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Expression and Enzymatic Analysis of the Human Cytochrome CYP1A2 in *Saccharomyces cerevisiae*

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To develop a broadly applicable assay system for studying human CYP1A2, we cloned the cDNA of *CYP1A2*1A* (wild-type) into pYES2/CT vector for galactose-inducible expression in budding yeast *Saccharomyces cerevisiae*, which was already integrated with CYP450 Oxidoreductase (POR) gene. Transformed yeast produced large quantities of microsome-bound CYP1A2*1A enzymes as determined by western blotting analysis; a 55 kDa protein was detected. The isolated S9 microsomes were collected to measure the kinetic constants of CYP1A2*1A enzymes in real-time assays using a fluormetric substrate CEC. It showed that the recombinant CYP1A2*1A enzyme possess evident activity, the Km value was about 7ìmol/L; we tested the inhibition of the recombinant *CYP1A2*1A* by a known inhibitor 7, 8-benzoflavon in the fluorescence assays; then we chose the specific inhibitors of other CYPs (2D6/3A4/2C8/2C9/2C19) to do the comparative trials, the results indicated the IC50 of 7, 8-benzoflavon is far lower than other inhibitors. Our study validated the feasibility of the established in vitro CYP1A2 detection system, which can guide the further study of high-throughput drug screening and drug-drug interaction.

Key words: CYP1A2*1A (wild-type), Enzyme Kinetics, Fluorescence Assay, Inhibition Assay, 3-Cyano-7-ethoxycoumarin (CEC), 7, 8-benzoflavon.

Cytochrome P450, also known as mixed function oxidase, single-dioxygenase or drug-metabolizing enzymes. Because there was a maximum absorption peak at the wavelength of 450 nm in reduction state in conjunction with CO, it was named P450 enzymes[1]. Cytochrome P450 mainly distributed in the endoplasmic reticulum and the mitochondrial inner membrane in eukaryotic cells[2]. Dioxygenase enzymes involved in a variety of reactions in vivo: metabolism of drugs and exogenous compounds, cholesterol, steroids, vitamin D3, the metabolism and synthesis of retinoic acid hydroxylation and other unknown roles[3]. Human cytochrome P450 superfamily consists of 57 genes, CYP1A2 belongs to the CYP1A subfamily of the CYP1 family, it was found primarily in the liver, accounted for 15% of total hepatic CYP enzymes; only second to CYP3A and CYP2C[4].CYP1A2 as a member of the cytochrome P450 superfamily involved in many of the former metabolic activation of carcinogens, including: aromatic compounds, heterocyclic amines, aromatic nitro compound, and mycotoxins. CYP1A2 metabolism of a variety of drugs, such as: caffeine, warfarin, theophylline, propranolol, mexiletine, verapamil, nifedipine, tacrine and other CYP1A2 is also responsible for a number of endogenous hydroxylation of sex hormones. Studies have found that the CYP1A2 activity is very different between the individuals, the factors for these differences may be involved in gender, race, genetic polymorphism and inducer, the genetic polymorphism of CYP1A2 may affect individual susceptibility to cancer individual difference of the

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effects of drug treatment. *CYP1A2*1A* is the wild-type allele of CYP1A2 for all SNPs, Individuals who are homozygous for the *CYP1A2*1A* allele are "rapid" caffeine metabolizers, whereas carriers of the variant *CYP1A2*1F* are "slow" caffeine metabolizers[5].

Flavonoids are a diet containing the chemical composition of plants, widely present in vegetables, nuts, fruits and herbal medicine that can induce or inhibit the activity of cytochrome P450. 7, 8- benzoflavon with a hydrophobic substituent has a high affinity to CYP1A2, which is CYP1A2 inhibitor[6]. Therefore, the study of 7, 8-benzoflavon inhibition to CYP1A2 has a positive meaning to mechanism of action of flavonoids in vivo.

We constructed *Saccharomyces cerevisiae* expression system for human CYP1A2, conducted fluorescence enzyme kinetic analysis and drugs inhibition reaction, initially established CYP1A2 enzyme detection system in vitro could lay the foundation for high-throughput drug screening platform, and to predict the relationship between CYP1A2 and precarcinogens, and to provide a model in vitro for the study of the carcinogenicity of certain chemicals. At the same time, the forecast of the role of CYP1A2 in drug metabolism, which provide a theoretical basis for clinical rational drug use and reduce adverse reactions, but also can promote the research and development of new drugs.

