Development and Application of TK6-derived Cells Expressing Human Cytochrome P450s for Genotoxicity Testing

Xilin Li,^{*} Si Chen,[†] Xiaoqing Guo,^{*} Qiangen Wu,[†] Ji-Eun Seo,^{*} Lei Guo,[†] Mugimane G. Manjanatha,^{*} Tong Zhou,[‡] Kristine L. Witt,[§] and Nan Mei^{*,1}

^{*}Division of Genetic and Molecular Toxicology and [†]Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas 72079; [‡]Center for Veterinary Medicine, U.S. Food and Drug Administration, Rockville, Maryland 20855; and [§]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

¹To whom correspondence should be addressed at Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079. Fax: 870-543-7132. E-mail: nan.mei@fda.hhs.gov.

ABSTRACT

Metabolism plays a key role in chemical genotoxicity; however, most mammalian cells used for *in vitro* genotoxicity testing lack effective metabolizing enzymes. We recently developed a battery of TK6-derived cell lines that individually overexpress 1 of 8 cytochrome P450s (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, and 3A4) using a lentiviral expression system. The increased expression and metabolic function of each individual CYP in each established cell line were confirmed using real-time PCR, Western blotting, and mass spectrometry analysis; the parental TK6 cells and empty vector (EV) transduced cells had negligible CYP levels. Subsequently, we evaluated these cell lines using 2 prototypical polyaromatic hydrocarbon mutagens, 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (B[a]P), that require metabolic activation to exert their genotoxicity. DMBA-induced cytotoxicity, phosphorylation of histone H2A.X, and micronucleus formation were significantly increased in TK6 cells with CYP1A1, 1B1, 2B6, and 2C19 expression as compared with EV controls. B[a]P significantly increased cytotoxicity, DNA damage, and chromosomal damage in TK6 cells overexpressing CYP1A1 and 1B1 when compared with EV controls. B[a]P also induced micronucleus formation in TK6 cells expressing CYP1A2. These results suggest that our CYP-expressing TK6 cell system can be used to detect the genotoxicity of compounds requiring metabolic transformation.

Key words: TK6 cells; cytochrome P450; TK6-derived cell lines; biotransformation; DNA damage; chromosomal damage.

Chemical risk assessment is undergoing a transition from relying on animal testing to the use of alternative methods and strategies that reduce animal testing (EPA, 2019; Grimm, 2020). *In vitro* genotoxicity testing has been and will be continually playing a key role in identifying DNA reactive chemicals for hazard identification. Currently, the bacterial gene mutation (Ames) test, mouse lymphoma thymidine kinase (Tk) gene mutation assay, *in vitro* micronucleus (MN) test, and metaphase chromosomal aberration test are the short-term assays

recommended by international authorities such as the Organization for Economic Co-operation and Development (OECD), the International Council for Harmonization (ICH) for *in vitro* genotoxicity assessment, and the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) (ICH, 2011; OECD, 2015; VICH, 2014). According to an international survey on the use of mammalian cell lines, the mouse lymphoma L5178Y cells and human lymphoblastoid TK6 cells are the most

commonly used cell lines for genotoxicity testing (Lorge *et al.*, 2016). However, these model cell lines largely lack metabolic capabilities, and thus require a source of exogenous metabolic activation for use in genotoxicity screening. Because chemicals can be converted into DNA reactive metabolites or detoxified into nonreactive products *in vivo*, current guidelines, such as the ICH S2R1 (ICH, 2011), require the incorporation of metabolic activation in bacterial mutation assays and *in vitro* mammalian cell assays. Accordingly, the S9 fraction of livers from male Sprague Dawley rats treated with Aroclor 1254 has been routinely used in standard *in vitro* genotoxicity tests as a source of metabolic activation (Hashizume *et al.*, 2010).

However, use of rat liver S9 to provide metabolic capability has some widely recognized drawbacks. For example, some genotoxic metabolites generated by S9 in culture medium may not enter the cellular compartments, preventing them from accessing the nucleus to induce genetic damage (Kirkland *et al.*, 2007). Aroclor 1254 preferably induces cytochrome P450 (CYP) 1A and 2B enzymes in the rat liver, whereas expression levels of other CYPs such as CYP1B1 are not increased. Notably, a number of prototypical procarcinogens, including polyaromatic hydrocarbons (PAHs), need to be bioactivated by CYP1B1 to elicit genotoxicity (Kirkland *et al.*, 2016). Thus, the enzymatic profile of rat liver S9 fraction is not an adequate representation of human CYP profiles (Ku *et al.*, 2007). A workshop convened by the European Center for the Validation of Alternative Methods (ECVAM) concluded that deficiencies in metabolism (leading to the reliance on rat liver S9), along with a lack of p53 function and DNA repair capability in some commonly used cell lines, are key factors responsible for the low specificity seen in standard *in vitro* genotoxicity assays (Kirkland *et al.*, 2007). In 2016, several governmental agencies (including the U.S. Environmental Protection Agency, the National Institutes of Health's National Center for Advancing Translational Sciences, and the National Toxicology Program) initiated a "Transform Tox Testing Challenge" to stimulate the development of *in vitro* testing methods incorporating metabolic competence (especially using human enzymes) for high-throughput toxicity screening (EPA, 2016).

