Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(11):39-44

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Inhibitory effect study of calf thymus DNA on catalytic activity of CYP450 enzyme in human liver microsomes

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ABSTRACT

The present study investigated the effect of calf thymus DNA (ctDNA) on human hepatic cytochrome P450s (CYP450s) in vitro. Specific substrate probes for each isoforms, CYP1A2, 2C9, 2C19, 2D6 and 3A4 were incubated using pooled human liver microsomes with or without ctDNA. Enzyme kinetics parameters K_i and IC_{50} values were estimated to determine the types and strength of inhibition. ctDNA could specifically inhibit the metabolism of CYP2C9 probe substrates, with the IC_{50}=0.9955 μg/ml, while was not able to inhibit CYP1A2, CYP2C19, CYP2D6 and CYP3A4 (IC_{50}＞100 μg/ml). The results showed that ctDNA was a potent, competitive inhibitor of CYP2C9 enzyme, has the metabolic interaction potential with the model drugs which are metabolism substrates of CYP2C9. These findings indicated that when the medical agents catalyzed mainly by CYP2C9 were co-administered in vivo in combination with adsorptive material in vitro, the potential inhibitory effect of ctDNA on enzyme activity and the following metabolism character changes of the former should be highly focused on.

Keywords: calf thymus DNA; Cytochrome P450; drug interactions; metabolism; inhibitory effect

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INTRODUCTION

Nowadays, drug-drug interactions (DDI) are of great interest to scientists involved in drug development, clinical research and toxicology evaluation, the majority of these interactions involve cytochrome P450 (CYP450). For the broad range of substrate specificity, CYP enzymes play an important role in Phase I metabolism involved in the biotransformation of drugs, other xenobiotics, as well as some endogenous substrates [1, 2]. Among numerous CYP enzymes identified to date, five human liver CYP isoforms, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 have been confirmed to be the most relevant isozymes in the metabolism of clinically commonly used drugs [3, 4]. Calf thymus DNA (ctDNA), as a biological macromolecule compound, is one of the absorptive materials which cleans up leprosy bacillus through effective physical adsorption, thus it is used to purify patients' blood in vitro and treat leprosy by hemodialysis [5, 6]. We investigated the in vivo pharmacokinetics and disposition property of ctDNA in previous study, to evaluate its safety of use as an immunoadsorptive carrier material for therapy [7, 8]. But in the practical application, the patients had leprosy or systemic lupus erythematosus (SLE) maybe undergo synergistic cure with other drugs together with ctDNA, or other kind of drugs were simultaneously or successively used against other disease and medical conditions[9-13]. Therefore, DDI may occur in potentially if some ctDNA fall off from the immunoadsorption column and go into blood circulatory system. To ensure the applying safety roundly，it is essential to conduct comprehensive inhibitory analysis for ctDNA with major drug-metabolizing CYPs[14-16].

The present study was carried out to determine the potential of nucleic acid compound, ctDNA, to inhibit the activity of human CYP enzymes that most commonly associated with the metabolic elimination of drugs: CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, followed by a full-scale characterization of the kinetics and type of

inhibition using classical probe substrates.

EXPERIMENTAL SECTION

Chemicals and reagents

The ctDNA was provided by Jianfan Biotechnology Company (Zhuhai, China). Acetparaphenetidine, diclofenac, mephenytoin, dextromethorphan, midazolam and testosterone were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and used as probe substrates. Acetaminophen, 4-hydroxydiclofenac, 4-hydroxymephenytoin, dextrorphan, 1-hydroxymidazolam and 6β-hydroxytestosteronein were purchased from Sigma-Aldrich (St. Louis, MI, USA) and used as metabolites of specific substrates. Chlorzoxazone, caffeine, nimodipine and diazepam were purchased from National Institute for the Control of Pharmaceutical and Biological Products and used as internal standard (IS). The α-naphthoflavone, sulphaphenazole and quinidine were purchased from Sigma-Aldrich, ticlopidine and ketoconazole were from National Institute for the Control of Pharmaceutical and Biological Products, which were all used as inhibitors. NADPH was purchased from Sigma-Aldrich. All other reagents were of analytical grade.

Human liver microsomes

Human liver microsomes (HLMs) were provided by the Research Institute for Liver Disease Co. (RILD) (Shanghai, China). The microsomes were prepared from ten individual human donor livers.

