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293FT is a highly suitable mammalian cell line for the *in vitro* enzymatic activity analysis of typical P450 proteins

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Mammalian cells have been widely used for the *in vitro* evaluation of the functional effect of allelic variants of cytochrome P450 (CYP). The aim of this study was to determine the most suitable mammalian cell line for the *in vitro* drug metabolism analysis of CYP variants. Three reported cell lines (COS-7, HepG2, 293T) and one fast-growing variant of the 293 cell line 293FT were transfected with vectors expressing green fluorescent protein or typical variants of CYP2C9, CYP2C19 or CYP2D6 to investigate the protein expression levels and the catalytic activity of expressed CYP allelic variants. The transfected 293FT cells had the highest protein expression level and exhibited the highest enzymatic activity, while HepG2 cells showed the lowest activity among the four tested cell lines. Simultaneously, 293FT cells still maintained the similar relative enzymatic ratio among three typical CYP2C9 variants to that of the commonly used COS-7 cells. In addition, 293FT cells could also be used for the *in vitro* functional evaluation of two other typical P450 proteins, CYP2C19 and CYP2D6. Therefore, the 293FT cell line is more suitable for the *in vitro* enzymatic activity analysis of typical P450 proteins than any other reported mammalian cell lines.

1. Introduction

Cytochrome P450 proteins (CYPs) are the most important drug-metabolizing enzymes (DMEs), which are responsible for the metabolism of both endogenous and exogenous compounds in human body. To date, 29 different types of CYP enzymes have been confirmed to be involved in the drug metabolism in humans (Sim and Ingelman-Sundberg 2013), of which CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6 and CYP1A2 play major roles in drug metabolism and are responsible for more than 90% of drugs metabolized by CYP (Ingelman-Sundberg et al. 2007). Previous studies have shown that these molecules show genetic polymorphism and have a close association with the individual susceptibility for adverse drug reactions and drug response, among which three CYP2 family members, CYP2C9, CYP2C19 and CYP2D6, are particularly polymorphic and exert the most important clinical consequences (Cavallari et al. 2011; Johansson and Ingelman-Sundberg 2011; Zuo et al. 2012). To evaluate the functional effects of the allelic variants of CYP, particular for low-frequency alleles, *in vitro* studies using cDNA expression systems have been proved to be the fast and efficient tools than analyzing the drug metabolism in patients with variant alleles of CYPs *in vivo* (Hiratsuka 2012).

Four different cDNA expression systems have been successfully used for the *in vitro* drug metabolism studies, including bacteria (Subramanian et al. 2012), yeasts (Neunzig et al. 2013), insect cells (Dai et al. 2013) and mammalian cells (Dai et al. 2014). Restricted by the posttranslational processing of expressed proteins in bacterial cells, cDNAs of CYPs must be modified prior to their introduction into the bacterial expression vector (Williams et al. 2003). In contrast, three other expression systems are based on eukaryote cells and can use the native cDNA for protein expression. Compared with yeast and insect cell expression systems, it is more relevant to assess the functional characteristics of human CYP variant proteins in mammalian cell systems. Mammalian cells usually have adequate endogenous NADPH-CYP oxidoreductase (OR) and cytochrome b_5 levels to support CYP activities and have been widely used for *in vitro* drug metabolism studies on CYP2C9, CYP2D6 and CYP2B6 allelic variants (Hiratsuka 2012). The African green monkey kidney-derived cell lines, COS-1 or COS-7, are the most commonly used mammalian cell lines due to their specific features, such as ease of culturing, high transfection efficiency and high protein expression etc (Hiratsuka 2012). In addition, another kidney-derived cell line HEK293T and a human hepatocellular carcinoma-derived cell line HepG2 were also reported for the expression of recombinant CYPs (Herman et al. 2007; Rettie et al. 1994). However, a comparison of these three different types of mammalian cell lines has not been reported to date. In this study, we compared the protein expression levels in these three typical mammalian cell lines and one fast-growing variant of the 293 cell line 293FT and investigated their applicability for the *in vitro* functional analysis of allelic variants of cytochrome P450.