Materials and equipment

Strains and plasmids

E. coli (*Escherichia coli*) TOP10 F 'was purchased from Tiangen; Saccharomyces cerevisiae strain yCY3 (Saccharomyces cerevisiaeBJ5628) were purchased from ATCC, then integration POR gene which is oxidoreductase of CYP450, named yCY107; shuttle plasmid vector pYES2/CT purchased from Invitrogen .

Reagents

The primers used were synthesized by Beijing Bioko; DNA polymerase 2 × Taq Mastermix plus share in the days of Time Inc.; DNA restriction cut of enzyme Xho I Kpn I share in TaKaRa; T4 DNA connection enzymes purchased at Promega; gel extraction reagents kit and plasmid extraction kit was purchased from Shanghai Hua Shunsheng Biological Engineering Co., Ltd.; enzyme reaction product purification kit from Qiagen; yeast extract, tryptone were purchased from Oxoid Company; Yeast nitrogen base (YNB), (D) galactose were purchased from Amresco Company; (D) glucose were purchased from Sigma; DNA molecular weight standards and pre-stained protein molecular weight standards were purchased from TaKaRa and NEB; the PVDF membrane by OSMONICS Inc. production ; anti-V5 monoclonal antibody was purchased from Invitrogen; HRP-conjugated goat anti-mouse IgG was purchased from Beijing Ding Guo; the chemiluminescence SuperSignal ® West Femto Trial Kit purchased at Pierce; the Bradford Reagent Share Sigama; glucose 6 - phosphate, 6 - phosphate dehydrogenase, NADP +, fluorogenic substrate CEC (3-cynao-7-ethoxycoumarin) were purchased from BD Biosciences; 6 - phosphate, glucose 6 - phosphate dehydrogenase, NADP +, fluorogenic substrate CEC (3-cynao-7-ethoxycoumarin) were purchased from BD Biosciences; the 7,8-benzoflavon purchased from Sigma.

Medium and buffers

LB medium [7]; YPD medium; Yeast uracil auxotrophic growth medium (SD Uracil-medium); Yeast uracil auxotrophic induction medium (SG Uracil-medium); pyrolysis buffer; 2 × SDS gel sample buffer; microsomal buffer.

The main instrument

Tgradient 96-PCR amplification was purchased from Biometra; the RS232C-type nucleic acid protein detector was purchased from Eppendorf; ultrasonic cell grinder JY92-a! was purchased from Bio-Technology Co., Ltd. of Ningbo Chi; continuous spectrum fluorescent meter Gemini XPS / EM share in Molecular Devices; 3310 double-beam UV spectrophotometer purchased from Hitachi; black flat-bottomed 96-well plates were purchased from Costar.

Experimental methods

Construction and identification of pYES2/CT-CYP1A2 recombinant expression vector

CYP1A2-WT gene amplification

Referring to the Genbank whole genome sequence of CYP1A2, the Poligonucleotide primers (named WP1/WP2) were designed. in the 5 'ends of primer, restriction enzyme sites of Kpn`!and Xho I were added, respectively. The reaction conditions as follows: 94 ° C hot start for 5 min; 94 ° C denaturation for 30 s, 60 ° C annealing for 30 s, 72

° C extension for 1 min, 30 cycles; Last 72 ° C extension for 10 min, 4 ° C preservation.

Construction and identification of recombinant expression vector of pYES2/CT-CYP1A2

The PCR products of CYP1A2-WT cDNA template and plasmid vector pYES2/CT were conducted double digestion using KPN I, Xho I after gel purified, digestion products were purified and mixed with T4 DNA ligase overnight under 4 ° C, ligation products were transformed to TOP10 F 'competent cells, coated on LB plates containing ampicillin to select the positive clones. Plasmids were extracted, identified by PCR electrophoresis recombinants, and then send the bacilli to the Beijing AUGCT company sequencing verify.

