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In vitro metabolic characteristics of cytochrome P-450 2A6 in Chinese liver microsomes

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KEY WORDS cytochrome P-450 CYP2A6; coumarins; glucuronosyltransferase; liver microsomes; rifampicin

ABSTRACT

AIM: To investigate the metabolic characteristics of cytochrome P-450 CYP2A6 in human liver microsomes. **METHODS**: Cytochrome P-450 enzyme activities were measured by biochemical assays. Xenobiotics were employed to observe their effects on CYP2A6 *in vitro*. The kinetics of coumarin 7-hydroxylase was determined, and the correlation between CYP2A6 and UDP-glucuronosyltransferase (UGT) was analyzed. **RESULTS**: CYP2A6 activities of human liver microsomes were from 0.47 to 4.14 $\mu mol \cdot min^{-1} \cdot g^{-1}$, with a 8.8-fold variation. The K_m and V_{max} of CYP2A6 ranged from 0.25 to 1.56 $\mu mol \cdot L^{-1}$ and 1.41 to 8.70 $\mu mol \cdot min^{-1} \cdot g^{-1}$, respectively. CYP2A6 activity was markedly inhibited (>50 %) by pilocarpine, diethyldithio carbamic (DDC), and rifampicin, the IC_{50} was 5.31 $\mu mol \cdot L^{-1}$, 156.35 $\mu mol \cdot L^{-1}$, and 38.81 $\mu mol \cdot L^{-1}$, respectively. α-Naphthoflavone, sulfaphenazole, troleandomycin (TAO), ketoconazole, phenobarbital, prednisolone, and azithromycin had little or no effects on coumarin 7-hydroxylation. A significant correlation was observed between CYP2A6 and UGT_2 ($r = 0.9453$, $P < 0.05$). **CONCLUSION**: CYP2A6 activity and kinetics exhibited a considerable variation in human liver microsomes *in vitro*, and a significant correlation was existed between CYP2A6 and phase Ⅱ enzyme UGT_2. Not only pilocarpine, CYP2A6 specific inhibitor, but also rifampicin and DDC inhibited CYP2A6 activity selectively.

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INTRODUCTION

Cytochrome P-450 (CYP) superfamily plays a key role in the metabolism of numerous exogenous and endogenous compounds[1]. CYP2A6, constituting about 5 % of total hepatic CYP, has been known to catalyze the metabolic activation of several precarcinogens and premutagens, including the liver-specific precarcinogens aflatoxin B_1, several nitrosamines, and certain clinically used drugs[2]. Recently, CYP2A6 has further received a lot of attention, because it is the principle human nicotine C-oxidase and the determinant factor in the smoking behavior and is tobacco-dependent[3,4]. However, it has been point out that CYP2A6 revealed not only large interspecies but also pronounced interindividual variability. Thus, it would be obviously meaningful to evaluate the metabolic activities of CYP2A6 in different population. The specific reaction catalyzed by CYP2A6 is coumarin 7-hydroxylation in human. In the present study, human liver microsomes were used to evaluate the CYP2A6 enzyme kinetic behavior and the effects of CYP selective chemical inhibitors and substrates on the activities of CYP2A6.

MATERIALS AND METHODS

Chemicals and reagents Resorufin, 7-ethoxyresorufin, coumarin, 7-hydroxycoumarin, uridine 5-diphosphoglucouronic acid (UDPGA), 7-hydroxy-4-methylcoumarin, 4-phenylphenol, pilocarpine, α-naphthoflavone, sulfaphenazole, diethyldithio carbamic sodium (DDC), troleandomycin (TAO), ketoconazole, rifampicin, prednisolone, isocitric acid, isocitric dehydrogenase, NADP, and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). Other chemicals were obtained from the following sources; cytochrome C from Boehringer Mannheim (Germany); NADPH from Serva (Heidelberg, Germany); erythromycin from AMSERCO (USA);

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Triton X-100 from Bio-Rad Co (USA); phenobarbital from Hongguang Pharmaceutical Factory (Jilin, China); azithromycin from Binhu Pharmaceutical Co (Wuhan, China).