Enzyme kinetics essay

All the standard solutions of each substrate were manipulated by serial dilution to concentrations gradient: phenacetin, 20-4000 μmol/L; diclofenac, 50-4000 μmol/L; mephenytoin, 50-2000 μmol/L; dextromethorphan, 1-500 μmol/L; midazolam, 1-400 μmol/L; testosterone, 50-4000 μmol/L. After incubating with HLMs at 37 ℃ for optimizing time, the formation rates for specific metabolites were observed and analyzed to estimate the kinetics parameter of substrates.

Optimizing of incubation time

The final concentrations of microsome proteins and substrates in reaction systems were fixed; metabolism reactions were initiated and terminated at different time to determine the formation amounts of individual metabolite, to evaluate the linear timelines of metabolism mediated by CYPs for each probe substrate.

Inhibition of CYP450s by ctDNA

The CYP enzymatic activities were determined by O-deethylation of phenacetin for CYP1A2, 4-hydroxylation of diclofenac for CYP2C9, 4-hydroxylation of mephenytoin for CYP2C19, O-demethylation of dextromethorphan for CYP2D6, 1-hydroxylation of midazolam and 6β-hydroxylation of testosterone for CYP3A4. For each inhibition study, preliminary incubation experiments were carried out using a single CYP-specific substrates concentration lower than its K_m (the Michaelis constant), specifically, phenacetin-20 μM; diclofenac-10 μM; mephenytoin-20 μM; dextromethorphan-5 μM; midazolam-2 μM and testosterone-20 μM, in the presence of a range of ctDNA concentrations (1.0–200 μg/ml) in the incubation mixture. Probe substrates were incubated with HLMs (0.2 mg/ml), 5 mM $MgCl_2$ and 1.0 mM NADPH in a total volume of 200 μl of 150 mM phosphate buffer (pH 7.4), in the presence and absence of ctDNA. After preincubated for 5 min in an incubator shaker at 37 ℃, the reactions were initiated by adding NADPH.

Known specific inhibitors were used in parallel as positive controls: α-naphthoflavone for CYP1A2, sulfaphenazole for CYP2C9, ticlopidine for CYP2C19, quinidine for CYP2D6 and ketoconazole for CYP3A4. The K_i (Equilibrium dissociation constant of reversible inhibitor) values of ctDNA were not determined if their IC_{50} (the half maximal inhibitory concentration of a substance) were higher than 100 μM. Inhibitor and substrate concentrations for determining K_i value were selected based on the preliminary study. The range of concentrations of phenacetin (0–0.6 μM) and ctDNA (1.0–200 μg/ml) were used for estimation of K_i value. All measurements were performed in triplicate.

Data analysis

The K_i values obtained from secondary Lineweaver–Burk plots were used as initial estimates for the determination of the exact K_i values by nonlinear least square regression analysis using GraphPad (Prism5.0). The data were all statistically processed by one-way analysis of variance.

RESULTS AND DISCUSSION

Method validation

The LC–MS/MS method described above showed good separation of analyte and internal standard from other indigenous substance in HLMs, thus the developed method had high specificity with no interference for sample determination. The calibration curves were linear for all the metabolites in selected concentration range with the correlation coefficients (r) higher than 0.99. The intra-day and inter-day precisions were below 15%, and the accuracies were within 85–115%. The results were all within the acceptable range and accordance with the requirements for biological sample determination.

Optimizing of incubation time for probe substrates

The optimizing results about incubation time investigated for marker substrates of CYP450s were listed in Table 1, indicating each special compound selected as probe substrate had the different optimal incubation time.