Construction, expression and identification of the recombinant yeast strains

Preparation of yeast competent cells

The monoclonal of Saccharomyces cerevisiae strain yCY107 inoculated in 100 mL of YPD medium at 28 ° C, 280rpm cultured overnight, OD600 value 0.5-1.0, 3000 rpm centrifugation for 5 min and the supernatant was discarded, cells were washed with the right amount of pre-cooling sterile water twice, collected by centrifugation, washed with 1M sorbitol solution 5mL pre-cooling again, resuspended pre-cooling and then 500μL 1M in sorbitol solution, prepared competent cells stored in the ice.

Construction of the recombinant yeast strains

500ng recombinant plasmid pYES2/CT-CYP1A2 was electroporated into integrated the POR gene of Saccharomyces cerevisiae strain, electric conversion conditions: voltage 200V capacitor 25μF, resistance 200Ù electricity into cup diameter of 2 mm. Take 200μL conversion broth SD Uracil-plate filter, 28 ° C inverted culture 2d, observe the conversion.

Expression of the recombinant yeast strains

6 monoclonal colonies were picked from the transformation plates in 2mL SD Uracil-medium, 28 ° C, 280 rpm oscillation grown to OD600 = 1.5 ~ 2.0, sterile water to wash the cells twice the amount of bacteria connected to the 6mL SG Uracil-the culture medium, the starting OD600 = 0.2 ~ 0.5,28 !, 280rpm oscillation to an OD600 of 1.8-2.0, 4 ° C, and the 5000 rpm collected by centrifugation.

Western blotting identification of the target protein expression

Per 1.5mL centrifuge tube added 200μL 2 ×Pyrolysis buffer, then resuspended by adding an equal volume diameter of 0.5 mm glass beads, severe Vortex 1min, ice bath for 1 min, for a total of 20 times. 4 ° C, 1000g centrifugation for 10 min, the supernatant was collected. 10μL was taken for SDS-PAGE, transferred to PVDF membrane, anti-V5 antibody monoclonal was used as the first antibody, HRP-labeled goat anti-mouse IgG as the second antibody, western blot hybridization was conducted, chemical luminescence chromogenic, developing film exposure until clear bands show.

Microsomal enzymatic analysis

Preparation of yeast microsomes

Moderate amounts of microsomal buffer resuspend the yeast cells by adding an equal volume of glass beads of 0.5mm diameter, the yeast cells lysis by Bead Beater. Cracking conditions: 110V/400W and role 30s, intermittent 2min, repeated 15 times, the ice bath was maintained. 4 ° C, 1000g centrifugation for 10 min, the supernatant (S9 portion), 4 ° C, 10000g centrifugation for 50min, the supernatant was discarded. With Moderate amounts of microsomal buffer resuspend and percussion precipitation suspension, 0.5 mL centrifugal tube aliquots and stored at -80 ° C.

Determinations of microsomal protein concentration

Bradford assay was used to determine microsomal protein content, bovine serum albumin (BSA) was adopted to construct standard curve, microsomal buffer diluted BSA to the following gradient: 0/0.1/0.2/0.4/0.6/0.8/1.0mg/mL, diluted BSA 5μL joined 250μL Bradford reagents, after reaction for 5min in the dark at room temperature, A595 values measured with a UV spectrophotometer at 595nm wavelength. linear regression equation obtained bovine serum albumin. In another test, 5μL dilution microsomal suspension (1:5 / 1:10 / 1:20 / 1:40) added to 250μL Bradford reagents for determination of the concentration, measured A595 and calculated the average, according to the linear regression equation, protein content of the sample was obtained.

To establish CHC standard curve

the appropriate the CHC concentration range was selected, 96-well plates in the continuous spectrum fluorescence spectrometer, excitation light of 409 nm and emission at 460nm light to read the fluorescence absorption value. Concentration

gradient was calculated corresponding to the average value of the fluorescence absorption result. According to the standard curve, the RFU values transformed into concentration units of CHC

Enzyme activity detection

CYP1A2-specific fluorescent substrates CEC prepared for 1A2-WT microsomal enzyme activity detected. Activity assay methods consulted M. Teresa Donato, et al [8]. the reaction system of per hole as follows: 20μL PB (potassium phosphate buffer, pH 7.4), 5μL 20 x co-factor, 1μL 100 G6PDH substrate CEC (final concentration of 20μM) and 10μL 10 × microsome solution with deionized water to top up to 100μL . The substrate was added to premix, 37 ° C incubated for 10 min in a fluorescence spectrometer in a continuous spectrum, and then add to 10μL for microsomal to the first hole-fold dilution, the final concentration gradient: 2/1/0.5/0.25 / 0.125 mg, and set up empty plasmid pYES2/CT as a negative control. Excitation wavelength 409nm, emission wavelength 460nm does Kinetics detecting fluorescence signals 60min. Each experiment is repeated at least three times, with at least two batches of induction of microsomal protein was prepared the same detection.

