doi:10.3969/j.issn.1674-5817.2010.02.003

Validation of Alternative Method for Evaluating Effects of Drug Metabolism on Cytotoxicity

ZHOU Jing-jing[1], YANG Ming[2], MAO Yu-chang[3], LU Jie-qi[2], YANG You-ming[3], HU Zhuo-han[1, 3]

(1. Department of Pharmacological, School of Pharmacy, Fudan University,Shanghai 201203, China;
2. Pudong Entry-Exit Inspection & Quarantine Bureau of China, Shanghai 200135, China.
3. Research Institute for Liver Diseases (Shanghai) Co., Ltd., Shanghai 201203, China)

[Abstract] **Objective** With increased concerns and gradual progress on the ethical use of experimental animals in the past decades, the development, and validation of new and revised non-animal and reliable alternative methods have been approached by academia, industry and government regulators in order to reduce the number of animals, and refine the procedures to lessen or eliminate pain and distress to animals, and replace animals with non-animal systems. According to the methods recommended by "The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)" in USA, priority for alternative methods has been placed on basal cytotoxicity methods and additional efforts are on incorporating *in vitro* cytotoxic data with *in vitro* parameters of absorption, distribution, metabolism, excretion, and toxicity (ADMET). This study was to validate an alternative ADMET method by using human primary hepatocytes. **Method** Human liver microsomes were used to evaluate the effects of natural products on CYP450 isoforms. Basal cytotoxicity of 5 imported natural products was estimated by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and effects of metabolism by CYP450 isoforms on cytotoxicity of natural products was evaluated by using chemical inhibitors of CYP2A6, 2C9, 2C19, and 3A4 preincubation with human primary hepatocytes. **Results** The inhibitory potential (IC_{50}) of natural products (TA-07-004, TA-07-005) on CYP1A2 were 0.22% and 0.03%, CYP 3A4 were 0.49% and 0.20% of the initial concentration, respectively.The hepatotoxicity, IC_{50} values were 0.37%, 0.26%, 0.62%, 0.19% of original concentrations for TA-07-001, TA-07-002, TA-07-004, and TA-07-005, respectively. No cytotoxicity was observed for TA-07-003. Preincubation of selective inhibitors for CYP450 isoforms with primary hepatocytes showed that cytotoxicity of TA-07-005 was reduced by inhibition of CYP2A6, 2C9, 2C19, 3A4, suggesting that TA-07-005 might be bioactivated by CYP450. **Conclusions** The *In vitro* hepatotoxic model with in vitro metabolism assay provided a valuable and feasible alternative procedure for estimating metabolism dependent toxic potentials of test materials as natural products.

[Key words] Alternative methods; Natural product; Cytochrome P450 (CYP450s); Cytotoxicity; Bioactivation; Human primary hepatocytes.

[CLC number] Q95-33 **[Document code]** A **[Article ID]** 1674-5817(2010)02-0087-08

[Recieved date] 2009-09-08

[Fund project] Science and Technology Plan Project from Shanghai Inspection and Quarantine Bureau (No.HK047-2007).

[Author] ZHOU Jing-jing (1984-), Female, Master Degree candidate, E-mail: zhoujj@ rild-biotech.com;
YANG Ming (1956-), Male, Associate Chief Physician, E-mail: Yangm@ shciq. gov.cn

[Corresponding author] HU Zhuohan (1953-), Male, Professor, E-mail: huzh@ rild- biotech.com, Tel: 021- 50802259

Introduction

The United States federal agencies and the European Unions respectively established the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the European Center for the Validation of Alternative Methods (ECVAM) with the objective of developing, and validating alternative methods and, where feasible, ensuring that they reduce, refine, or replace the use of animals. Based on the efforts contributed by ICCVAM, U.S. laws (42 USC 289 d, 7USC2123) was issued[1], and require that alternatives must be considered before using animals for research and testing. Such alternative methods including new or revised test assays will reduce the number of animals to the minimum, refine procedures to lessen or eliminate pain and distress to experimental animals, and replace animals with non-animal systems or one animal species with a phylogenetically lower animal species, which are all contributions to the 3Rs (Reduction, Refinement, and Replacement)[2].