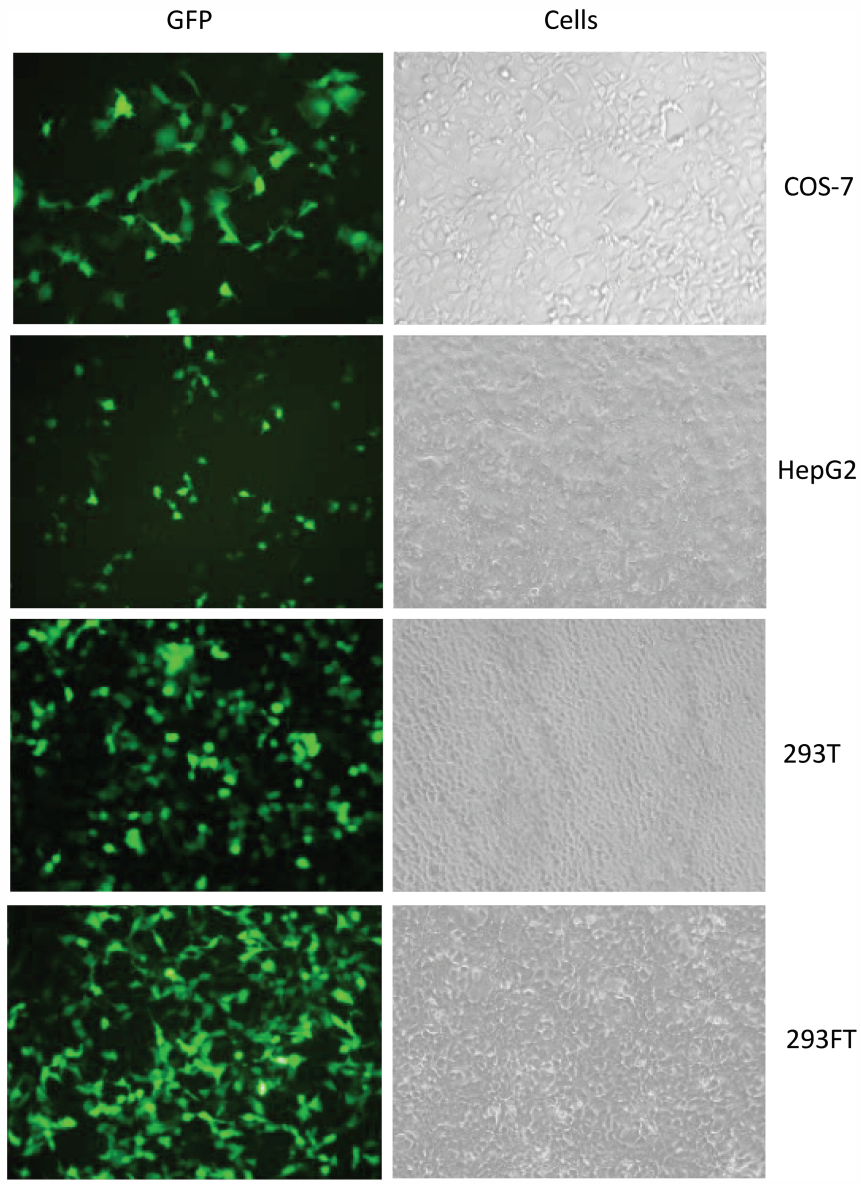

Fig. 1: 293FT cells have higher transfection efficiencies compared to other cell lines. A GFP-expression plasmid pEGFP-C1 was introduced into COS-7, HepG2, 293T and 293FT cells respectively to monitor the external gene expression efficiency of each cell line. Twenty-four hours after transfection, the signals were visualized using an inverted fluorescence microscope.

2. Investigations and results

2.1. 293FT cell exhibits highest capacity for exogenous gene expression

To date, COS-7 is the most widely used mammalian cell line for *in vitro* CYPs enzymatic activity analysis (Hiratsuka 2012). HepG2 and 293T cells have also been reported for the *in vitro* catalytic ability analysis of CYP2C9 protein (Herman et al. 2007; Rettie et al. 1994). In this study, these three cell lines and one HEK 293 cell variant 293FT cell line were transfected with plasmid expressing green fluorescent protein (GFP) to visualize the exogenous protein expression level. As illustrated in Fig. 1, HepG2 cells and 293T cells demonstrated the lowest and highest GFP expression levels among the three reported cell lines, respectively, whereas 293FT cells showed the highest signals in all of the tested cell lines. These results indicated that more exogenous proteins can be detected in transfected 293FT cells than in any other previously reported cell lines.