Enzyme kinetics analysis

The final concentration gradient of substrate CEC as follows: 160/80/40/20/10/5/2.5/ 1.25μM, final concentration for microsomal 1mg/ mL. The experimental conditions are the same as the enzyme activity detection. Each experiment is repeated at least three times, with at least two batches of microsomal protein was prepared the same detection. The experimental data was collected and processed with fluorescence values of the experimental group. Kinetic parameters Km and Vmax values obtained by nonlinear regression using GraphPad software (Prism version 4.03, Graphpad Software, Inc. San Diego, CA).

Inhibition experiment of recombinant human CYP1A2 wild-type [9]

7, 8-benzoflavon is a known CYP1A2 inhibitor, a low concentration of 7; 8-benzoflavon can produce a strong inhibitory effect of CYP1A2. According to the FDA guidance document[8], Ki value of 7, 8-benzoflavon to CYP1A2 is 0.01, so the concentration range set as (8.5E-05 ~ 5μM), the concentration of substrate CEC set as 10μM microsomal concentration of 1mg/mL.The positive control (no inhibitor) and negative control (no inhibitor / reaction terminated after adding the substrate and microsomes) were set in the assay. The gradient dilution premix (including 20μL 1mol / L potassium phosphate buffer solution pH 7.4, 10μL co-factor, Two microliters 40U/mL 6 - glucose phosphate dehydrogenase, deionized water filled) was added into the hole and incubated for 15min, then the mixture of enzyme and substrate 100μL was mixed. Reaction conducted for 20min and the termination buffer was added to stop the reaction. Values were read by a continuous spectrum fluorescence analyzer. The collected data were analyzed and IC50 was calculated by a method, referring to Crespi CL[11].

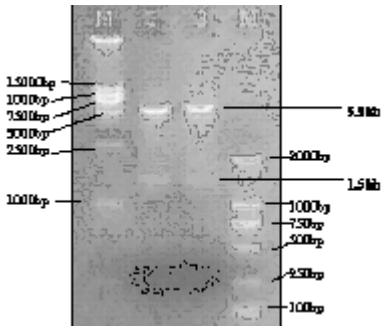
Specific experiment of inhibition

The specific inhibitor (Quinidine / Ketoconazole / Quercetin / Sulfaphenazole / Tranylcypromine) of different CYPs (2D6, 3A4, 2C8, 2C9, 2C19) were used to compare the inhibition effect with 7, 8-benzoflavon. The inhibitor concentration range is set to (0.00217 ~ 128μM), and other reaction conditions mentioned above, the IC50 was calculated and the inhibitory effect of different inhibitors were assessed.

RESULTS

Construction and identification of pYES2/CT-CYP1A2 recombinant expression vector

The recombinant plasmid digested by *Kpn/* and *Xho/,* two fragment of about 5.9 kb and 1.5Kb were available, which in line with the expected results. Gene sequences of cDNA sequences was



M: DL15000/DL2000 marker; 2/3: the restricted fragment of pYES2/CT-CYP1A2

Fig.1 Restriction digestion identification of pYES2/ CT-*CYP1A2*1A* clone with *Kpn'!*and *Xho'!*

consistent with 1A2 * 1A (wild-type gene) by sequencing, but there is a difference of one base: 1365bp T> C, but its encoded amino acid did not change.