ICCVAM's Test Method Evaluation Report for *in vitro* cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests recommended the future studies. One of the recommendations was to use *in vitro* cell-based test methods for incorporating evaluations of ADME (absorption, distribution, metabolism, excretion) to provide improved estimations of acute toxicity hazard and extrapolate from *in vitro* toxic concentrations to equivalent doses *in vivo* [3].

Cryopreserved human primary hepatocytes are widely accepted as a unique *in vitro* tool for estimation of both cytotoxicity, metabolism and metabolic interactions[4], because they play a fundamental role as a major organ for metabolizing xenobiotics by drug metabolizing enzymes including cytochrome P450 (CYP450), a superfamily of enzymes predominantly expressed in the liver [5, 6,7].

Combining *in vitro* cytotoxicity assay with *in vitro* metabolism assay provides researchers an opportunity to estimate drug metabolism related toxicity.

This study aimed to validate an alternative ADMET method for the estimation of CYP450 related hepatotoxicity of imported natural products by using human primary hepatocytes in order to improve the assessment (support speeding estimation) of imported goods and reduced (with reducing) the use of laboratory animals.

1 Materials and methods

1.1 Reagents, Chemicals and Test Materials

Probe substrates, metabolites, selective inhibitors, and cofactor of CYP450s including phenacetin, testosterone, quinidine, sulfaphenazole, tranylcypromine, itraconazole and NADPH (β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt) were purchased from Sigma. 6β-hydroxyl-testosterone and acetaminophen were purchased from Daiichi Pure Chemicals (Tokyo, Japan).

Reference compounds and reagent for cytotoxicity including tamoxifen and DMSO (dimethylsulfoxide) were purchased from Sigma. MTT[3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] was purchased from Fluka.

As test materials, five natural products (TA-07-001, TA-07-002, TA-07-003, TA-07-004, TA-07-005) were provided by Pudong Entry-Exit Inspection & Quarantine Bureau of Shanghai, China.

1.2 Equipments and Instruments

Plate Reader (Thermo labsy stems), High Performance Liquid Chromatography (HPLC, Agilent series 1100), HWS-12 waterbath (Shanghai permanent science and technology (science) Ltd, Steri-Cycle CO_2 Incubators (Thermo Fisher Scientific Inc) were used in this Study

1.3 Test Systems

Human liver microsomes (Polled from 10 donors) and primary hepatocytes were prepared under Chinese Donation Regulation with informed consent and has been tested negative serologically for HIV, Human hepatitis B (HBV), Human hepatitis C (HCV) and rapid plasma reagin (RPR). Liver microsomes were prepared by differential centrifugation of liver homogenates[8] and hepa-

tocytes were prepared using a method modified from Li [9]. The *in vitro* reagents from human and animal liver tissues were previously characterized for enzyme activities of CYP450s.

1.4 Preparation of Solutions

The natural products were diluted with 0.1 mol/L Tris buffer to the required concentrations. The concentrations were 0.1%, 0.2%, 0.5%, 1%, 2% of the initial concentration.

1.5 Procedures

1.5.1 CYP450 Characterization Each different concentration of substrate solution was added into respective wells. Liver microsome solution was added to each well. Samples were pre-incubated for 15 minutes at 37℃ in an incubator. The probe substrate reaction was begun by adding NADPH (the cofactor of cytochrome P450 oxidases) to each well. Final concentrations were 0.5 mg/ml protein and 0.5 mmol/L NADPH. After incubating samples for 60 minutes at 37℃, cold methanol was added including internal standard to each well to stop the reaction .After vortexing and centrifuging at 3 000 r/min for 15 minutes, 200 μl of the supernatants were transferred to high performance liquid chromatography (HPLC) vials for assay.

1.5.2 CYP450 Inhibition The microsomes were pre-incubated with the natural products (TA-07-004, TA-07-005) respectively, at 37℃ for 15 minutes, while 0.1 mol/L tris buffer was added to the negative control group to make up the volume . Then all the groups were incubated with selective substrates of phenacetin (CYP1A2) and testosterone (CYP3A4) respectively, in presence of NADPH, at 37℃ for 30 minutes. Final concentrations were 0.5 mg/ml protein and 0.5 mmol/L NADPH. Incubations were stopped by adding 0.5 ml cold methanol. Samples were transferred to centrifuge tubes, centrifuged at 3 000 r/min for 15 minutes, and 500 μl of the supernatants were transferred to vials for assay.

