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Development of an optimized cytotoxicity assay system for CYP3A4-mediated metabolic activation via modified *piggyBac* transposition

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ABSTRACT

Drug-induced hepatotoxicity is often caused by cytochrome P450 (CYP)-dependent metabolism of drugs into reactive metabolites. Assessment of hepatotoxicity induced by bioactive compounds is hampered by low CYP expression within in vitro cell lines. To overcome this limitation, *piggyBac* transposition and monoclonal expansion were used to generate a HepG2 cell line with stable and homogenously high expression of CYP3A4, a prominent CYP isoform. Our studies demonstrate the generated line's constant CYP3A4 expression and activity for over 40 cell passages; to date, it has been in subculture for more than a year without addition of Puromycin. This cell line was utilized to evaluate cytotoxicity of two bioactive (troglitazone and acetaminophen) and two non-bioactive (citrate and galactosamine) compounds by MTT assay. Cell viability significantly decreased upon treatment with bioactive drugs. Moreover, cell lines used in the present study were more sensitive to toxic effects of troglitazone than previously reported. Therefore, this HepG2 cell-based assay system may provide a suitable hepatic model for predicting CYP3A4-mediated hepatotoxicity during preclinical drug development.

1. Introduction

Drug-induced liver injury is a major cause of post-market drug withdrawals. Many forms of drug-induced liver injury originate from intermediate reactive metabolites, some of which are produced by drug-metabolizing enzymes specific to humans (Eno and Cameron, 2015). Species-related differences in drug-metabolizing enzymes make it difficult to predict human hepatotoxicity using animal-based studies (Grillo, 2015). Therefore, it is essential to detect potential hepatotoxicity mediated by drug metabolism prior to animal safety studies and clinical trials.

Primary human hepatocytes are considered the "gold standard" for performing in vitro drug metabolism and hepatotoxicity studies. However, use of these cells is often unfeasible because of scarcity, phenotypic instability, significant variability between human hepatocytes from different donors and numerous other reasons (Bale et al., 2014). Recently, several attempts have been made to differentiate functional hepatocytes from embryonic stem cells or adult induced pluripotent stem cells (iPSCs). Although these cells may provide a stable source of hepatocytes in the future, current differentiation processes are inefficient and yield heterogeneous cell populations, which limit their routine use in hepatotoxicity assessments (Lu et al., 2015; Mann, 2015; Suter-Dick et al., 2015).

In recent years, metabolically competent human hepatocytes have been developed by transfecting hepatic cells with complementary DNA (cDNA) encoding for drug-metabolizing enzymes and other genes involved in drug metabolism (e.g., glutathione transferases) (Gomez-Lechon et al., 2014). Some of the most widely used lines are HepG2 transformants stably expressing individual CYPs. Yoshitomi et al. established a series of HepG2 cell lines that stably express single CYPs, including CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Yoshitomi et al., 2001). Bioactive compounds acetaminophen, cyclophosphamide and benz[a]anthracene proved significantly more cytotoxic in transgenic HepG2 cells than wild-type HepG2 cells. Other hepatotoxicity studies applied adenovirus-mediated transient expression of CYP enzymes to hepatic cells (Kwon et al., 2014; Lahoz et al., 2013; Tolosa et al., 2012, 2013). By transducing cells with individual or mixtures of recombinant adenovirus-encoding CYPs, a battery of hepatic cell lines overexpressing

Abbreviations: CYP, cytochrome P450; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; iPSCs, induced pluripotent stem cells; cDNA, complementary DNA; PB, piggyBac; PBase, piggyBac tranposase; BSO, L-buthionine-(S,R)-sulfoximine; CMV, cytomegalovirus promoter; RFP, red fluorescent protein; PURO, Puromycin; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum; qRT-PCR, quantitative real-time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBST, Tris–HCl buffered saline with 1‰ Tween; PBS, phosphate buffered saline; GSH, glutathione; DMSO, dimethyl sulphoxide.

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CYPs were established and used successfully for hepatotoxicity screening of bioactive drugs. Hepatic cell lines, manipulated for stable or transient expression of CYPs, provide sensitive assays systems for in vitro screening of metabolism-mediated toxicity. However, expression mediated by recombinant adenovirus is transient and new infections must be performed for each experiment. Further, heterogeneity in gene expression limits adenovirus-related strategies, as stability is one of the most important parameters to validate for any in vitro liver toxicity platform (Bale et al., 2014).

Traditionally, stable transgene expression has been accomplished with plasmids, retroviral or lentiviral vectors. However, plasmid- or viral vector-mediated transgene expression often occurs at low levels and decreases over time because of low copy numbers and/or chromatin remodeling repression. The *piggyBac* (PB) transposon has emerged as a promising non-viral vector system for efficient gene transfer into mammalian cells; it works through a "cut and paste" mechanism. The PB transposon system has two major components: a donor plasmid (or transfer vector), carrying the gene of interest flanked by two terminal repeat domains; and a helper plasmid, expressing PB transposase (PBase) to catalyze movement of transposons (Chen et al., 2015). Apart from distinct advantages over lentiviral and/or retroviral systems, including large cargo size, multiple copy integration and lack of a genetic footprint, the PB system also limits undesired genomic effects on human cells (Chen et al., 2015; Smith et al., 2015).