Table 1. Optimal time for incubating CYP450s-specific substrates

CYP450s	probe substrates	metabolites	Incubating time (min)
CYP1A2	acetparaphenetidine	acetaminophen	20
CYP2C9	diclofenac	4-hydroxydiclofenac	15
CYP2C19	mephenytoin	4-hydroxymephenytoin	30
CYP2D6	dextromethorphan	dextrorphan	15
CYP3A4	midazolam	1-hydroxymidazolam	5
	testosteronein	6β-hydroxytestosteronein	10

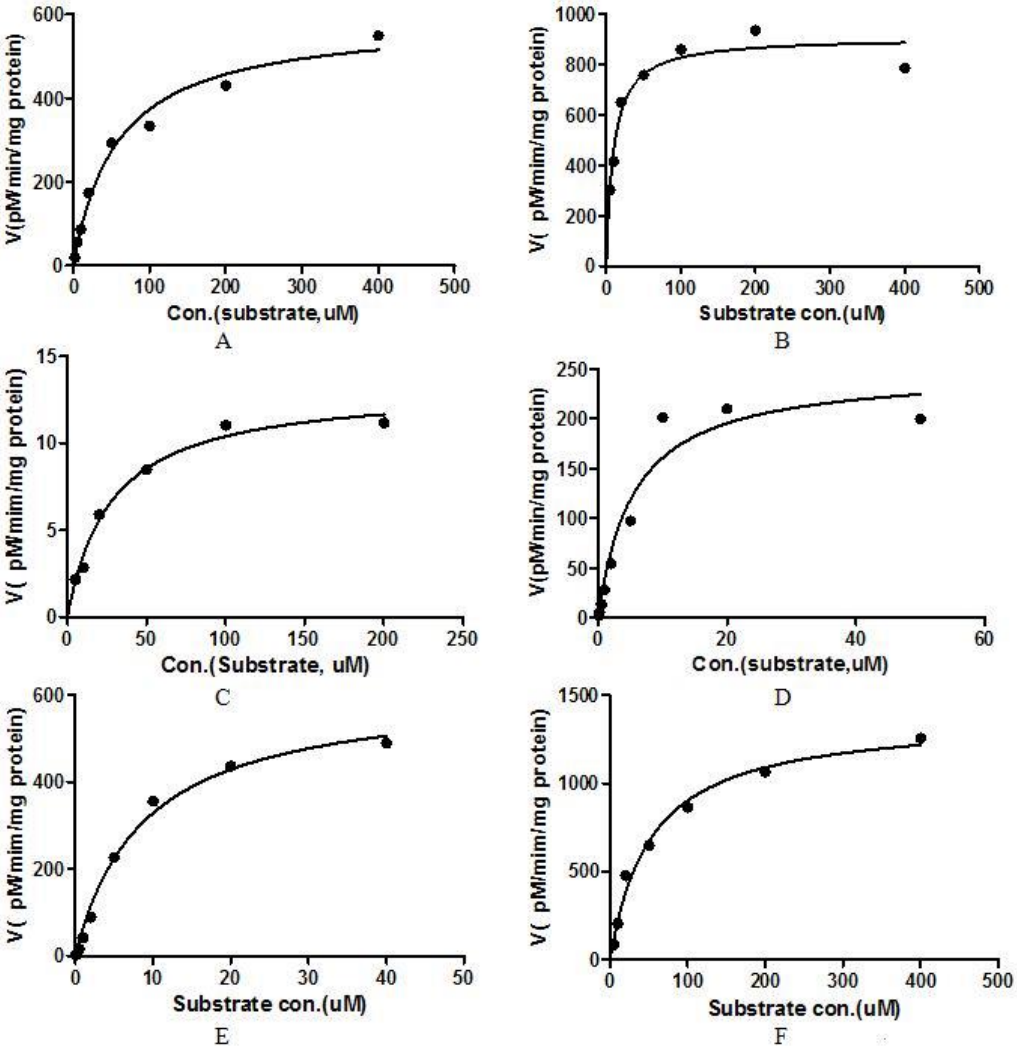

Fig.1. Catalyzed dynamic characteristics of CYP450s-specific substrates in HLMs

A: acetparaphenetidine; B: diclofenac; C: mephenytoin; D: dextromethorphan; E: midazolam; F: testosteronein

Enzyme kinetics study

The corresponding initial velocities of enzyme reaction for each concentration of substrate were calculated. In the range of concentrations used in this study, the reaction kinetics from six probe substrates characterized in HLMs all accorded with the dynamic characteristics of Michaelis-Menten hyperbola, which were showed in Fig 1. The kinetics parameters, K_m and V_{max} were obtained by nonlinear regression and listed in Table 2.