1.5.3 Cytotoxicity The cryopreserved human primary hepatocytes were thawed by transferring the vial from liquid nitrogen directly into 37℃ waterbath and shaken constantly for 30 s. The thawed cell suspension was transferred into centrifuge tubes and centrifuged at 50 × *g* for 5 minutes at 4℃. The resulting pellets were resuspended with incubation media. The viability of post-thaw primary hepatocytes was determined by the trypan blue exclusion method and hepatocytes were accepted for experimental use if viability was more than 70%.

Test articles or tamoxifen dosing solutions were added to a 96-well plate with hepatocytes of 350 000 cells/ml, and incubated at 37℃ with 5% CO_2 for 4 h.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) with final concentration of 5 mg/ml was added per well for another 3 h incubation at 37℃ with 5% CO_2. 150 μl of DMSO was added per well, and the plate was shaken on an orbital shaker at 50 r/min at room temperature for 10 minutes. The optical density was measured for each sample at wavelength of 570 nm.

1.5.4 Metabolism pathways involved in cytotoxicity Suspended human hepatocytes were plated at 350 000 cells per well into 96-well plates on the day of experimentation, and pre-incubated with the selective inhibitors of CYP450 isoforms (tranylcypromine of 73.66 μmol/ml for CYP2A6, sulfaphenazole of 79.52 μmol/ml for CYP2C9, quinidine of 144.93 μmol/ml for CYP2C19, and itraconazole of 14.17 μmol/ml for CYP3A4) at 37℃ for 15 minutes, respectively.

None of the inhibitors at the concentrations and incubation-times had obvious cytotoxicity towards the human hepatocytes. Then, each different concentration of natural products solution was added into the respective wells. The wells of the positive control group and the negative control group were added with tamoxifen of 6.25, 12.5, 25, 50, 100, 200 μg/ml and 0.1 mol/L.Tris buffer to make volume up respectively. All above groups were incubated at 37℃ in a humidified, 5% CO_2 atmosphere for 4 h.

1.6 Bioanalysis: HPLC for CYP450 isoform activity

The activity of CYP1A2 was determined by analysing the production of acetaminophen by a validated HPLC method under the following conditions: analytical column-Phenomenex Nucleosil C_{18}(250 mm × 4.60 mm i.d, 5 μm); mobile phase: solvent A-93.906% deionized water, 6% acetonitrile; 0.094% trifluoroacetic acid,

solvent B-100% acetonitrile; flow rate-1 ml/min.; gradient programme-initially 100% solvent A, ramp to 25% solvent B (and 75% solvent A) at 7.6 minutes. and back to 100% solvent A at 17.6 minutes,. Total run time 23 minutes. Injection volume was 50 μl. Ultraviolet detection at 245 nm.

The activity of CYP3A4 was determined by analysing the production of 6 β-hydroxy-testosterone by a validated HPLC method under the following conditions: analytical column-Luna phenyl-hexyl (150 mm × 4.60 mm i.d, 5 μm); mobile phase: solvent A-10% acetonitrile, 15% methanol, 75% deionized water; solvent B -10% acetonitrile, 60% methanol, 30% deionized water; flow rate-1 ml/min.; gradient programe-initially 100% solvent A the next 10 minutes for, ramp to 35% solvent B (and 65% solvent A) over the next 13 minutes, ramp to 90% solvent B over the next 1 minutes, ramp to 100% solvent B for 4 minutes, and back to 100% solvent A at 28 minutes. Total run time 31 minutes. Injection volume was 50 μl. Ultraviolet detection at 247 nm.

1.7 Parameter calculations and Statistics

The following equation was used for calculating relative activity of CYP3A4 and 1A2:

Relative activity (%) = The peak areas of metabolite formations (with the natural products added)/the peak areas of metabolite formations (Negative control) × 100(%).

The following equation was used for calculating cell viability:

Cellular viability (%) = $Absorbance_{treatment}$/ $Absorbance_{negative}$ × 100(%).

The following equation was used for calculating IC_{50} for CYP450 inhibition and cytotoxicity by by GraphPad Prism version 4.0 (GraphPad Software, San Diego CA):

Y=Bottom +(Top-Bottom)/(1+10^·[($LogIC_{50}$-X)• HillSlope)],

Where X is the logarithm of concentration, Y is the response, Bottom is the minimum response, Top is the maximum response, $LogIC_{50}$ is logarithm of X at the response midway between TOP and Bottom, and Hillslope describes the steepness of the curve.