Western blotting of target protein expression

Western blotting results showed that six positive monoclonal had expressed target protein,

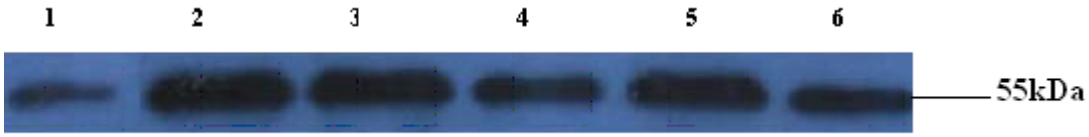


Fig. 2. Western-blotting analysis of CYP1A2*1A protein expression 1~6: The six yeast transformants of CYP1A2*1A;

a molecular weight of about 55 kDa, the same size as expected, and the expression levels between the different transformants varied modestly.

Microsomal enzymes activity assay

Fluorescent substrates CEC was used for the preparation of microsomal CYP1A2-WT by repeatedly activity detection, showed a more significant catalytic activity, within 60min, the RFU value with time linearly; provided microsomal concentration gradient range (2 / 1 / 0.5 / 0.25mg/ mL), analysis of the different concentrations and the RFU linear relationship shows that the microsomal concentration of 1mg/mL linear Preferably, the highest efficiency.

Enzyme kinetics analysis

After determine the enzymatic reaction conditions, the substrate concentrations were ranging from 1.25 to 160 microns. The samples incubated at 37 ° C for 60min in the continuous spectrum fluorescent microplate reader to record fluorescence data and draw the particulate enzyme kinetics curves of CYP1A2-WT. Application GraphPad software (Prism version 4.03, Graphpad Software, Inc. San Diego, CA) by nonlinear regression obtained kinetic parameters Km value (6.71 ± 1.53) micromol / L, Vmax value was (6.11 ± 1.06) RFU / min / mg.

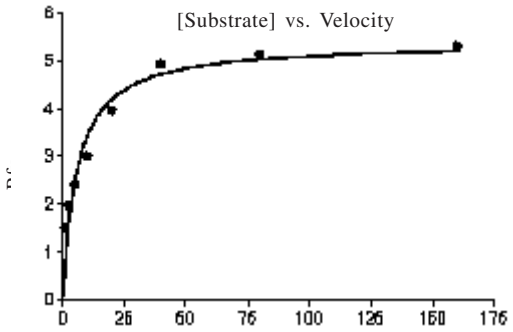


Fig. 3. Kinetic analysis of recombinant CYP1A2*1A protein

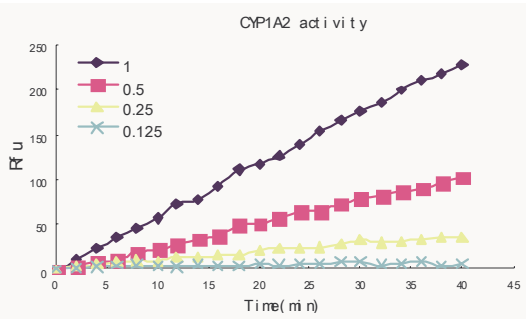


Fig. 4. Microsomal enzyme activity assayof recombinant CYP1A2*1A protein

Table 1. The type of the specific CYPs inhibitors and their IC50 values

CYPs	CYP2D6	CYP3A4	CYP2C8	CYP2C9	CYP2C19
Inhibitor	Quinidine	Ketoconazole	Quercetin	Sulfaphenazole	Tranylcypromine
IC50	>128μM	20.3μM	30.1μM	121.6μM	0.013μM

Drugs that inhibition assay

7, 8-benzoflavon of wild strains of recombinant human CYP1A2 inhibition assay

Select the substrate CEC has a concentration of 10μM, the concentration of microsomal for 1mg/mL 7, 8-benzoflavon concentration range from 8.5E-05 to 5μM, experiments show that 7, 8-benzoflavon had a strong inhibitory effect on CYP1A2, calculated, IC50 is 0.07.

Specific inhibition assay

The specific inhibitor (Quinidine / Ketoconazole / Quercetin / Sulfaphenazole / Tranylcypromine) of different CYPs (2D6, 3A4, 2C8, 2C9, 2C19) were used to compare the inhibition effect with 7, 8-benzoflavon.The results show that, except tranylcypromine Tranylcypromine(2C19 inhibitor) has a strong inhibitory effect to microsomal 1A2-WT, other inhibitor of other CYPs is not obvious.