Preparation of human liver microsomes Human liver (HL) samples were obtained from patients undergoing surgery with hepatic tumors in Zhongnan Hospital, Wuhan University, approved by the Academic Committee and the Ethics Committee of Medical College, Wuhan University. Patients aged from 37 to 50 year-old, male, were not habitual consumers of cigarettes or alcohol. HL-4 received liver local injection of cisplatin, 5-fluorouracil, and mitomycin C. Microsomes were prepared as described elsewhere[5]. The washed microsomes were resuspended at a protein concentration of 13 – 17 $g \cdot L^{-1}$ in 0.25 $mol \cdot L^{-1}$ sucrose and frozen at – 80 ℃ until used. Protein concentration and P-450 content were determined by the methods of Lowry[6] and Omura and Sato[7], respectively.

Enzyme assays Coumarin 7-hydroxylation (COH) was assayed fluorometrically with λ_{ex} = 390 nm and λ_{em} = 450 nm according to Bourrie[8]. Coumarin concentrations ranged from 0.5 to 50 $\mu mol \cdot L^{-1}$. 7-Ethoxyresorufin *O*-deethylation (EROD), aniline hydroxylation (ANH), and erythromycin demethylation (ERD) were assayed for the determination of the activities of CYP1A1, CYP2E1, and CYP3A, respectively[9].

Pilocarpine, ketoconazole, α-naphthoflavone, sulfaphenazole, TAO, DDC, rifampicin, phenobarbital, prednisolone, and azithromycin were added to microsomal incubation at various concentrations. Incubations contained microsomal protein 0.2 mg, potassium phosphate 50 $mmol \cdot L^{-1}$ (pH 7.4), KCl 50 $mmol \cdot L^{-1}$, $MgCl_2$ 2.5 $mmol \cdot L^{-1}$, and the NADPH-generating system (NADP 0.4 $mmol \cdot L^{-1}$, isocitric acid 10 $mmol \cdot L^{-1}$, isocitric dehydrogenase 0.3 U) in a final volume of 1 mL. Incubation mixtures containing DDC and TAO were preincubated at 37 ℃ for 30 min, because these inhibitors are mechanism-based. All other incubation mixtures contained substrates and inhibitors were without previous incubation. Except DDC, which was dissolved in potassium phosphate buffer 100 $mmol \cdot L^{-1}$ (pH 7.4), other compounds were dissolved in methanol in a final concentration of 1 % (v/v).

Uridine diphosphate glucuronosyltransferase (UGT) activities were determined using microsomal protein 0.5 mg, 7-hydroxy-4-methylcoumarin 0.5 $mmol \cdot L^{-1}$, and 4-phenylphenol 0.5 $mmol \cdot L^{-1}$ as the substrates for UGT_1 and UGT_2, respectively[10]. All enzymatic assays were measured as duplicates.

Data analysis Results were expressed as $\bar{x} \pm s$. Lineweaver-Burk plots were constructed for the determination of K_m and V_{max}. The CYP2A6 mediated coumarin 7-hydroxylase activities in the presence of inhibitors were expressed as a percentage of control values (3.71 $\mu mol \cdot min^{-1} \cdot g^{-1}$). The IC_{50} values for inhibitors were determined by linear regression analysis.

RESULTS

Coumarin (1,2-benzopyrone) is mainly hydroxylated in 7-position of cumarin by CYP2A6 in human liver. In the present study, COH exhibited the highest activity and the largest variability in the cytochrome P-450 enzyme activities tested, ranged from 0.47 to 4.14 $\mu mol \cdot min^{-1} \cdot g^{-1}$ with a 8.8-fold variation. Comparatively, CYP1A1 (EROD), CYP2E1 (ANH), and CYP3A (ERD) activities, exhibited 4.3, 2.0, and 1.8-fold variation, respectively (Tab 1). Both K_m and V_{max} values of coumarin 7-hydroxylation exhibited a 6.2-fold difference. But the intrinsic clearance (Cl_{int}) of coumarin *in vitro* was fairly constant ranging from 3.69 to 10.4 $L \cdot min^{-1} \cdot g^{-1}$ microsomal protein, with a 2.8-fold difference (Tab 2, Fig 1).

Pilocarpine is a potent inhibitor of CYP2A6-mediated coumarin 7-hydroxylation. Linear regression analysis indicated the IC_{50} of pilocarpine was 5.31

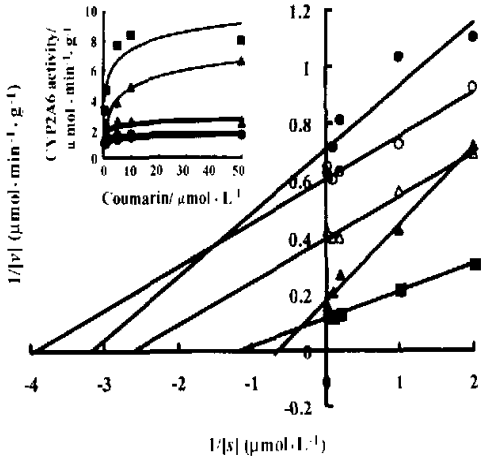

Fig 1. The individual variation of CYP2A6 activities in Chinese liver microsomes. $n = 5$. (●) human liver 1; (▲) human liver 3; (■) human liver 4; (◡) human liver 33; (△) human liver 34.