2.2. 293FT cell is suitable for catalytic activity assessment of CYP2C9 variants in vitro

To explore the efficacy and applicability of 293FT cells in the *in vitro* assessment of CYP proteins, cDNAs coding for wild-type CYP2C9*1 or two commonly defective CYP2C9 variants, CYP2C9*2 and CYP2C9*3, were transfected into above 4 different mammalian cell lines. As demonstrated in Fig. 2A, 293FT cells showed the highest CYP2C9 expression level in all tested cell lines. Furthermore, CYP2C9-transfected 293FT cells exhibited the strongest catalytic activity for the probing substrate diclofenac (Figs. 2B and 2C). When CYP2C9*1 was expressed in cells, 293FT cells exhibited an approximately 2.5-fold higher metabolic ratio value compared to that of COS-7 cells. Moreover, the relative ratio for the catalytic activity of three common CYP2C9 variants (*1,*2 and *3) was still similar between 293FT cells and COS-7 cells. These data indicated that 293FT cells have the highest exogenous protein expression capacity while still maintain the relative enzymatic ratio

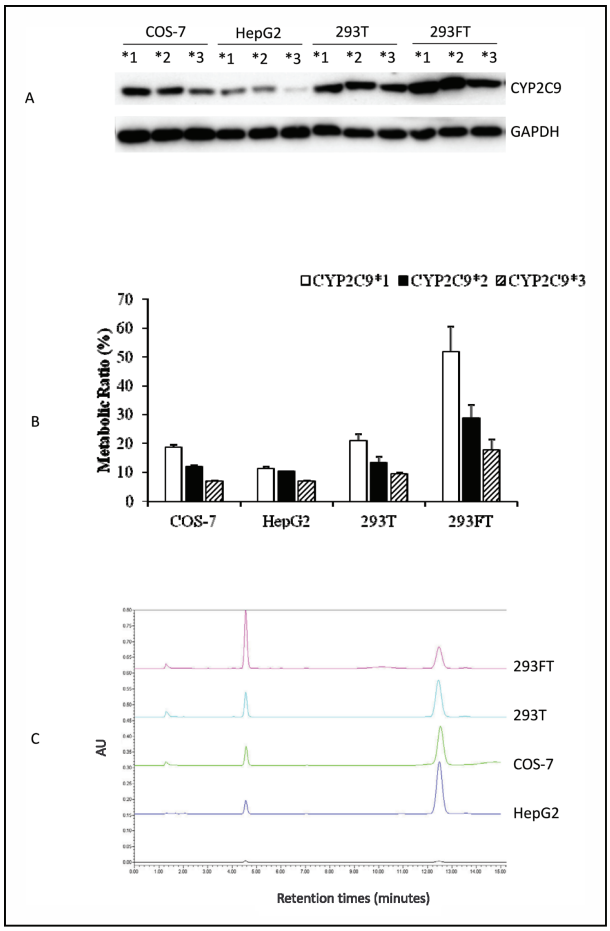

Fig. 2: CYP2C9-transfected 293FT cells exhibit higher catalytic activity than that of other cell lines. A: cDNAs coding for wild-type (*1) and two commonly defective CYP2C9 variants (*2, *3) were introduced into 4 different mammalian cell lines. The protein expression level was detected using western blotting analysis of recombinant CYP2C9 proteins. B: The catalytic activity of expressed wild-type CYP2C9*1 and two defective variants CYP2C9*2 and CYP2C9*3 were analyzed using the typical CYP2C9 probe substrate diclofenac. Values were calculated as described in the materials and methods section and the data were presented as the mean ± SD (n = 3). C: The chromatographic results of the drug metabolic ability of the expressed CYP2C9 wild-type protein toward the CYP2C9-specific substrate diclofenac, in which the retention time for hydroxylated diclofenac and diclofenac was 4.6 and 12.5 min, respectively.

when different types of CYP2C9 variants are introduced into cells.

2.3. 293FT cell is suitable for in vitro enzymatic activity assessment of other CYP proteins

To confirm whether 293FT cells could be used for the *in vitro* catalytic activity assessment of other CYP proteins, common CYP2C19 and CYP2D6 variants were also expressed and analyzed in this cell line. As shown in Fig. 3, these two typical CYP proteins could be highly expressed in 293FT cells and successfully metabolize the probing substrate (*S*) mephenytoin or bufuralol to its corresponding hydroxylated metabolite. In addition, the relative enzymatic activities of the typical CYP2C19 and CYP2D6 variants were in good accordance with previously reported results (Sakuyama et al. 2008; Wennerholm et al. 2001).