Table 2. Kinetic parameters of CYP450s-specific substrates in HLMs

CYP450s	Substrates	Km	Vmax
		μmol/L	pmol/min/mg protein
CYP1A2	acetparaphenetidine	94.06	185.9
CYP2C9	diclofenac	9.801	908.8
CYP2C19	mephenytoin	27.63	13.25
CYP2D6	dextromethorphan	5.901	1165
CYP3A4	midazolam	8.788	616.7
CYP3A4	testosteronein	52.76	1378

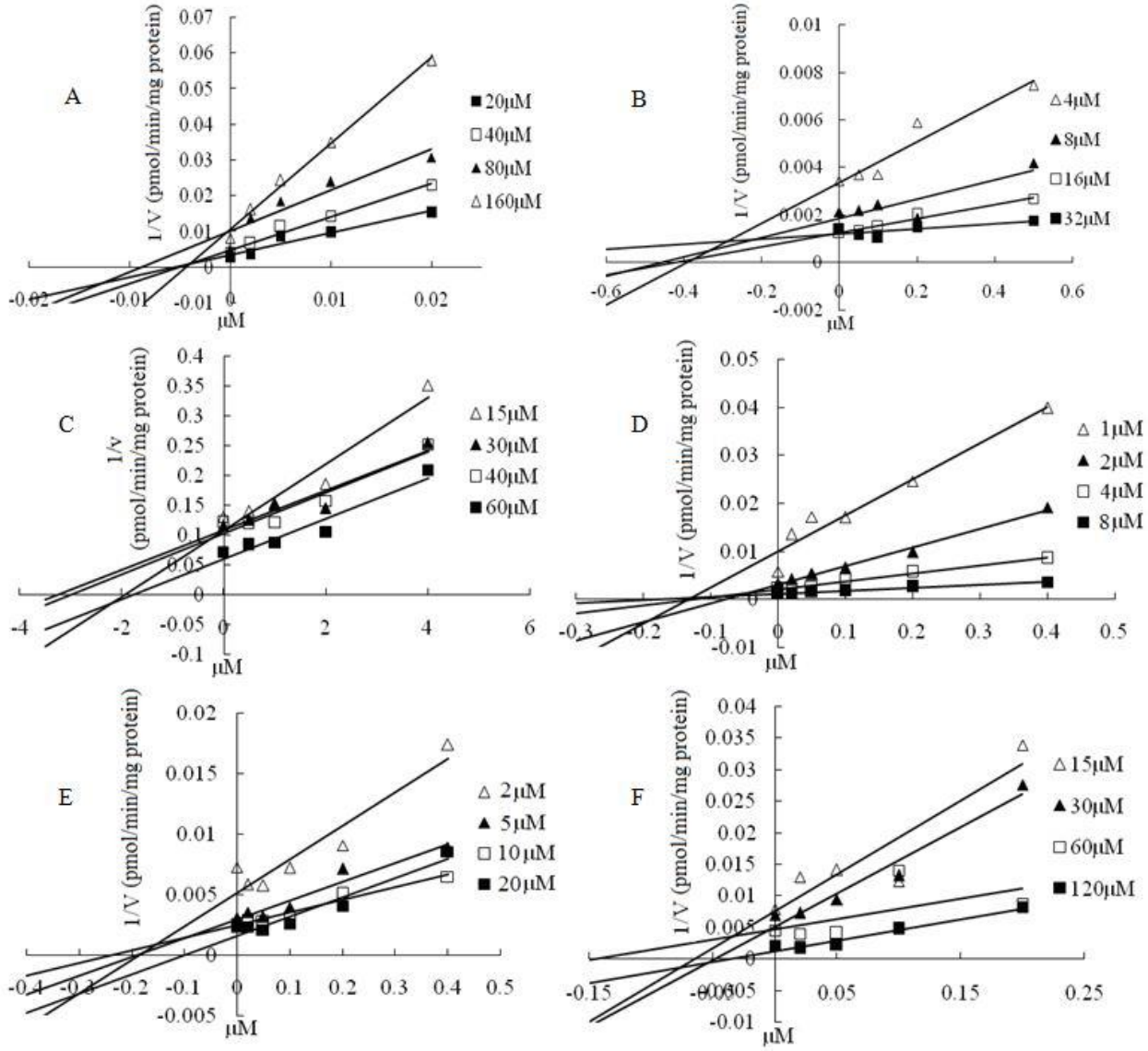

Fig.2. Dixon plots illustrating the effects of specific inhibitor on the metabolism of each probe substrate in human liver microsomes, by using formation rates of metabolites versus the inhibitor concentrations. Inhibitors