Tab 1. Cytochrome P450 enzyme activities in Chinese liver microsomes. Hepatic microsomal fractions were incubated with various cytochrome P-450 probes in the presence of NADPH generating system, and the rate of the reaction was determined on the basis of metabolite appearance. $n=5$. $\bar{x} \pm s$.

Samples	CYP2A6/μmol·min^{-1}·g^{-1}	CYP1A1/μmol·min^{-1}·g^{-1}	CYP2E1/μmol·min^{-1}·g^{-1}	CYP3A/μmol·min^{-1}·g^{-1}	P-450 content/μmol·g^{-1}	Cyto b$_5$ content/μmol·g^{-1}	Cyto C-reductase/μmol·min^{-1}·g^{-1}
HL-1	0.47	0.12	0.59	0.18	0.36	0.33	170
HL-3	4.14	0.13	0.90	0.32	0.30	0.12	167
HL-4	3.91	0.03	0.87	0.33	0.38	0.33	142
HL-33	0.62	0.04	0.65	0.32	0.19	0.46	94
HL-34	0.86	0.04	1.20	0.28	0.26	0.53	78
$\bar{x}$	2.0	0.07	0.84	0.29	0.30	0.41	130
s	1.9	0.05	0.24	0.06	0.08	0.09	42

Tab 2. Kinetic parameters of CYP2A6 in male Chinese liver microsomes. Each sample was incubated with coumarin ranged from 0.5 to 50 μmol·L^{-1}. $n=5$. $\bar{x} \pm s$.

Samples	Age/a	K_m/μmol·L^{-1}	V_{max}/μmol·min^{-1}·g^{-1}	Cl_{int} (V_{max}/K_m)/L·min^{-1}·g^{-1}
HL-1	41	0.32	1.41	4.41
HL-3	50	1.56	5.76	3.69
HL-4	19	0.84	8.70	10.4
HL-33	37	0.25	1.65	6.60
HL-34	45	0.38	2.53	6.66
$\bar{x}$	44	0.67	4.01	6.34
s	6	0.55	3.15	2.60

μmol · L^{-1}. Rifampicin and DDC also markedly inhibited CYP2A6 activity (>50 %), the IC_{50} were 38.81 μmol · L^{-1} and 156.35 μmol · L^{-1}, respectively (Fig 2). α-Naphthoflavone, an inhibitor of CYP1A1/2, has slightly inhibitory effect on CYP2A6 activity, while sulfaphenazole, ketoconazole, TAO, phenobarbital, prednisolone, and azithromycin had little or no effect on CYP2A6 activity (Fig 3).

UGT is the major phase Ⅱ metabolic enzyme involved in coumarin metabolism[11]. The metabolites of coumarin 7-hydroxylation were excreted as the glucuronide in urine. The UGT_1 and UGT_2 activities ranged from 2.8 to 5.09 and from 0.27 to 1.0 μmol · min^{-1} · g^{-1}, respectively, as we have measured. A parallel change of enzyme levels between coumarin 7-hydroxylase and UGT_1, UGT_2 were observed in human liver microsomes. Correlation analysis indicated a significant correlation between COH and UGT_2 ($n=5$, $r=0.9453$, $P<0.05$) (Fig 4). There was an obvious correlation tendency between COH and UGT_1, however, it exhibited