3. Discussion

To assess the functional characterization of the variant alleles, especially for low-frequency alleles, on CYP activity *in vitro*, several studies have used various heterologous expression systems, including bacteria, yeasts, insect cells and mammalian cells (Hiratsuka 2012). Compared with other expression systems, mammalian cells have adequate endogenous OR and cytochrome b_5 to support CYP activities and are much closer to the inherent environment of human proteins; thus it is much more relevant to assess the potential effects of recombinant human CYP variant proteins in the mammalian cell system.

The African green monkey kidney-derived cell lines, COS-1 or COS-7, are the most widely used mammalian cell, due to its simplicity for culturing and the fact that cDNAs do not need to be modified before they are inserted into the expression vectors. They have been successfully used for the *in vitro* functional analysis of CYP proteins by expression of different CYP2C9 (Guo et al. 2005a, 2005b; Niinuma et al. 2013), CYP2D6 (Gaedigk et al. 2002; Marcucci et al. 2002; Sakuyama et al. 2008), CYP2B6 (Crane et al. 2012; Honda et al. 2011; Watanabe et al. 2010), CYP2E1 (Bansal et al. 2013; Hanioka et al. 2003), CYP1A2 and CYP3A4 (Takeda et al. 2005). Another kidney-derived cell line HEK293T and the human hepatocellular carcinoma-derived cell line HepG2 cells were also reported to be used for the *in vitro* functional assessment of CYP2C9 variants (Herman et al. 2007; Rettie et al. 1994). However, no reports have been published regarding the comparison of the protein expression levels among these three cell lines. In this study, we found that 293T cells exhibited the highest protein expression ability for manually introduced genes (Fig. 1).

Similar to 293T, 293FT is also a human embryonic kidney cell line and is commonly used for the production of lentiviral or retrovirus stocks (Hua et al. 2013; Ye et al. 2013). It is a fast-growing variant of 293 cell line and stably expresses the SV40 large T antigen and E1A adenovirus gene. According to the description of the manufacturer, 293FT cells can produce higher levels of protein than other 293 cell lines and the COS cell lines which also stably express the T antigen. Our results also confirmed that higher levels of protein could be detected in 293FT cells compared to 293T cells, COS-7 cells or HepG2 cells (Figs. 1, 2A). Recently, an increasing number of articles have been published about the use of 293FT cells to obtain high levels of expressed protein (Hsu et al. 2013; Qian et al. 2013b), but no one used 293FT cells as the mammalian cell expression platform for CYP protein enzymatic ability analysis *in vitro*. Here, our results indicated that 293FT cells produced the highest expression level of exogenous CYP2C9 protein in all tested cell lines, while still maintained the relative ratio for the catalytic activity of both typical CYP2C9 variants*2 and *3 to wild-type CYP2C9*1 (Fig. 2). In addition, due to the higher protein expression capacity, transfected 293FT cells could be used directly for *in vitro* functional analysis in twenty-four hours later after transfection and exhibited similar enzymatic ability to that of COS-7 cells being transfected after forty-eight hours (Dai et al. 2014).

To investigate whether 293FT cells are also suitable for the *in vitro* functional analysis of other CYP proteins, typical CYP2C19 and CYP2D6 variants were also expressed in these cells to analyze their catalytic activity toward the corresponding probing substrates. As shown in Fig. 3, both of these two CYP wild-type proteins could be highly expressed in 293FT cells and exhibited high enzymatic activities *in vitro*. To date, no reports have been documented using COS or other mammalian cells to study the potential effects of CYP2C19 variants, although yeast or *E. coli* have been successfully used. Hiratsuka (2012) hypothesized that expressing recombinant CYP2C19 protein in mammalian cells might be difficult due to the short half-life of human CYP2C19 protein. In this study, we demonstrated that recombinant CYP2C19 protein could be highly expressed in 293FT cells similar to other typical polymorphic CYP proteins, such as CYP2C9 and CYP2D6. *CYP2C19*3* is one prevalent defective allele observed in Asian subjects with a guanine to