Standard deviation and mean value were performed on Microsoft Excel 2003.

2 Results

2.1 Characterization of Hepatocyte and Liver Microsomes

Pooled human liver microsomes (Lot OHJZ) were characterized by determining enzyme kinetics of CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A4, the values of K_m and V_{max} were showed in Table 1, suggesting that the test system be validated for the study of inhibition.

Cyropreserved human primary hepatocytes (Lot MJLK) were checked for clinical viral test and characterized for enzyme activities of CYP1A2, 2A6, 2D6 and 3A4 and results were showed in Table 2, suggesting that the test system was validated for this metabolism study.

2.2 CYP450 Inhibition

The results showed that the inhibitory potential (IC_{50}) of natural products (TA-07-004, TA-07-005) on CYP1A2 were 0.22% and 0.03%, CYP 3A4 were 0.49% and 0.20% of the initial concentration, respectively (Table 3). It indicated that the natural products (TA-07-004, TA-07-005) had obvious inhibition on CYP1A2 and CYP3A4.

2.3 Hepatotoxicity

Tamoxifen showed a dose-dependent cytotoxicity for both suspended and attached cryopreserved human hepatocytes with IC_{50} values of 84.54 and 64.66 μg/ml, respectively, indicating that the test system was validated for this study (Table 4).

The suspended and attached cryopreserved human hepatocytes were used for testing cytotoxicity of the natural products. The cell viability (IC_{50}) were presented and plotted in Table 4, indicating that cytotoxity of the tested natural products was of the order of TA-07-004 <TA-07-001 <TA-07-002 <TA-07-005. Therefore, the most cytotoxic TA-07-005 was selected for further investigating effects of CYP450 metabolism on cytotoxicity.

Table 1 Characterization of pooled human liver microsomes (Lot:OHJZ)

CYP450 Isoform	Phenotyping Reaction	Enzyme Kinetics Km (μmol/L)		Enzyme Activity (pmole • mg^{-1} • min^{-1})		
			96% confident range	Substrate (μmol/L)	Mean	SD
1A2	Phenacetin deethylation	35.5	30.3～40.6	100	256	7.17
2A6	Coumarin 7-hydroxylation	6.81	5.63～8.25	25	29.9	1.61
2C9	Diclofenac 4'-hydroxylation	57.5	50.3～65.8	100	36.3	0.65
2C9	Tolbutamide 4-hydroxylation	52.5	43.3～63.6	100	6.2	0.36
2C19	S-mephenytoin 4'-hydroxylation	7.07	6.29～7.96	50	42	0.98
2D6	Dextromethorphan O-demethylation	61.4	51.3～73.5	100	811	69.4
2E1	Chlorzoxazone 6-hydroxylation	62.4	47.6～81.9	100	190	12.3
3A4	Testosterone 6β-hydroxylation	4.94	4.12～5.92	12.5	337	12.2
3A4	Midazolam 1'-hydroxylation	35.5	30.3～40.6	100	256	7.17

Table 2 Characterization of cryopreserved human primary hepatocytes (Lot: MJLK)

Viability(%)	Genotyping of 2D6 mutant 10*	Rate of Metabolic Activity of P450s (pmoles • 10^{-6} • min^{-1})			
		1A2	2A6	2D6	3A4
84	10*/10*	67	8	3	26

Table 3 Inhibitory of TA-07-004 and TA-07-005 on CYP1A2 and CYP3A4 activity (mean ± SD, *n*=3)

Concentration (%)	Relative activity of CYP3A4 % NC		Relative activity of CYP1A2% NC	
	TA-07-004	TA-07-005	TA-07-004	TA-07-005
0.1	58.21 ± 3.84	64.88 ± 2.10	59.44 ± 0.87	28.86 ± 3.73
0.2	48.95 ± 2.29	49.84 ± 1.31	41.93 ± 0.66	20.06 ± 1.75
0.5	32.44 ± 2.69	32.08 ± 0.46	18.76 ± 0.07	10.82 ± 0.43
1	23.25 ± 0.84	17.62 ± 1.62	6.44 ± 0.03	6.49 ± 0.43
2	10.05 ± 1.15	7.15 ± 0.78	0.85 ± 0.06	ND
IC_{50} (%)	0.49	0.20	0.22	0.03
95% Confidence	0.30～0.78	0.16～0.25	0.20～0.25	0.02～0.04