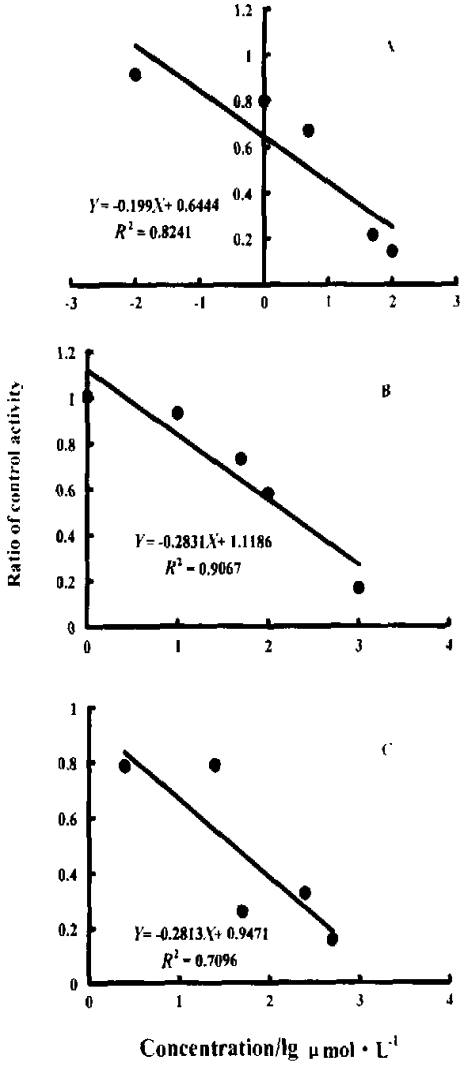

Fig 2. Linear regression analysis from the plot of the logarithm of inhibitor concentration versus percentage of the COH activity. Control activity of COH was 3.71 μmol ·min^{-1}·g^{-1}. A: Pilocarpine; B: Diethyldithio carbamic; C: Rifampicin.

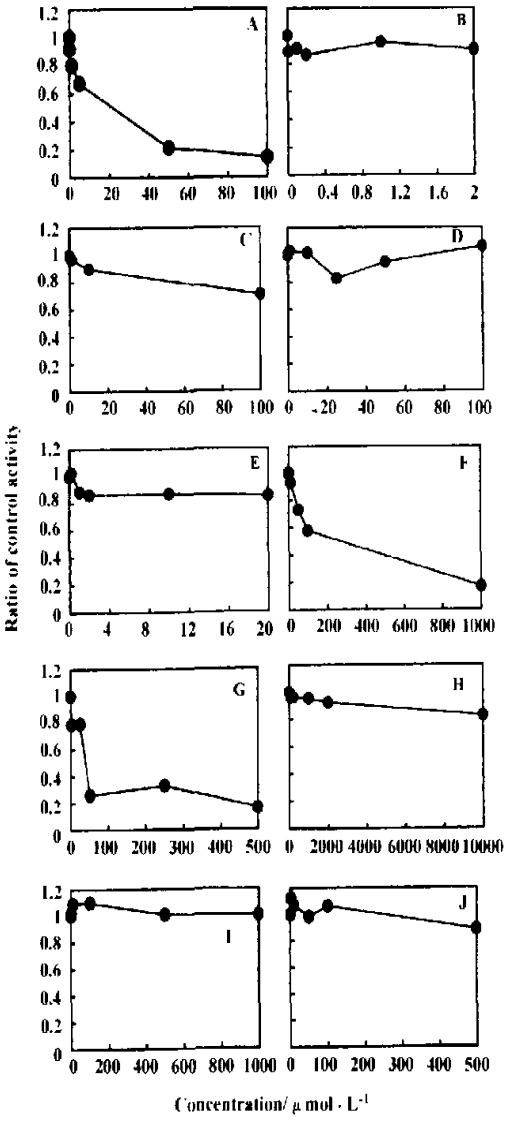

Fig 3. ***In vitro*** **effects of xenobiotics on CYP2A6 activity in human liver microsomes (HL-3). Control activity of COH was 3.71 μmol·min^{-1}·g^{-1}. A: Pilocarpine (0.01 – 100 μmol·L^{-1}); B: Ketoconazole (0.01 – 2 μmol·L^{-1}); C: α-Naphthoflavone (0.01 – 100 μmol·L^{-1}); D: Sulfaphenazole (1 – 100 μmol·L^{-1}); E: Troleandomycin (0.2 – 20 μmol·L^{-1}); F: Diethyldithio carbamic (1 – 1000 μmol·L^{-1}); G: Rifampicin (2.5 – 500 μmol·L^{-1}); H: Phenobarbital (100 – 10000 μmol·L^{-1}); I: Prednisolone (1 – 1000 μmol·L^{-1}); J: Azithromycin (1 – 500 μmol·L^{-1}).**

no statistical significance ($n = 5$, $r = 0.870$, $P > 0.05$).