DISCUSSIONS

Heterogonous expression of the application of CYPs

In order to achieve a reasonable dosing to ensure maximum therapeutic effect of the drug and safety, we need to create a human drug metabolism model in vitro, In general, the model is divided into two types: one is the simulation of a complex system of drug metabolism process, such as human liver cells and human hepatocellular carcinoma cell cultures, the method used in vitro incubation liver microsomes incubation etc.; the other type is simulation of simple reaction system, such as heterologous expression of the purified enzyme from these organizations. The simple model is useful when a lot of steps and identification of drug metabolism enzymes involved. CYPs metabolic substrates vary widely, and each CYPs has its specific metabolic spectrum. In addition, the construction of the CYPs protein can also be used to study the nature of the enzyme, the action mechanism of the enzyme, and the enzyme structure-activity relationship. Many bioinformatics researchers used laboratory recombinant homologues CYPs metabolism data to speculate the specific sites and effect relationship of substrate role of CYPs. The data obtained from the model will be more accurate and reliable[12]. To the 1990s, with the rapid development of molecular biology, biochemistry, CYPs expressed in heterologous expression systems, has become the main methods and means to get a single pure enzyme[13].

Heterologous expression systems selection

Heterologous expression system can be divided into two categories: prokaryotic expression system and eukaryotic expression systems according receptor cells. Prokaryotic expression system generally do not contain introns, there is no post-transcriptional processing system does not recognize, and to cut off the intron, the lack of posttranslational processing system, can not be further modified protein translation processing. Eukaryotic expression system consists of three types: yeast expression systems, mammalian cell expression system, insect cell expression system. Mammalian cells, insect cell culture system technical requirements, the time is long and expensive, is the possibility of contamination of their own endogenous cytochrome P450 in mammalian cells, there is a fatal flaw. In this study the integration of a POR gene of Saccharomyces cerevisiae BJ5628 cells expressing CYP1A2-WT cDNA, because the yeast system has certain advantages compared to other expression systems. First, yeast expression systems have the the modification system after the transcription and translation of the eukaryotic expression system, of exogenous genes in certain post-translational processing capacity, harvest exogenous protein having a certain degree of folding processing and glycosylation; there is no possibility of contamination of the endogenous, only two cytochrome strain Saccharomyces cerevisiae, and the activity is very low[14]. Third, yeast growth cycle is short; the culture method is relatively simple.

Detection system selection

In recent years, the international development of techniques and methods for CYP450 detection metabolism, such as those radiolabeled metabolites measurement method based on the separation of the radiation[15], high performance liquid chromatography[16] and liquid chromatography / mass spectrometry analysis (LC / MS detection method)[17]. However, these complicated technology and expensive equipment does not apply to high-throughput drug screening. Recently, a number of research results showed that

the fluorescence-based detection system with high sensitivity, high throughput and simple operation, can be used in preliminary screening of new drugs on the P450 enzyme inhibition[18]. In this study, the enzyme kinetics analysis result of microsomal CYP1A2 wild-type (fluorogenic substrate CEC) was close to the Km values obtained with BD and NovaScreen published values.

Inhibitor selection

Flavonoids are widespread phytochemical substances which has a variety of biological activity. It can induce or inhibit the activity of a variety of cytochrome P450. According to FDA, the standard inhibitor of recombinant CYP1A2 is furafylline [10]. In this study, 7, 8-benzoflavon was taken as the inhibitor of CYP1A2 catalyzes substrate CEC transformed. The result showed that 7, 8-benzoflavon had strong inhibition effect to CYP1A2. The CYPs inhibitors comparative experimental results showed that, except tranylcypromine (2C19 inhibitor) had inhibited microsomal 1A2-WT; the inhibition effect of other CYPs was not obvious. It is considered that we can take the 7, 8-benzoflavon as a specific inhibitor in our *Saccharomyces cerevisiae* expression system and use it as a positive control in the subsequent drug screening experiments.

In summary, we used the yeast *Saccharomyces cerevisiae* expression system to obtain a stable active *CYP1A2*1A* (wild type) enzyme, which can be used for the study of drug metabolism. Our next experiment step, we will select different alleles of CYP1A2, complete expression and enzymatic analysis, and establish a platform for effective screening of drugs and compounds.

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