From the standpoint of metabolism, human primary hepatocytes are considered the "gold standard" because they generally retain the activity of major drug metabolism enzymes (Guo *et al.*, 2020). However, due to their limited availability, phenotypic instability, short life span, and interindividual variability, it is not feasible to use them in high-throughput genotoxicity tests. Although other immortalized hepatic cell lines such as HepG2 cells are readily available, they express low or negligible levels of drug metabolism enzymes (Westerink and Schoonen, 2007), limiting their application for screening compounds that require metabolic activation. In addition, some evidence suggests the use of HepG2 cells may result in a reduced sensitivity to genotoxins in the MN assay (Fowler *et al.*, 2014). Recently, some genetically modified HepG2 cell lines expressing human drug metabolizing genes have been developed and used for assessing drug metabolism-associated toxicity (Chen *et al.*, 2018; Wu *et al.*, 2016; Xuan *et al.*, 2016). Human embryonic kidney cells (HEK293T) have also been engineered to transiently express human CYPs via mRNA transfection (DeGroot *et al.*, 2018). However, their application may be restrained in high-throughput genotoxicity screening.

The human lymphoblastoid TK6 cell line was established in 1978 from the parent cell line WI-L2 that was derived from a 5-year-old male with hereditary spherocytosis (Lorge *et al.*, 2016). Due to TK heterozygosity on human chromosome 17 in these

cells as well as the presence of the hypoxanthine phosphoribosyltransferase (HPRT) gene on the X chromosome, TK6 cells have been widely used for TK and HPRT gene mutation assays. TK6 cells, which are grown in suspension, are also one of the most frequently used cell lines for the *in vitro* chromosome aberration, MN, and comet assays. Recent whole-genome sequencing indicated negligible variability between TK6 and the human reference genome (Revollo *et al.*, 2016). TK6 cells express wild-type (WT) p53 protein with a near diploid karyotype, and therefore they are more physiologically relevant than other commonly used cell models. These features make TK6 cells a useful cell model for *in vitro* genotoxicity testing (Kirkland *et al.*, 2007; Whitwell *et al.*, 2015). Results of genotoxicity tests performed in TK6 cells showed a better correlation with results obtained in primary human lymphocytes compared with rodent and HepG2 cell lines (Fowler *et al.*, 2014).

It has been demonstrated that human TK6 cells have negligible expression of the major CYPs responsible for metabolic transformation of chemicals. For example, the well-characterized genotoxicant, benzo[a]pyrene (B[a]P), failed to induce micronuclei, and HPRT mutations in TK6 cells because CYP1A1, 1B1, and 1A2 are not expressed (Shah *et al.*, 2016). In an effort to develop a p53- and metabolically competent cell model for screening genotoxic compounds, we created a set of TK6 cell lines that expresses 8 CYPs (1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, and 3A4) individually using a lentiviral expression system to identify the specific CYP isoforms responsible for the bioactivation of genotoxic chemicals of interest. We confirmed the mRNA and protein expression levels, and enzymatic activity of each CYP in the corresponding cell line. Because PAHs are one of the most well-studied procarcinogens that require biotransformation by CYPs (mainly CYP1A1 and 1B1) to exert their genotoxicity, we selected 2 representative PAHs, 7,12-dimethylbenz[a]anthracene (DMBA) and B[a]P and further tested them using our cell system.

MATERIALS AND METHODS

Test chemicals. B[a]P (> 96% purity), DMBA (> 95% purity), phenacetin, 7-ethoxy-4-(trifluoromethyl)coumarin, coumarin, bupropion, diclofenac, omeprazole, and midazolam were all purchased from Sigma-Aldrich (St. Louis, Missouri). The chemicals were stored at -20°C and protected from light.

Cell culture and treatment. Human lymphoblast TK6 cells were purchased from ATCC (Manassas, Virginia) and cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with L-glutamine (Gibco, Gaithersburg, Maryland), 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, Georgia), 100 U/ml penicillin (Gibco), and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco). Cells were routinely maintained in a density of 2×10^5 to 1.5×10^6 cells/ml and the number of passages did not exceed 10. Unless otherwise specified, cells at the density of $2 \times 10^5/\text{ml}$ were treated with different concentrations of DMBA and B[a]P dissolved in dimethyl sulfoxide (DMSO) for 24 h. The final concentration of DMSO did not exceed 1%.

The 293T cell line required for lentiviral packaging was obtained from Biossetia, Inc (San Diego, California) and propagated in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acids. All cells were grown at 37°C in a humidified atmosphere with 5% CO_2 .

Construction of CYP-expressing TK6 cell lines. A total of 8 human CYP cDNAs either amplified from cDNA libraries or prepared by gene synthesis were individually subcloned into the lentiviral expression vector pLV-EF1 α -MCS-IRES-Bsd (Biosettia) along with an in-frame c-Myc extension added to the carboxyl terminus of each recombinant CYP protein. The identities and structures of each of the recombinant CYP-expression vectors were confirmed by nucleotide sequencing using EF1 α -3' and IRES-5' primers for the 5'- and 3'-ends of the CYP gene inserts, respectively. The production of lentivirus was conducted following the manufacturer's instructions. Briefly, 293T cells were seeded at a density of 1.8×10^6 cells per well in 6-well tissue culture plates and incubated overnight. On the following day, each of the 8 recombinant lentiviral CYP-expression vectors, as well as the lentiviral control vector, was cotransfected with 3 lentiviral packaging vectors (pVSV-G, pRSV-Rev, and pMDLg/pRRE) into the 293T cells using Lipofectamine 2000 transfection reagent (Life Technologies). At 48 h post-transfection, lentiviral-containing supernatants were harvested from each well and stored at -80°C . Lentiviral titers were measured using a functional lentivirus titrating kit from Biosettia. For lentiviral transduction, TK6 cells were seeded at a density of 2.5×10^5 cells per well in 6-well tissue culture plates and then were infected with lentivirus at a multiplicity of infection of 10 with $8\mu\text{g/ml}$ polybrene (Sigma) by centrifuging at $1000 \times g$ for 60 min at room temperature. The transduction medium was replaced with fresh complete medium after centrifugation. Forty-eight hours after transduction, transduced cells were selected in the presence of $8\mu\text{g/ml}$ blasticidin to generate stable TK6 cell lines expressing individual CYPs or the control vector.