A-α-naphthoflavone, B-sulfaphenazole, C-ticlopidine, D-quinidine, E and F-ketoconazole; metabolizing reactions: A-acetparaphenetidine to acetaminophen, B-diclofenac to 4-hydroxydiclofenac, C-mephenytoin to 4-hydroxymephenytoin, D- dextromethorphan to dextrorphan, E-midazolam to 1-hydroxymidazolam, F- testosteronein to 6β-hydroxytestosteronein

Each point represents the mean of triplicate determinations.

Inhibition effect of specific inhibitors on CYP450s

The six probe substrates were co-incubated with their own specific inhibitor in HLMs, respectively. The formation

rates of metabolites at a variety of substrate and inhibitor concentrations were determined and Dixon plots for the inhibition of CYP isoforms were constructed, presented in Fig. 2. The inhibition rate constant K_i of each inhibitor for CYP isoforms were determined based on the analysis of nonlinear regression and GraphPad software for inhibition data, and presented in Table 3. The results showed the K_i values for specific substrates of major CYP isoforms obtained in this study were in general agreement with the values in previously reported literature. Therefore, the in vitro test model can be well used to evaluate the effect of ctDNA on the activity of human CYP enzymes.

Table 3. The Ki values of suppressing effect for CYP450s-specific substrates

CYP450s	substrates	inhibitors	Ki(μmol/L)	
			measured values	reference value *
CYP1A2	acetparaphenetidine	α-naphthoflavone	0.0068	0.01
CYP2C9	diclofenac	sulphaphenazole	0.31	0.3
CYP2C19	mephenytoin	ticlopidine	1.17	1.2
CYP2D6	dextromethorphan	quinidine	0.12	0.027～0.4
CYP3A4	midazolam	ketoconazole	0.102	0.0037～0.18
CYP3A4	testosteronein	ketoconazole	0.096	

**Note：The reference values are all drawn from official website of Food and Drug Administration: http://www.fda.gov/cder/drug/drug interactions/default.htm-overview*

Table 4. The IC_{50} values for the inhibition of calf thymus DNA on CYP450s activities

Substrates	CYP450 isoforms	IC50 (μg/ml)
acetparaphenetidine	CYP1A2	>100
diclofenac	CYP2C9	0.9955
mephenytoin	CYP2C19	>100
dextromethorphan	CYP2D6	>100
midazolam	CYP3A4	>100
testosteronein	CYP3A4	>100

Inhibition of CYP450 activities by ctDNA in HLMs

The capabilities of ctDNA to inhibit individual CYP enzymes were examined by the probe reaction assays with HLMs. IC_{50} values were determined by plotting with the formation rates of each probe metabolites in incubation system versus the logarithm being concentration gradient of ctDNA, and listed in Table 4. It could be inferred that ctDNA strongly inhibited the reaction catalyzed by CYP2C9 in HLMs, with the IC_{50} value of 0.9955 μg/ml. For the rest of four isoforms, CYP1A2，CYP2C19，CYP2D6 and CYP3A4, the obtained IC_{50} value of ctDNA were all higher than 100 μg/ml, indicating their catalytic activity in vivo would not be inhibited by ctDNA. This suggests that, if ctDNA fall off and further go into the circulatory system, it maybe interact with the drugs reside in body of patients which are metabolized by CYP2C9 [17-18].

CONCLUSION

In summary, systematic research was conducted in this paper to evaluate and predict drug interactions promoted by ctDNA. Our data indicated that ctDNA potentially possessed high potency to inhibit the activity of CYP enzymes. Careful monitoring would be indispensable for the concomitant use of ctDNA with other drugs that are metabolized primarily by CYP2C9. Coupling with the former pharmacokinetics study in animals, we have provided the comprehensive in vivo and in vitro pharmacokinetics information for ctDNA, which can be used as beneficial guidance for the safe and reasonable application of this absorptive material in therapeutic practice.

Acknowledgements

This work was financially supported by the doctoral scientific research start-up foundation from Henan University of Technology (No: 2012BS034).

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