DISCUSSION

It is known that CYP2A6 is responsible for the

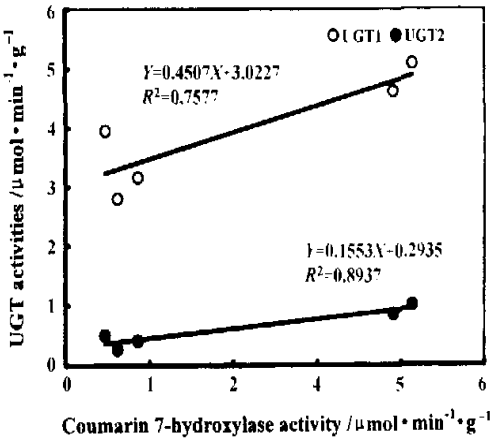

Fig 4. The correlation between coumarin 7-hydroxylase and uridine diphosphate glucuronosyltransferase (UGT) in Chinese liver mirosomes. UGT_1 was assayed with 7-hydroxy-4-methylcoumarin 0.5 mmol·L^{-1}; UGT_2 with 4-phenylphenol 0.5 mmol·L^{-1} as substrates.

clearance of many drugs and environmental chemicals, specially, in determining an individual smoking behavior. An aim of this study was to ascertain the kinetic characteristics and the extent of individual variation of CYP2A6 in human hepatic microsomes. The data presented in this report revealed that the V_{max} and K_m values of COH were consistent with previous reports[12,13], and its activity exhibited a rather large variability (9-fold variation). Studies using microsomes in Japanese subjects revealed that the CYP2A6 individual variability was also apparent[14]. We have observed (unpublished data) urine excretion of 7-hydroxycoumarin in 50 healthy volunteers in Chinese population, the variability *in vivo* was 16-fold. The pronounced individual variability in CYP2A6 level and activity with some livers completely lacking the enzyme can be attributed to polymorphism in CYP2A6 gene, and CYP2A6 activity is also modified by certain drug and environmental factors[4]. Thus, the variation presented in our data is not surprising.

With regard to evaluation of the significance of inhibitory effect, it has been pointed out[15], that the enzyme source used in the study may determine the inhibitory effect toward CYP enzyme, especially when human liver microsome used for the assay, may contribute potentially to a particular CYP enzyme in the metabolism of drugs. In this study, CYP2A6 levels were inhibited by rifampicin in a concentration-dependent manner in human liver microsomes. Moreover, the IC_{50} value of rifampicin (38.81 μmol·L^{-1}) was lower than

the corresponding value of DDC (156.35 $\mu mol \cdot L^{-1}$), and the latter is known as the specific inhibitor of CYP2A6. Therefore, on our present experimental condition, the result suggests that rifampicin may appear to be an inhibitor of CYP2A6. However, it has been reported that CYP2A6 mRNA can be induced[16] or not[17] in human hepatocytes when treated with rifampicin. The reasons for this discrepancy are unknown, which indicates that further studies are desirable to fully clarify this important issue.

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中国人肝微粒体细胞色素 P450 2A6 的体外代谢特征

R96 A

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关键词 细胞色素 P-450 CYP2A6；香豆素类；葡萄糖醛酸转移酶；肝微粒体；利福平

目的：观察人肝微粒体 CYP2A6 动力学特征。**方法**：采用生化分析法，体外研究化学异物对 CYP2A6 酶活性的影响。测定香豆素 7-羟化酶的动力学参数。同时分析 CYP2A6 与Ⅱ相酶 UGT 之间的相关性。**结果**：CYP2A6 活性差异 8.8 倍，K_m 和 V_{max} 分别为 0.25-1.56 $\mu mol \cdot L^{-1}$、1.11-8.70 $\mu mol \cdot min^{-1} \cdot g^{-1}$。匹鲁卡品、二乙基二硫代氨基甲酸盐、利福平明显抑制 CYP2A6 活性，IC_{50} 值分别为

5.31 $\mu mol \cdot L^{-1}$、156.35 $\mu mol \cdot L^{-1}$和 38.81 $\mu mol \cdot L^{-1}$. α-萘黄酮、磺胺苯吡唑、醋竹桃霉素、酮康唑、泼尼松龙和阿奇霉素对香豆素 7-羟化反应几乎无影响. CYP2A6 与 UGT_2 之间存在显著相关性($r = 0.9453$, $P < 0.05$). **结论**：中国人细胞色素 P1502A6 酶活性及动力学参数存在个体差异，CYP2A6 与 UGT_2 之间有显著相关. 除匹鲁卡品有 CYP2A6 选择性抑制作用外，利福平和 .乙基 .硫代氨基甲酸盐也明显抑制 CYP2A6 活性.

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