Cell viability and cellular ATP level. Cells were seeded in 24-well plates at the density of 2×10^5 cells/ml. After 24 h of treatment, cell viability and cellular ATP level were determined as described previously using a CellTiter-Blue cell viability assay kit (Promega) and a CellTiter-Glo luminescent cell viability assay kit (Promega), respectively (Guo et al., 2018; Seo et al., 2019). Both assays provide measures of cell viability, but they evaluate 2 different endpoints, thus providing orthogonal measures for confirmation of responses. The relative cell viability and cellular ATP content were calculated by comparing the signal intensity of the treated cells to that of the controls.

RNA extraction and quantitative real-time PCR. A total of 2×10^6 TK6 cells were used for RNA extraction using the RNeasy Mini kit (Qiagen, Valencia, California). The quantity and purity of RNA were measured with a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). cDNA was synthesized from $2\mu\text{g}$ of total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR) was used to examine the expression of individual CYP enzymes in TK6 cell lines at the mRNA level. Reactions were performed in a $20\text{-}\mu\text{l}$ volume according to the manufacturer's protocol of FastStart Universal Probe Master (Rox) (Roche Applied Science, Indianapolis, Indiana) using a Bio-Rad CFX Real-Time PCR system (Bio-Rad Laboratories, Hercules, California) under the following conditions: 10 min at 95°C followed by 40 cycles of 15 s at 95°C , and 1 min at 60°C . The following TaqMan probes (Applied Biosystems) were used: human CYP1A1 (Hs01054796_g1), human CYP1A2 (Hs00167927_m1), human CYP1B1 (Hs00164383_m1), human CYP2A6 (Hs00711162_s1), human CYP2B6 (Hs03044634_m1), human CYP2C9 (Hs00426397_m1),

Table 1. Quantitative RT-PCR Analysis of mRNA Expression in CYP-transduced TK6 Cells

CYP	Relative CYP mRNA Levels ($-\log_2$ Fold-change) ^a	
	vs parental TK6 cells	vs EV cells ^b
CYP1A1	13.73 ± 0.09	13.97 ± 0.31
CYP1A2	14.42 ± 0.80	16.09 ± 0.11
CYP1B1	15.28 ± 0.77	15.24 ± 0.10
CYP2A6	10.98 ± 1.23	12.03 ± 0.26
CYP2B6	10.02 ± 0.19	10.30 ± 0.39
CYP2C9	14.95 ± 0.90	15.75 ± 0.04
CYP2C19	13.51 ± 0.77	14.31 ± 0.28
CYP3A4	13.08 ± 0.90	13.87 ± 0.06

^aFor details of calculation of the relative CYP mRNA levels, see "RNA extraction and quantitative real-time PCR" section.

^bEV, TK6 cells transduced with empty vector.

human CYP2C19 (Hs00426380_m1), human CYP3A4 (Hs00604506_m1), and human GAPDH (Hs02758991_g1). Quantitation cycle (Cq) was used to determine the relative expression levels of target genes and Cq values were normalized to that of the endogenous control GAPDH. Cq values above 35 were deemed nondetectable (McCall et al., 2014). To calculate a relative amount, a Cq value of 35 was assigned to samples with nondetectable signals. The following formula was used to calculate the relative amount of the transcripts in the CYP-transduced TK6 cells (CYP) to the parental TK6 cells (parental) or to the empty vector (EV) transduced TK6 cells (Table 1). $\Delta\Delta\text{Cq} = \Delta\text{Cq (CYP)} - \Delta\text{Cq (parental or EV)}$. Note: $\Delta\text{Cq} = \text{Cq(CYP)} - \text{Cq(GAPDH)}$; the fold-change for each CYP-transduced TK6 cells relative to the parental or EV TK6 cells = $2^{-\Delta\Delta\text{Cq}}$; and $-\log_2$ fold-change = $\Delta\Delta\text{Cq}$ (Table 1).

Western blot analysis. A total of 1×10^7 TK6 cells were lysed in RIPA buffer with Halt Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, Massachusetts) for protein extraction. The protein concentrations of cell lysates were determined using the BCA assay (ThermoFisher Scientific). Equivalent amounts ($20\mu\text{g}$) of total protein were used and standard Western blot procedures were performed. Primary antibodies against CYP1A1, 1B1, 1A2, 2A6, 2B6, 2C9, 2C19, 3A4, phosphorylation of histone 2A.X ($\gamma\text{H2A.X}$), and GAPDH (loading control) were diluted in blocking reagent at a concentration of 1:1000. Secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Dallas, Texas) were incubated at a dilution of 1:10 000. The protein signals were determined by FluorChem E System and quantified by AlphaView SA (ProteinSimple, San Jose, California).

