.002 (13)	0.003 (20)	0.015
		0.070 (67)	0.014 (13)	0.020 (19)	0.104
CYP2B1-1,2	96.1	0.023 (100)	ND	ND	0.023
		0.207 (100)	ND	ND	0.207
CYP2B1-1,2,3	93.6	0.013 (100)	ND	ND	0.013
		0.120 (100)	ND	ND	0.120

Hep G2 cells expressing P450 isozyme were disrupted by sonication, and the microsomal fraction was used as a source of enzyme. Each value in the first row ([a]) for each virus represents the mean formation rate of duplicate determinations at 0.2 mM toluene, and that in the second row ([b]) represents the mean formation rate of duplicate determinations at 5.0 mM. The numbers in parentheses represent percentage of the metabolite. ND, not detected.

In Situ *Metabolism of Toluene by cDNA-Expressed Human CYPs*

Catalytic activities of four CYP forms, with the activity for toluene metabolism in microsomes from cell lysate, were also investigated using cultured cells (Table 5). The order of activity for the formation of benzyl alcohol by four CYP forms was similar to that by microsomes, except for CYP2B6 and CYP2E1: in the cell culture system, the formation was greater by CYP2B6 than by CYP2E1, but the result was reversed in the cell lysate system. It is of particular interest that in the cell culture system, *o*- and *p*-cresol could not be detected in any dish containing CYP forms. However, when the medium was treated with 15% hydrochloric acid and placed in boiling water for 1 hr, according to Wang and Nakajima [23], *o*- and/or *p*-cresol in the medium incubated with the above CYP forms, except for CYP2C8, were detected at a ratio similar to that seen using the cell lysates.

Relationship Between the Structure of CYP2B1 cDNA and the Activity for Toluene Metabolism

To investigate the effects of substitution of some amino acids in CYP2B1 cDNAs on the metabolism of toluene,

TABLE 5. Toluene metabolism in a cell culture system including cDNA-expressed human P450

Recombinant virus	Benzyl alcohol	*o*-Cresol	*p*-Cresol	Total
	(nmol/2 hr/dish)			
CYP1A2	22 ± 4 (100)	ND	ND	22 ± 4
CYP2B6	166 ± 17 (100)	ND	ND	166 ± 17
CYP2C8	36 ± 3 (100)	ND	ND	36 ± 3
CYP2E1	44 ± 5 (100)	ND	ND	44 ± 5

Hep G2 cells, cultured in dishes containing DMEM and 10% fetal bovine serum, were infected with vaccinia virus encoding one of the P450 isozymes, and toluene was added directly to the dishes. Two hours after incubation in a CO_2 incubator at 37°, the concentrations of the three metabolites were determined. P450 contents ranged from 135 to 172 pmol/57-cm^2 dish. Each value represents the mean ± SD of triplicate determinations. The numbers in parentheses represent percentage of the metabolite. ND, not detected.

metabolites of toluene by CYP2B1 variants were measured using microsomes from the cell lysate. CYP2B1-2 catalyzed the formation of benzyl alcohol and *o*- and *p*-cresol, but the amount was about 30% that of CYP2B1. In contrast, CYP2B1-1,2 and CYP2B1-1,2,3 retained the activity to form benzyl alcohol to a greater extent than did CYP2B1-2, but completely lost the activity to form *o*- and *p*-cresol.

DISCUSSION

In human liver microsomes as well as in cDNA-expressed human CYPs, the major metabolite of toluene was benzyl alcohol, a finding which is similar to that for rodents [7, 24]. Of eleven human CYP forms, CYP2E1 was the most active in formation of benzyl alcohol in microsomes from the cell lysate, whereas CYP2B6 was the most active in *in situ* cell culture systems. A similar result was seen in the metabolism of other organic solvents [20]. The metabolic activity from the *in situ* system may more accurately reflect metabolism *in vivo* than that obtained from the cell lysate. However, the results from the latter may be more accurate for studying the true activity of individual CYP forms in the formation of *o*- and *p*-cresol, because the effects of conjugation reactions are excluded in Hep G2 cells [25]. This is supported by our finding that free *o*- and *p*-cresol were not detected in the medium of the *in situ* cell culture system (Table 5). Benzyl alcohol diffuses through the cell membrane without conjugation with an endogenous substrate, but *o*- and *p*-cresol probably do not pass through the membrane without being conjugated. CYP2E1, CYP2B6, CYP1A2, and CYP2C8, which are expressed in human liver [20], mediate the formation of benzyl alcohol, and all but CYP2C8 also catalyzed the formation of *o*- and/or *p*-cresol, suggesting that these are the principal CYP forms involved in metabolism of toluene in the human liver.

It is noteworthy that CYP2B1 significantly catalyzed the formation of *o*- and *p*-cresol from toluene, whereas CYP2B6 only slightly catalyzed the formation of *p*-cresol. In the metabolic assay of toluene using CYP2B1 variants, CYP2B1-2 catalyzed the formation of *o*- and *p*-cresol, whereas CYP2B1-1,2 and CYP2B1-1,2,3 did not, suggesting that Leu-58 of CYP2B1 influences the formation of toluene ring products. The 58th, 114th and 282nd amino acids of CYP2B6 cDNA are Phe, Ile and Glu [12], similar to that of CYP2B1 variant CYP2B1-1 [19], and also rather similar to CYP2B1-1,2 and CYP2B1-1,2,3. While CYP2B1-1 protein is unstable and could not be detected by immunoblot analysis [19], that of CYP2B6 is stable. These results suggest that (i) the low activity of CYP2B6 for ring oxidation of toluene is due, at least in part, to the amino acid residue at the 58th amino acid: the introduction of Leu-58 into CYP2B6 cDNA in place of Phe-58 may lead to the high production of *o*- and *p*-cresol, and (ii) the binding site of the toluene side chain to CYP2B1 differs from that of the toluene ring. The question as to why the replacement of the Leu-58 of CYP2B1 by Phe influences toluene ring oxidation may be that the Leu-58 is located at a different corner of the substrate channel from that of Ile-114, and replacement of Leu-58 by Phe destabilizes the helix [26, 27]. The substitution thus increases hydrophobicity in the center of the sequence linking helices A and B [28].

CYP2E1 catalyzed the formation of benzyl alcohol and *p*-cresol but not *o*-cresol, which is in good agreement with that of rat CYP2E1 [7]. Some differences in the contribution of CYP forms to toluene metabolism have been seen between humans and rats: rat CYP2C11/6 significantly catalyzes the ring products of toluene [7], but human CYP2C8 and CYP2C9 do not; human CYP1A2 mediates toluene ring products, but not rat CYP1A2 [7]. These results indicate that use of human CYPs is helpful for classifying the role of individual CYPs in toluene metabolism.

In conclusion, of the eleven human CYP forms, five (CYP1A2, CYP2B6, CYP2E1, CYP2C8, and CYP1A1) contributed to the formation of benzyl alcohol, *o*-cresol and/or *p*-cresol. In the human liver, the first four in the list may play an important role in the formation of benzyl alcohol, and the first three in that of *o*- and/or *p*-cresol.

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