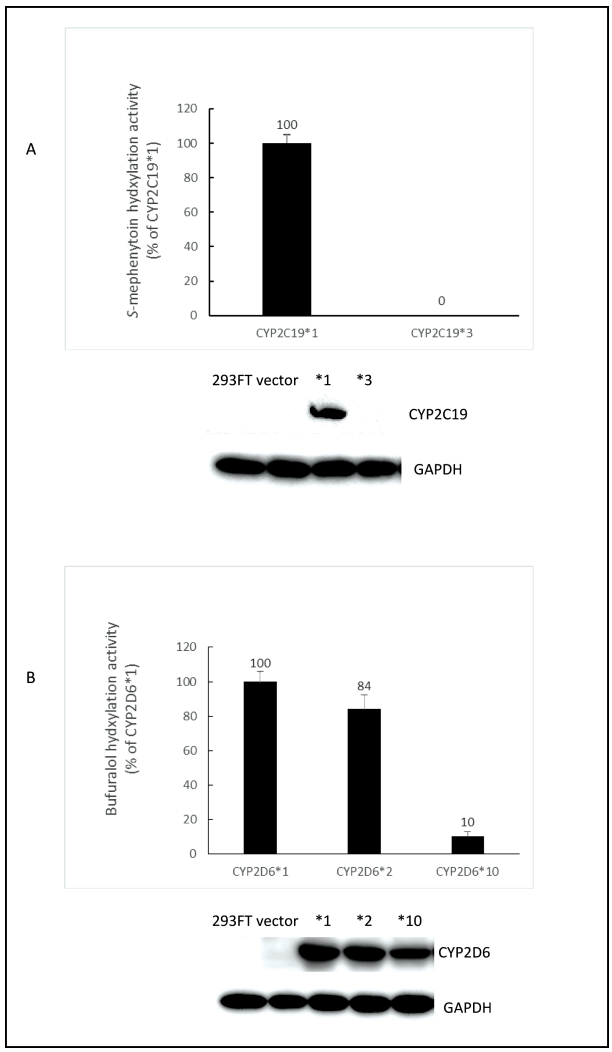

Fig. 3: 293FT cells can be used to analyze the *in vitro* enzymatic ability of two other important P450 proteins. The catalytic activities of expressed CYP2C19 (A) or CYP2D6 (B) variants toward the probe substrates (*S*)-mephenytoin or bufuralol were calculated as the percentage of the wild-type protein. The bottom panel shows the expression level of CYP proteins using the western blotting analysis.

adenine mutation at position 636 of exon 4 in CYP2C19, which results in a premature stop codon and produces a truncated and inactive protein (De Morais et al. 1994; Hu et al. 2012). Our data showed that the introduced *CYP2C19*3* cDNA could not express detectable full-length CYP2C19 protein in 293FT cells and exhibited no catalytic activity toward the probe substrate (*S*)-mephenytoin *in vitro* (Fig. 3). CYP2D6*2 and CYP2D6*10 are the most common defective alleles in Asian populations and occur with a frequency of 10% and 43%, respectively, in the Chinese population (Qian et al. 2013a). When expressed in COS cells, the allelic variant CYP2D6*2 exhibited slightly decreased catalytic activity toward the probe substrate bufuralol, while the variant CYP2D6*10 exhibited extremely low enzymatic activity compared with that of wild-type CYP2D6*1 (Sakuyama et al. 2008; Wennerholm et al. 2001). As shown in Fig. 3, our results were consistent with that of previous studies. Taken together, our data inferred that 293FT cells may also be used for *in vitro* drug metabolism analysis of other P450 proteins besides CYP2C9.

In summary, we compared four different mammalian cell lines for their protein expression capacity and investigated their applicability for the *in vitro* assessment of the potential effects of allelic variants of cytochrome P450. Our data indicated that 293FT can express the highest level of CYP proteins, while still maintain the relative enzymatic ratio of typical CYP allelic isoforms toward CYP specific substrates *in vitro*; thus, it is a more suitable mammalian cell line for the *in vitro* enzymatic activity analysis of P450 proteins than any other reported mammalian cell line.

4. Experimental

4.1. Chemicals and materials

COS-7 and 293T cell lines were purchased from ATCC (Manassas, VA, USA), and the 293FT cell line was obtained from Invitrogen (Carlsbad, CA, USA). The HepG2 cell line was donated by Dr. Guo-Ping Li from Beijing Institute of Geriatrics. Diclofenac was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 4-Hydroxydiclofenac, (*S*) mephenytoin, 4-hydroxymephenytoin, bufuralol and 1-hydroxybufuralol were obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). PrimeSTAR GXL DNA Polymerase, restriction enzymes and the DNA ligation kit were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). PEGFP-C1 and pIRES vectors were purchased from Clontech (Mountain View, CA, USA). Rabbit polyclonal anti-CYP2C9 and anti-CYP2D6 antibodies were both purchased from AbD serotec (Kidlington, Oxford, UK). Rabbit monoclonal anti-CYP2C19 antibody was purchased from Epitomics (Burlingame, CA, USA). Rabbit monoclonal anti-GAPDH antibody was obtained from Abmart (Shanghai, China). The Super Signal West Pico Trial kit was obtained from Thermo (Rockford, IL, USA). High-pressure liquid chromatography-grade solvents were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey, USA). All other chemicals and solvents that were used in this study were of the highest grade or analytical grade that was commercially available.