Cyp activity measurement by UPLC-MS. CYP450 activities in TK6 cells were measured by ultraperformance liquid chromatography coupled with mass spectrometry (LC-MS) as described previously (Seo et al., 2019). Briefly, 5×10^5 cells were incubated with 1 ml of FBS free medium containing the substrate for each corresponding CYP (Supplementary Table 1): $25\mu\text{M}$ phenacetin (CYP1A1 and 1A2), $20\mu\text{M}$ 7-ethoxy-4-(trifluoromethyl) coumarin (7-EFC, CYP1B1), $20\mu\text{M}$ coumarin (CYP2A6), $25\mu\text{M}$ bupropion (CYP2B6), $20\mu\text{M}$ diclofenac (CYP2C9), $10\mu\text{M}$ omeprazole (CYP2C19), and $50\mu\text{M}$ midazolam (CYP3A4), at 37°C in a humidified atmosphere with 5% CO_2 . The incubation time was 24 h for phenacetin and 2 h for all other substrates. At the end of the incubation, the medium was collected, and then $40\mu\text{l}$ of the

medium was diluted by 160 μ l of ice-cold acetonitrile (ie, 1:4 dilution) followed by centrifugation at 14 000 rpm for 5 min to precipitate proteins. Two microliters of the supernatants were injected onto a Waters ACQUITY UPLC coupled with a QDa Mass Detector. The monitored metabolites were eluted on an ACQUITY UPLC HSS T3 column (2.1 mm $\times$ 50 mm, 1.8 μ m) at 40°C with mobile phases of 0.1% formic acid in LC-MS grade water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) at a flow rate of 0.5 ml/min. Elution started with 0% Solvent B followed by a linear gradient of 0%–100% Solvent B in 1.2 min, then dropping back to 0% Solvent B in 0.1 min, and maintained for 1.2 min to re-equilibrate the column. The eluates were detected by QDa with an electrospray ion source with single ion recording. The monitored (M+H)⁺ ions were *m/z* 152 for acetaminophen (the metabolite of phenacetin by CYP1A1 or 1A2, [Supplementary Table 1](#)), *m/z* 256 for hydroxy bupropion (the metabolite of bupropion by CYP2B6), *m/z* 312 for 4-hydroxydiclofenac (the metabolite of diclofenac by CYP2C9), *m/z* 362 for 5-hydroxyomeprazole (the metabolite of omeprazole by CYP2C19), and *m/z* 342 for 1-hydroxymidazolam (the metabolite of midazolam by CYP3A4). In addition, 2 (M-H)[−] ions were monitored as *m/z* 161 for 7-hydroxycoumarin (the metabolite of coumarin by CYP2A6) and *m/z* 229 for 7-hydroxy-4-(trifluoromethyl) coumarin [the metabolite of 7-ethoxy-4-(trifluoromethyl) coumarin by CYP1B1].

High-throughput micronucleus assay. The high-throughput micronucleus analysis was conducted using a FACSCanto II flow cytometer equipped with a High Throughput Sampler (BD Biosciences, San Jose, California). Cells were seeded in 24-well plates at the density of 2×10^5 cells/ml. After 24 h of treatment, cells were resuspended in fresh media and transferred to a 96-well plate (about 5×10^4 cells/well). Subsequently, the cells were stained and lysed following the protocol described in the *in vitro* MicroFlow Kit (Litron Laboratories, Rochester, New York). For the flow cytometry analysis, the stopping gate was set to record 10 000 intact nuclei ([Guo et al., 2016](#)) and threshold parameters were set as recommended in the instruction manual. The percentage of micronucleated (%MN) cells was calculated as the ratio of micronucleus events to the total number of nucleated events. The relative survival (as an indicator of cytotoxicity) was calculated by comparing the events of nucleated cell count of treated cells to that of vehicle controls at a specified time point.

Statistical analysis. All data were expressed as mean $\pm$ 1 standard deviation (SD) from at least 3 independent experiments. One-way ANOVA followed by Dunnett's post hoc test or 2-way ANOVA (for cytotoxicity data) followed by Bonferroni post hoc test was used to compare the differences between groups. These analyses were performed using GraphPad Prism version 6.0 for windows (GraphPad Software, La Jolla, California). For all tests, a *p* < .05 was deemed statistically significant.

RESULTS

Confirmation of CYP Expression in Newly Developed TK6-Derived Cell Lines

We observed that parental TK6 cells and the TK6 cells transduced with EV had nondetectable mRNA levels of CYP1A2, 1B1, 2C9, 2C19, and 3A4; whereas CYP1A1, 2A6, and 2B6 gene expression were low but detectable by qPCR. There were no significant differences in CYP mRNA levels between parental TK6 cells and EV control transduced-TK6 cells, suggesting that the lentiviral

expression vector did not affect the overall production of CYPs in TK6 cells. However, significantly increased expression of each CYP mRNA in the corresponding cell line was detected. The $-\log_2$ fold-change of all the tested CYPs was above 10 (ie, increased more than 2^{10} -fold) in the CYP-expressing cell lines as compared with parental or EV controls ([Table 1](#)).

We also characterized the protein expression of the CYPs in our cell system by Western blot ([Figure 1A](#)). Both parental TK6 cells and EV controls had negligible protein expression of all CYPs studied. As expected, the specific CYP protein along with Myc (as transfection tag) was strongly expressed in the corresponding CYP-expressing cell lines. To further confirm the metabolic functions of CYPs in the stably transduced TK6 cells, we measured the enzymatic activity of each CYP using their corresponding substrate. [Figure 1B](#) shows the representative UPLC-MS chromatograms for the assessment of enzymatic activity. The substrate and metabolite of each CYP are shown in [Supplementary Table 1](#). Although the EV controls had no detectable activity for the enzymes studied, all CYP-expressing TK6 cells showed conversion of the substrate to the corresponding metabolite. Taken together, we used 3 different endpoints to ensure that our recombinant lentivirus system efficiently transduced functional human CYPs individually into TK6 cells.