Note: ND, not detectable

Table 4 Hepatotoxicity of the natural products on human primary hepatocytes (*n*=3)

Test articles	Cellular Viability (%)			
	Suspended hepatocytes		Attached hepatocytes	
	IC_{50}	95% Confidence	IC_{50}	95% Confidence
Tamoxifen	84.54	57.88～123.50	64.66	49.59～84.31
TA-07-001	0.37	0.17～0.78	0.93	0.52～1.65
TA-07-002	0.26	0.19～0.36	0.83	0.66～1.05
TA-07-003	ND*	ND	1.01	0.95～1.07
TA-07-004	0.70	0.41～0.91	1.36	1.07～1.72
TA-07-005	0.19	0.12～0.31	1.26	1.03～1.53

Note: μm/ml and % for Tamoxifen and percentage of original concentration for natural products; ND, not detectable

2.4 Identification of CYP450s for affecting hepatoxicity

Natural product TA-07-005 showed a dose dependent cytotoxicity with IC_{50} of 0.21% of its original concentration in incubation with cryopreserved human primary hepatocytes. After pre-incubation of human

primary hepatocytes with selective inhibitors, tranylcypromine for CYP2A6, sulfaphenazole for CYP2C9, quinidine for CYP2C19, and itraconazole for CYP3A4, the IC_{50} values were 0.44%, 0.52%, 0.56%, and 0.49%, respectively. The IC_{50} values with pre-incubation CYP450 inhibitors were over 200% higher than that of vehicle control, suggesting that TA-07-005 might bioactivated by CYP2A6, 2C9, 2C19 and 3A4(Table5 and Figure 1).

3 Discussion

In recent years, *in vitro* procedures have become alternatives to animal experiment, especially the use of human hepatocytes has been found to contribute significantly to understand drug metabolism, drug-drug interaction, hepatotoxicity, and so on[9,10,11]. In this study, we used similar *in vitro* approaches to evaluate the role of metabolism in the cytotoxicity of natural

Table 5 Effects of CYP450 on cytotoxicity of TA-07-005 (*n*=3)

Dosing Level (% of original concentration)	Cellular Viability (%)				
	Vehicle control	Inhibition of CYP2A6 by Sulfaphenazole	Inhibition of CYP2C9 by Tranylcypromine	Inhibition of CYP2C19 by Quinidine	Inhibition of CYP3A4 by Itraconazole
0.1	113.90 ± 9.82	119.6 ± 10.85	99.67 ± 9.16	116.30 ± 6.36	115.63 ± 11.60
0.2	85.27 ± 9.47	126.6 ± 5.89	106.9 ± 16.20	95.67 ± 27.28	105.63 ± 13.25
0.5	55.70 ± 23.21	67.1 ± 11.8	81.10 ± 16.93	90.97 ± 16.17	73.33 ± 30.09
1	36.70 ± 6.94	64.8 ± 16.04	49.33 ± 9.60	48.07 ± 7.48	36.70 ± 4.31
2	49.60 ± 1.13	42.4 ± 4.71	49.00 ± 3.00	49.17 ± 3.66	45.10 ± 6.42
IC_{50} (%)	0.21	0.44	0.52	0.56	0.49
95% Confidence	0.18～0.23	0.26～0.52	0.46～0.58	0.45～0.71	0.44～0.56
% of NC	100	212	252	273	240