4.2. Plasmid construction and plasmid transfection

To express CYP proteins in mammalian cells, dual-expression plasmid pIRES was used to express the internal control protein Gluc and target the CYP protein simultaneously. The coding regions of Gluc and CYPs genes were amplified and inserted into the multiple cloning site B and site A as previously described (Dai et al. 2014). To ensure that no errors were introduced during PCR amplification, all of the cDNA regions in each of the vectors were sequenced using the CEQ DTCS Quick Start Kit on the CEQ 8000 Genetic Analysis System (Beckman&Coulter, Brea, CA, USA).

One day before transfection, approximately 3×10^6 cells/well were plated on a 6-well plate to allow the cells to grow to 80-90% confluency at the time of the transfection. On the next day, 4 μg of expression plasmid were transfected into each cell type using 10 μL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. To inspect the transfection efficiency, pEGFP-C1 vector was introduced into each cell lines and protein expression level was visualized with inverted fluorescence microscope XDS-500 (Nikon, Chiyoda-ku, Tokyo, Japan) 24 h after transfection.

4.3. Protein expression level quantification using Western blotting analyses

Twenty-four hours after transfection, the cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% NP-40, 0.01 mg/mL aprotinin and 1 mM phenylmethanesulfonyl fluoride. Next, 20 μg of total cellular lysates were applied to 12% SDS-PAGE and electro-transferred onto PVDF membranes. Anti-CYP2C9, CYP2C19, CYP2D6, or GAPDH antibody was used as the primary antibody with a dilution from 1:1000 to 1:5000. After incubation at 4 °C overnight, goat anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated secondary antibody was applied with a dilution of 1:2000 and incubated at room temperature for 1 h. The Super Signal West Pico Trial Kit was then used to visualize the blot signals.

4.4. Drug metabolic ability analysis using intact cells

Twenty-four hours after transfection with dual-expression plasmids in 6-well plates, the cells were digested with trypsogen and re-suspended in 300 μL DMEM culture medium supplemented with 10% FBS, 10,000 U/mL penicillin, 10,000 μg/mL streptomycin and 100 μmol/L diclofenac, 20 μmol/L (*S*)-mephenytoin or 10 μmol/L bufuralol. Then samples were suspension cultured on a shaking table at 300 rpm in a CO_2 incubator for 2 h, and the reactions were terminated using 1 mL ice-cold ethyl acetate with the addition of 40 μL 0.1% trifluoroaceticacid (TFA) or 20 μL 0.1 M NaOH. Followed by centrifugation at 10000 × g for 10 min at 4 °C, the supernatants were extracted, and the organic layer was dried under nitrogen. The dried samples were re-dissolved by the mobile phase and injected into the high-performance liquid chromatography system e2695 separation module (Waters, Milford, MA, USA).

Separation was performed on a ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm, Agilent, Santa Clara, CA, USA) according to the mobile phrase of (A) 0.1% trifluoroaceticacid (B) water and (C) acetonitrile (20:35:45, V/V) at a flow rate of 1.0 mL/min for diclofenac detection. The retention times of hydroxy diclofenac and diclofenac were 4.6 min and 12.5 min, respectively. For (*S*)-mephenytoin detection, the extracted samples were separated by gradient elution with the linear gradient as follows: 0 min to 15 min, 20% solvent A, 45% to 35% solvent B and 35% to 45% solvent C; 15 min to 22 min, 20% solvent A, 35% to 45% solvent B and 45% to 35% solvent C. The samples were monitored at 210 nm at 30 °C, and the retention times of 4-hydroxymephenytoin and (*S*)-mephenytoin were 8.5 min and 15.2 min, respectively. For bufuralol detection, the linear gradient mobile phase was the following: 0 min-10 min, 20% solvent A, 62% to 45% solvent B, 18% to 35% solvent C; 20% A, 45% B and 35% C maintained for 4 min; 14 min-15 min, 20% solvent A, 45% to 62% solvent B, 35% to 18% solvent C. The samples were monitored at 246 nm at 30 °C, and the retention times of hydroxybufuralol and bufuralol were 9.9 min and 13.3 min, respectively. The drug metabolic ability of each variant was calculated as previously described (Dai et al. 2014).

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