Effects of CYPs on the Cytotoxicity of DMBA

It is widely accepted that excessive cytotoxicity can contribute to the false positive rate in *in vitro* genotoxicity tests. Therefore, concentration range-finding tests were conducted to develop a proper concentration range to avoid excessive cytotoxicity (data not shown). The highest concentration selected for DMBA was 100 μ M with a 24-h incubation time. At this concentration, cellular ATP levels (CellTiter-Glo) decreased to about 70% of the DMSO control levels in both parental TK6 cells and EV controls. The decrease in ATP content was the result of the DMBA exposure, and not the effect of lentiviral transduction, as no significant difference was observed between ATP levels in EV controls and in parental TK6 cells at any of the concentrations tested ([Figure 2A](#)). Compared with EV controls, TK6 cells overexpressing CYP1A1 had significantly lower ATP levels at all concentrations tested; at 100 μ M DMBA, ATP levels were $53\% \pm 4\%$ of the DMSO control. Small but inconsistent decreases in ATP levels were seen with CYP1B1 and 2B6 overexpression ([Figure 2A](#)). The other 5 CYPs had no significant effect on DMBA-induced reduction of cellular ATP content when compared with EV controls.

The results from the CellTiter-Blue viability assay were similar to those from the ATP content assay. CYP1A1 and 1B1 significantly increased the cytotoxicity of DMBA compared with EV controls; cytotoxicity in the CYP1B1-expressing cells was increased at all concentrations, except the lowest concentration of 1.56 μ M ([Figure 2B](#)). These results indicate that these 2 CYPs play critical roles in metabolism of DMBA. Cell viability in the CYP2B6 and 2C19 overexpressing cells was also found to be lower compared with EV controls at concentrations above 12.5 μ M. Other CYPs including CYP1A2, 2A6, 2C9, and 3A4 had negligible effects on the viability of TK6 cells exposed to DMBA.

Effects of CYPs on the Cytotoxicity of B[a]P

B[a]P was less cytotoxic to TK6 cells than DMBA. Exposure to 100 μ M B[a]P for 24 h decreased ATP levels in parental TK6 cells and EV controls to $88\% \pm 3\%$ and $86\% \pm 3\%$ of DMSO controls, respectively. No significant reductions in ATP levels were seen in any of the CYP-expressing cell lines following treatment with B[a]P, compared with the EV controls ([Figure 3A](#)). However, in the CellTiter-Blue assay, a significant increase in B[a]P-induced

Figure 1. A, Characterization of CYP protein expression in stably transduced TK6 cells. The protein levels of CYP isoforms ranging in molecular weight between 45 and 60 kDa were detected by Western blotting. Myc was used as a tag for transfection and GAPDH (37 kDa) was used as a loading control. Similar results were obtained from 3 independent experiments. B, Chromatograms of LC-MS showing the enzymatic activities in the TK6 cells transduced with various CYPs. Abbreviations: APAP, acetaminophen; 7-HFC, 7-hydroxy-4-(trifluoromethyl) coumarin; 7-HC, 7-hydroxycoumarin; HB, hydroxy bupropion; 4-HD, 4-hydroxydiclofenac; 5-HO, 5-hydroxyomeprazole; 1-HM, 1-hydroxymidazolam.

cytotoxicity was seen in cell lines overexpressing CYP1A1 and CYP1B1, although the responses were not concentration related (Figure 3B). We also found B[a]P significantly decreased the cell viability in CYP2C19-expressing cells, but only at 50 μ M concentration.

DMBA-Induced Micronuclei in CYP-Expressing TK6 Cells

DMBA has been well documented to induce micronuclei only after metabolic activation (Hégarat et al., 2014; Thougard et al., 2014). In this study, the highest concentration of DMBA selected for the high-throughput flow cytometric micronucleus assay was 25 μ M, determined by the results of the initial cytotoxicity investigations. Relative cell survival at this concentration was above 45% for all independent experiments (Figure 4B).

Representative gating diagrams of EV controls, and CYP1A1- and 1B1-overexpressing cells treated with 25 μ M DMBA are shown in Figure 4A. In EV controls, no change was observed in the %MN cells following exposure to DMBA. Concordant with the results from the cell viability measurements (Figure 2), relative survival values confirmed that CYP1A1 was the most effective CYP in reducing viability of TK6 cells following exposure to DMBA (Figure 4B). The %MN was also increased 7.9- and 9.4-fold over controls in CYP1A1-overexpressing cells treated with 1 and 25 μ M DMBA, respectively. In CYP1B1-expressing cells, smaller but still highly significant increases in %MN were observed at these same 2 concentrations with less cytotoxicity. These results are consistent with DMBA bioactivation by CYP1A1 and 1B1. In addition, DMBA, at 25 μ M, significantly increased %MN in CYP2B6- and 2C19-overexpressing cells, indicating that these 2

Figure 2. Effects of DMBA on cytotoxicity in the TK6 cells stably transduced with various CYP450s. CYP cDNAs or EV-transduced TK6 cells were treated with DMBA at the indicated concentrations for 24 h. The cellular ATP level (A) and cell viability (B) were determined by the CellTiter-Glo assay and CellTiter-Blue assay, respectively. The data points represent the means $\pm$ SD from at least 3 independent experiments. * indicates $p < .05$ for a treatment group versus the corresponding EV cells at the same concentration. Abbreviation: WT, wild-type TK6 cells.