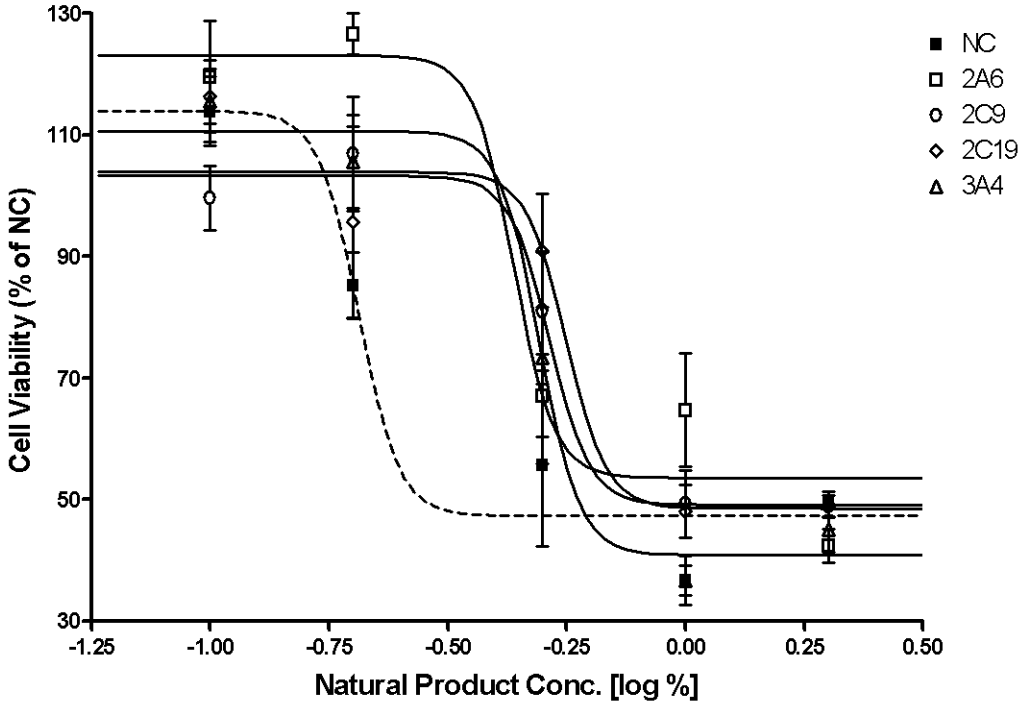

Figure 1 Effects of inhibition of CYP450s on the cytotoxicity of TA-07-005 to human hepatocytes. NC: vehicle control; CYP2C9: preincubation with sulfaphenazole; CYP2A6: preincubation with tranylcypromine; CYP2C19: preincubation with quinidine; CYP3A4: preincubation with itraconazole

products. Primary hepatocytes, including cryopreserved ones, represent a self-contained system with complete, functional, physiological levels of enzyme pathways and cofactors [12]. Therefore, the cryopreserved human primary hepatocytes have been used in this study to determine the effects of cytochrome P450 on cytotoxicity of natural products. The cytotoxicity of natural products on suspended hepatocytes was compared with those of attached hepatocytes in this study. The results showed that suspended hepatocytes were more sensitive than attached ones when they were dosed with the natural products.

Preincubation of primary hepatocytes with selective inhibitors of CYP450s provided an opportunity to identify CYP450s that bioactivate or detoxify the cytotoxicity of test articles. The cytotoxicity of TA-07-005 was attenuated by preincubation of hepatocytes with sulfaphenazole (inhibitor of CYP2A6), tranylcypromine (inhibitor of CYP2C9), quinidine (inhibitor of CYP2C19), and itraconazole (inhibitor of CYP3A4) significantly, IC_{50} values were over 200% of vehicle control. This result suggested that TA-07-005 be subject to metabolism by CYP2A6, 2C9, 2C19 and 3A4, and be bioactivated.

This alternative method of combining a cytotoxic assay with a metabolism approach by using human primary hepatocytes was validated for estimating the cytotoxicity of a test article, and further evaluating the role of metabolism-either bioactivation or detoxification.

Future validation should include more metabolic pathway(s) such as CYP1A2, 2D6, 2E1, and phase II conjugation enzymes. If involved, elucidation of toxic metabolite(s) should also be considered.

[Reference]

[1] Halle W. The Registry of Cytotoxicity: Toxicity testing in cell cultures to predict acute toxicity (LD_{50}) and to reduce testing in animals[J]. Altern lab Anim, 2003, 31(2):89-198.

[2] Manciocco A, Chiarotti F, Vitale A, *et al*. The application of Russell and Burch 3R principle in rodent models of neurodegenerative disease: the case of Parkinson's disease [J]. Neurosci Biobehav Rev, 2009, 33(1):18-32.