Thus, in the present study, we utilized a modified PB transposon to develop an optimized in vitro cell-based system containing a high level of sustained CYP3A4 expression. Moreover, this cell system was applied to investigate in vitro hepatotoxicity of drugs. Our results demonstrate the potential of this system to assess CYP3A4-mediated hepatotoxicity in response to new drugs and their active metabolites.

2. Materials and methods

2.1. Materials

Culture media and complements were Gibco® brand (Life Technologies, Carlsbad, CA, USA). Troglitazone and L-buthionine-(S,R)-sulfoximine (BSO) were acquired from Toronto Research Chemicals, Inc. (Canada). Acetaminophen, testosterone and galactosamine were obtained from Aladdin Industrial Corporation (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from Calbiochem (Merck Millipore, Germany). All other chemicals and solvents were of the highest grade commercially available.

2.2. Construction of plasmid PB–CYP3A4

The PB transposon system used in this study contained two vectors. First, a PB donor vector (System Biosciences, Mountain View, CA, USA) as shown in Fig. 1A was constructed using two expression cassettes. The first contained a multiple cloning site located downstream of the CMV promoter, allowing for cloning of human CYP3A4 cDNA, and a second further downstream, contained an EF1 alpha promoter to drive expression of red fluorescent protein (RFP) (mRuby) and Puromycin (PURO) genes to facilitate double selection in mammalian cells. Human CYP3A4 cDNA (GenBank ID: 1576) was amplified and then cloned into PB donor vector using restriction sites for Nhe I and Not I. Donor vector expression cassettes were flanked by genomic insulator elements and PB ITR sequences to promote stabilization, mobilization and integration, respectively. Second, a helper vector (System Biosciences) featuring an optimized Super PBase was used to support hyperactive transposon integration activity in mammalian cells (Fig. 1B).

Both plasmids for cell transfection were prepared using NucleoBond® Xtra Midi EF Kit (Macherey-Nagel, Germany) according to manufacturer instructions.

2.3. HepG2 cell line and culture conditions

HepG2 cell line was obtained from ATCC (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.4. Establishment of isogenic HepG2 cell lines stably expressing CYP3A4 by monoclonal expansion

GenJet™ In Vitro DNA Transfection Reagent (SignaGen Laboratories, Rockville, MD, USA) was used as the transfection reagent. One day before transfection, 0.3×10^6 HepG2 cells were seeded onto each well of a six-well plate. When cultures reached 70–80% confluence, cells were co-transfected with 1.5 μg PB–CYP3A4 plasmid and 0.5 μg helper vector plasmid expressing Super PBase. 48 h post-transfection, cells were suspended at a density of 1000 cells/ml and seeded into 10 cm dishes (20 cells per dish) containing fresh medium supplemented with 4.0 μg/ml Puromycin (Gibco). Culture media was changed every two days. Isolated, Puromycin-resistant and RFP-positive colonies were trypsinized and transferred to 24-well plates using cloning rings for monoclonal expansion. Colonies exhibiting high purity were selected for subsequent real-time PCR and enzyme activity analyses. Stability of CYP3A4 expression within generated cell clones was validated for individual cell populations.

2.5. Quantitative real-time PCR

Total RNA was extracted from cells using Takara MiniBEST Universal RNA Extraction Kit (Takara, Kusatsu, Japan) according to manufacturer protocols. RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm with a NanoVue™ Plus Spectrophotometer (GE Healthcare Life Science, Buckinghamshire, UK). For quantitative real-time PCR (qRT-PCR), cDNA was synthesized from 2 μg total RNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara). SYBR® Premix Ex Taq™ II (Takara) was used for qRT-PCR. PCR reactions were performed in a LightCycler®96

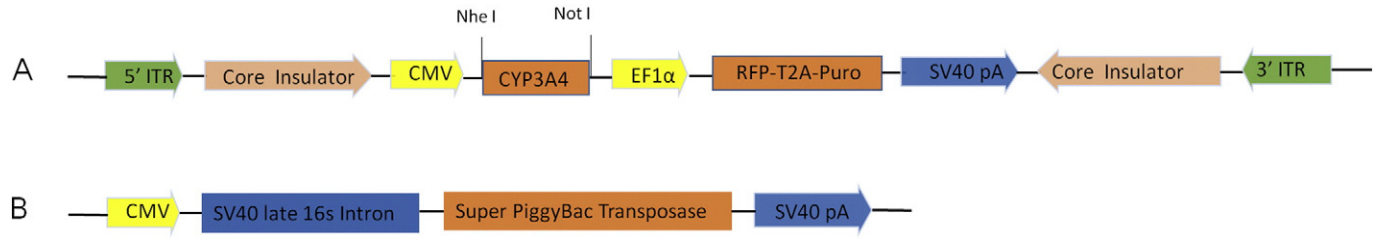

Fig. 1. Schematic diagram of the plasmids. A. Donor plasmid: PB–CYP3A4 contains two expression cassettes (CMV-CYP3A4 and EF1-RFP-Puro) flanked by genomic core insulator, PB 5-ITR sequences and 3-ITR sequences. CMV, cytomegalovirus promoter; EF1, human elongation factor-1 alpha promoter; T2A, insect virus *Thosea asigna* 2A sequences. B. Helper vector: *PiggyBac* (PB) transposase expression plasmid. The PB transposase expression vector features optimized Super *PiggyBac* Transposase to support hyperactive transposon integration activity in mammalian cells.