Figure 3. Effects of B[a]P on cytotoxicity in the TK6 cells stably transduced with various CYP450s. CYP cDNAs or EV-transduced TK6 cells were treated with B[a]P at the indicated concentrations for 24 h. The cellular ATP level (A) and cell viability (B) were determined by the CellTiter-Glo assay and CellTiter-Blue assay, respectively. The data points represent the means $\pm$ SD from at least 3 independent experiments. * indicates $p < .05$ for a treatment group versus the corresponding EV cells at the same concentration. Abbreviation: WT, wild-type TK6 cells.

CYPs are also capable of converting DMBA to DNA reactive metabolites.

B[a]P-Induced Micronuclei in CYP-Expressing TK6 Cells

As shown in Figure 5, B[a]P is metabolized to DNA reactive metabolites by CYP1A1 and 1B1. A concentration-dependent increase in %MN in CYP1A1-overexpressing cells was observed after treatment with B[a]P; highly significant increases in %MN were observed at concentrations of 1 and 25 μ M, with fold-changes in %MN exceeding 5.9. In CYP1B1-expressing cells, significant increases in %MN were also seen at the 2 highest concentrations of B[a]P tested. In cells expressing CYP1A2, statistically significant increases in %MN were seen at the 2 lower concentrations of B[a]P tested.

DNA Damage Induced by DMBA and B[a]P in CYP-Expressing TK6 Cells

Phosphorylation of the histone H2A.X (γ H2A.X) is a sensitive biomarker reflecting various types of DNA damage, particularly DNA double-strand breaks (Audebert et al., 2010). We performed Western blotting using the anti- γ H2A.X antibody to validate CYP bioactivation of DMBA and B[a]P in our TK6 cell system. As shown in Figure 6A, treatments with DMBA did not induce detectable DNA damage in parental TK6 (WT) or EV control cells. DMBA (25 μ M) significantly induced γ H2A.X protein expression in CYP1A1-, 1B1-, 2B6-, and 2C19-overexpressing cells (Figure 6B). These results are consistent with our observations in the MN assay, and thus confirmed the involvement of these 4 CYPs in the bioactivation of DMBA. Moreover, we showed CYP1A1 and 1B1 were the predominant enzymes required for B[a]P-induced DNA damage in our cell system (Figure 6C).

We further investigated the concentration-dependent effects of DMBA and B[a]P in TK6 cells expressing the bioactivating CYPs. As expected, both PAHs were efficiently bioactivated to DNA damaging metabolites by CYP1A1 and 1B1, with significant DNA damage seen at both the 1 μ M and the 25 μ M concentrations (Figure 7). Consistent with the results from the MN assay, CYP2B6 and 2C19 were active in the metabolism of DMBA only at the highest concentration (25 μ M).

DISCUSSION

Genotoxicity is a key component in a comprehensive chemical toxicity evaluation due to its role in carcinogenicity and other adverse human health effects (Guyton et al., 2018; Heflich et al., 2020). Investigators have numerous *in vitro* and *in vivo* assays available for characterizing the genotoxicity of compounds of interest. However, each assay has recognized benefits and limitations. A lack of “normal” metabolism and impaired DNA repair capability in conventional test cell lines, along with the high test concentrations typically employed, are the major factors that result in the low specificity in many *in vitro* standard genotoxicity tests. In addition, standard assay protocols that are low- and medium-throughput, are not suited for screening the thousands of compounds that remain uncharacterized. In an attempt to address this latter concern, the U.S. Tox21 Program used a battery of 5 qHTS *in vitro* assays to measure DNA damage-associated events and predict the genotoxicity of more than 10 000 compounds. However, these high-throughput *in vitro* screens also lacked effective metabolic capability. A recent report revealed the overall sensitivity of these qHTS assays was low, due in part to the absence of metabolic capability, and thus, these assays cannot replace traditional genotoxicity assays (Hsieh et al., 2019). Adding metabolic

competency may help improve the sensitivity of the current Tox21 qHTS assays. To address these challenges with existing test systems, we created a metabolically and DNA-repair-competent, human TK6 cell system, and evaluated its performance in a high-content MN assay (in combination with γ H2A.X protein quantification as confirmation).

To evaluate our cell system, we chose DMBA and B[a]P, 2 model PAHs that require metabolic activation to induce genotoxicity. Initially, CYP1A1 was thought to be the only CYP involved in metabolizing PAHs (Shimada et al., 1989). Additional research revealed that CYP1B1 also contributed to the conversion of DMBA and B[a]P to their epoxide intermediates and the highly reactive diol-epoxides that induce formation of DNA adducts (Baird et al., 2005; Shimada et al., 1996). Numerous studies have confirmed that CYP1A1 and 1B1 are the major enzymes responsible for the bioactivation of PAHs. For example, a recent study showed both CYP1A1 and 1B1 were responsible for the initiation of carcinogenesis by B[a]P through the generation of DNA reactive d($\pm$)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) (Shiizaki et al., 2017). Generation of 9-hydroxy-B[a]P, another intermediate metabolite that can form B[a]P DNA adducts, was also catalyzed by human CYP1A1 and 1B1 via an exogenous drug metabolizing system (Supersome) (Šulc et al., 2016). Similarly, these 2 CYPs are required for DMBA conversion to DMBA-3,4-diol-1,2-epoxide, a metabolite that induces DNA adduct formation (Quesnot et al., 2016). Using our metabolically competent TK6 cell model, we confirmed that CYP1A1 and 1B1 were the 2 main CYP isoforms required for producing genotoxic metabolites of both DMBA and B[a]P. Both indicators of genotoxic damage (MN and γ H2A.X) showed significant and similar results (Figures 4 and 5; Table 2) in our newly generated metabolically competent TK6 cell lines.