[3] ICCVAM. *In Vitro* Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests .NIH Publication No: 07-4519.Reaearch Triangle Park, NC: National Institute of Environmental Health Sciences. Available: *http://iccvam.niehs.nih.gov/methods/in vitro.htm* [accessed November 2006]

[4] Albert P. Li, Chuang Lu, Julie A, *et al*. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential[J]. Chem Biol Interact, 1999, 121(1): 17-35.

[5] Moschella C, Jaber BL. Interaction between cyclosporine and Hypericum perforatum (St.John's wort) after organ transplantation[J]. Am J Kidney Dis, 2001, 38(5):1105-1107.

[6] Vaes LP, Chyka PA. Interactions of warfarin with garlic, ginger, ginkgo, or ginseng: nature of the evidence[J]. Ann Pharmacother, 2000, 34(12):1478-1482.

[7] Izzo AA, Ernst E. Interactions between herbal medicines and prescribed drugs: a systematic review[J]. Drugs, 2001, 61 (15): 2163-2175.

[8] Meier PJ, Mueller HK, Dick B, *et al*. Hepatic monooxygenase activities in subjects with a genetic defect in drug oxidation [J]. Gastroenterology, 1983, 85(3): 682-692.

[9] Li AP, Roque MA, Beck DJ, *et al*. Isolation and culturing of hepatocytes from human livers[J]. Methods Cell Sci, 1992, 14: 139-145.

[10] Hengstler JG, Utesch D, Steinberg P, *et al*. Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction[J]. Drug Metab Rev, 2000, 32(1): 81-118.

[11] Mcginnity DF, Berry AJ, Kenny J R, *et al*. Evaluation of time-dependent cytochrome P450 inhibition using cultured human hepatocytes[J]. Drug Metab Dispos, 2006, 34(8): 1291-1300.

[12] Li AP. Human hepatocytes: isolation, cryopreservation and applications in drug development[J]. Chem Biol Interact, 2007, 168(1):16-29.

体外替代安全评价方法的验证-代谢性相互作用对细胞毒性的影响

周晶晶[1], 杨 明[2], 毛玉昌[3], 陆解旗[2], 杨幼明[3], 胡卓汉[1,3]
(1. 复旦大学药学院, 上海 201023; 2. 上海浦东出入境检验检疫局, 上海 200135;
3. 瑞德肝脏疾病研究(上海)有限公司, 上海 201023)

[摘要] **目的** 随着近20年实验动物伦理原则的逐步普及, 学术界、工业界和政府管理部门开始研究和推广动物试验的替代方法, 用于药品、食品和化妆品的安全评价。美国15个政府管理机构和研究机构成立了"替代评价方法协调委员会(ICCVAM)"并指定和验证了评价方法。作者按照ICCVAM推荐的方法, 应用体外细胞毒性评价方法和代谢性相互作用评价方法, 研究出入境天然产物制品的毒性作用及其代谢性活化或代谢性解毒机制。**方法** 用人肝微粒体, 研究了天然产物制品对肝细胞色素P450亚酶的作用。用超低温冻存的人肝原代细胞, 评价了5个进口天然产物制品的细胞毒性(MTT方法)。应用化学抑制法, 研究了细胞色素P450 2A6, 2C9, 2C19和3A4对天然产物制品细胞毒性的影响。**结果** 天然产物制品TA-07-004和TA-07-005对CYP1A2有明显的抑制作用, 它们的IC_{50}分别为初始浓度的0.22%和0.03%, 且它们对CYP3A4也有明显的抑制作用, 它们的IC_{50}分别为初始浓度的0.49%和0.20%。天然产物制品TA-07-003没有肝细胞毒性, 其余4个天然产物制品观察到有肝细胞毒性, 它们的IC_{50}分别为初始浓度的0.37%, 0.26%, 0.62%和0.19%。在研究与天然产物制品TA-07-005细胞毒性有关的细胞色素P450亚型酶中, 发现CYP2C9、CYP2A6、CYP2C19和CYP3A4活性被抑制后能减轻TA-07-005对肝细胞的毒性, 说明TA-07-005被CYP450生物活化后毒性增加。**结论** 用人原代肝细胞模型为基础的替代方法研究细胞毒性及代谢性相互作用对细胞毒性的影响是可行的。

[关键词] 替代方法; 天然产物制品; 细胞色素P450; 细胞毒性; 生物活化; 人原代肝细胞