With regard to metabolic activation of procarcinogens, a concept of “promiscuous activation” was proposed during an ECVAM workshop, where it was concluded that using high concentrations (10 mM or 2 mg/ml in current guidelines) of test chemicals in genotoxicity assays can lead to the involvement of enzymes that are unimportant in *in vivo* biotransformation, thereby contributing to false positive results *in vitro* (Kirkland et al., 2007). The panel suggested that if a compound is truly genotoxic *in vivo*, it should be detected as positive at concentrations less than 100 μ M using *in vitro* assays with appropriate bioactivation mechanisms. Therefore, avoiding high concentrations was a key consideration in our study. For determining appropriate concentration ranges in our studies, we measured cytotoxicity by 2 different endpoints using the CellTiter-Glo and CellTiter-Blue assays. Based on these results, we chose a maximum concentration of 25 μ M, which produced cytotoxicity values for DMBA and B[a]P that were less than 25% in all cell lines tested, except CYP1A1-overexpressing TK cells (Figure 2B).

Although some studies have reported that CYP2C19 and 3A4 can metabolize B[a]P to a certain extent (Šulc et al., 2016), we saw no increases in genotoxicity in these 2 expressing cell lines following B[a]P treatment. Consistent with our results, B[a]P was reported to induced negligible changes in *Umu* gene expression in *Salmonella typhimurium* NM2009 expressing human CYP2C19 and 3A4 (Shimada and Fujii-Kuriyama, 2004). We postulate that any B[a]P DNA reactive metabolites that may have been produced in CYP2C19- or 3A4-expressing cell lines were not present in sufficient amounts to induce any measurable genotoxicity at the concentrations we tested. It is also possible, particularly in p53 competent TK6 cells, that most of the low-level DNA damages that may have been induced in CYP2C19- and 3A4-expressing cells were repaired by p53-mediated mechanisms. In

Figure 4. Induction of micronuclei by DMBA in CYP-expressing TK6 cells. A, Representative gating diagrams show pattern of events in cells treated with 25 μ M DMBA for 24 h by flow cytometric analysis. The “Nucleated” region (dark green dots) indicates healthy cells’ nuclei. A stop mode was set to include at least 10 000 nuclei in this region. The “MN” region (pink dots) indicates micronucleus events. B, The TK6 cells transduced with different CYPs were exposed to various concentrations of DMBA for 24 h. Micronucleus frequency is presented as the percentage of micronuclei relative to intact nuclei (left y-axis and black bar) and cytotoxicity is indicated as the percentage of relative survival to control (right y-axis and red line), when compared with its DMSO control. The data points represent the means $\pm$ SD from at least 3 independent experiments and * indicates $p < .05$ for a treatment group versus the corresponding DMSO control.

Figure 5. Induction of micronuclei by B[a]P in CYP-expressing TK6 cells. A, Representative gating diagrams show pattern of events in cells treated with 25 μ M B[a]P for 24 h by flow cytometric analysis. The “Nucleated” region (dark green dots) indicates healthy cells’ nuclei. A stop mode was set to include at least 10 000 nuclei in this region. The “MN” region (pink dots) indicates micronucleus events. B, The TK6 cells transduced with different CYPs were exposed to various concentrations of B[a]P for 24 h. Micronucleus frequencies are presented as the percentage of micronuclei relative to intact nuclei (left y-axis and black bar) and cytotoxicity is indicated as the percentage of relative survival to control (right y-axis and red line), when compared with its DMSO control. The data points represent the means $\pm$ SD from at least 3 independent experiments and * indicates $p < .05$ for a treatment group versus the corresponding DMSO control.

Figure 6. γ H2A.X DNA damage response induced by DMBA or B[a]P in the CYP-expressing TK6 cells. Cells were exposed to 25 μ M DMBA (A and B) or B[a]P (C and D) for 24 h. Total cellular protein was extracted after the treatment, the levels of γ H2A.X were detected by Western blotting (A and C), and GAPDH was used as a loading control. Densitometric analyses quantified the γ H2A.X protein expression levels after DMBA or B[a]P treatments and results are expressed as the means $\pm$ SD from 3 independent experiments (B and D). Intensities of bands were normalized to the amount of GAPDH. * indicates $p < .05$ between the treatment group and corresponding DMSO control.

support of this notion, a recent study showed that following direct exposure to BPDE, a B[a]P metabolite, a 60% relative repair of DNA adducts was observed in TK6 cells after 24 h (Piberger et al., 2018), and functional p53 is required for nucleotide excision repair of DNA adducts (Akyüz et al., 2002; Seo and Jung, 2004). Because we used relatively low concentrations of the parent compound (B[a]P), p53-related DNA repair machinery may have efficiently removed any damage induced following metabolism of B[a]P by CYP2C19 and 3A4.

To further verify the functions of our cell lines, we made additional testing using chemicals from different categories, which include an alkylator (cyclophosphamide; metabolically activated by CYP2B6), 2 bioflavonoids (galangin and kaempferol; CYP1A1), and 2 pyrrolizidine alkaloids (lasiocarpine and riddelline; CYP3A4). The results obtained from the high-throughput MN assay (Supplementary Figure 1) indicate that our cell system is useful in detecting chemicals from different categories that require metabolic activation to elicit their genotoxicity. To the best of our knowledge, this is the first study to use TK6 cells expressing recombinant human CYPs in the assessment of the genotoxicity of specifically identified carcinogens that require

metabolic activation. The TK6 cell model is p53 competent, and accurate assessment of chemically induced cytotoxic and genotoxic potential in mammalian cells is dependent on the p53 functional status of the cell type that is used (Fowler et al., 2012; Petibone et al., 2017). Previously, Crespi et al. (1989, 1993) performed a series of studies to transfect human CYP genes into human lymphoblastoid AHH-1 cells and showed these recombinant cells were effective in detecting the mutagenicity of procarcinogens. However, AHH-1 cells and their derivative cells (including MCL-5 cells) contain a heterozygous mutation at the TP53 locus (Guest and Parry, 1999; Morris et al., 1996). Compared with TK6 cells, AHH-1 and MCL-5 cells showed a higher level of MN formation and lower cytotoxicity after 4-nitroquinoline 1-oxide treatment (Brüsehauer et al., 2016), suggesting that the mutation may compromise the function of p53 and thus restrain the application of these cell lines in genotoxicity tests.

In addition, our TK6 cell system has the advantage of identifying the specific CYPs that are responsible for chemical biotransformation. Previous studies tried to establish HepG2 cells expressing human CYPs for genotoxicity evaluations using the MN assay (Hashizume et al., 2009, 2010). Although the

Table 2. Summary of the Effects of CYPs on the Cytotoxicity and Genotoxicity of DMBA and B[a]P

CYP	DMBA			B[a]P		
	Cytotoxicity ^b	DNA damage (γ H2A.X)	Chromosomal damage (micronuclei)	Cytotoxicity	DNA damage (γ H2A.X)	Chromosomal damage (micronuclei)
EV ^a	–	–	–	–	–	–
CYP1A1	+	+	+	+	+	+
CYP1A2	–	–	–	–	–	+
CYP1B1	+	+	+	+	+	+
CYP2A6	–	–	–	–	–	–
CYP2B6	+	+	+	–	–	–
CYP2C9	–	–	–	–	–	–
CYP2C19	+	+	+	±	–	–
CYP3A4	–	–	–	–	–	–

^aEV, TK6 cells transduced with an empty vector.^bCytotoxicity was measured by the CellTiter-Blue and CellTiter-Glo assays; significant cytotoxicity in 1 of the 2 assays was required for a positive response to be indicated.

+, positive; –, negative; ±, equivocal.

Figure 7. Dose-dependent changes in γ H2A.X protein expression in the CYP-expressing TK6 cells exposed to DMBA (A and B) or B[a]P (C and D) for 24 h. Total cellular protein was extracted after the treatment, the levels of γ H2A.X were detected by Western blotting (A and C), and GAPDH was used as a loading control. Densitometric analyses were used to quantify γ H2A.X protein levels and results are expressed as the means $\pm$ SD of 3 independent experiments (B and D). * indicates $p < .05$ between the treatment group and corresponding DMSO control.

investigators successfully identified that CYP1A1 accounted for DMBA and B[a]P bioactivation, overexpression of all other CYPs individually in HepG2 cells increased the %MN more than 2- and 3-fold in DMBA- and B[a]P-treated cells, making it difficult to identify the true enzymes responsible for the bioactivation of these 2 PAHs. In addition, HepG2 cells have been shown to have low levels of CYP1A1, 1A2, 2C9, and 3A4 mRNA expression and enzymatic activity (Westerink and Schoonen, 2007), which may account for a roughly 3-fold increase in %MN even in the parental HepG2 cells exposed to B[a]P (Hashizume et al., 2009). We demonstrated that parental TK6 cells expressed negligible functioning CYPs by systematically characterizing the mRNA level, protein expression, and enzymatic activity of these cells (Figs. 1A and 1B). Although it is not appropriate to use parental TK6 cells to perform genotoxicity tests without an added metabolic activation system, the “null” CYP background of TK6 cells can be exploited as a unique advantage to help identify the specific CYP isoforms involved in chemical biotransformation.

One of the limitations of our new cell system is the absence of phase II enzymes, as parental TK6 cells have low or negligible levels of phase II enzymes. It has been well documented that 2 types of phase II enzymes, sulfotransferases (SULTs) and acetyltransferases (NATs), play a critical role in the bioactivation of xenobiotics (Glatt and Meinl, 2005; Sachse et al., 2016). Therefore, incorporation of human recombinant phase II enzymes in our CYP-transduced TK6 cells is an effort we would like to explore in the future. In addition, despite the advantage of identifying the specific CYPs that account for the metabolism of progenotoxicants, testing chemicals using each CYP-expressing cell line individually would be labor and time intensive. Developing cell lines expressing multiple key phases I and II enzymes simultaneously can provide an effective system for biotransformation and substantially increase testing efficiency. However, the specific CYPs responsible for the bioactivation cannot be identified using this approach.

In summary, we developed a set of TK6-derived cells expressing human cytochrome P450s for potential application in genotoxicity testing. This system was shown to detect the genotoxicity of 2 prototypical genotoxicants that require metabolic activation and to identify the specific CYPs responsible for their bioactivation. A broader set of compounds with well-characterized biotransformation pathways needs to be tested in the future to fully validate these cell lines.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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DECLARATION OF CONFLICTING INTERESTS

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