Comparison of drug metabolism and its related hepatotoxic effects in HepaRG, cryopreserved human hepatocytes, and HepG2 cell cultures

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Abstract

Differentiated HepaRG cells maintain liver-specific functions such as drug-metabolizing enzymes. In this study, the feasibility of HepaRG cells as a human hepatocyte model for in vitro toxicity assessment was examined using selected hepatotoxic compounds. First, basal drug-metabolizing enzyme activities (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, uridine 5'-diphospho-glucuronosyltransferase [UGT], and sulfotransferases [SULT]) were measured in HepaRG, human hepatocytes, and HepG2 cells. Enzyme activities in differentiated HepaRG cells were comparable to those in human hepatocytes and much higher than those in HepG2 cells, except for SULT activity. Second, we examined the cytotoxicity of hepatotoxic compounds, acetaminophen (APAP), aflatoxin B1 (AFB1), cyclophosphamide (CPA), tamoxifen (TAM), and troglitazone (TGZ) in HepaRG cells and human hepatocytes. AFB1- and CPA-induced cytotoxicities against HepaRG cells were comparable to those against human hepatocytes. Furthermore, the cytotoxicities of these compounds were inhibited by 1-aminobenzotriazole (ABT), a broad CYP inhibitor, in both cells and were likely mediated by metabolic activation by CYP. Finally, toxicogenomics analysis of HepG2 and HepaRG cells after exposure to AFB1 and CPA revealed that numerous p53-related genes were upregulated-and the expression of these genes was greater in HepaRG than in HepG2 cells. These results suggest that gene expression profiles of HepaRG cells were affected more considerably by the toxic mechanisms of AFB1 and CPA.
than the profiles of HepG2 cells were. Therefore, our investigation shows that HepaRG cells could be useful human hepatic cellular models for toxicity studies.

**Keywords**: Drug-induced liver injury, HepaRG cells, Metabolic activation, HepG2 cells, hepatocytes, metabolites
Introduction

One of the major obstacles to drug development and reasons for subsequent drug withdrawal from the market is toxicity issues.\(^1,2\) Drug-induced liver injury (DILI) is an example of one of the major toxicity incidences.\(^3\) Therefore, toxicity testing systems for risk assessment of DILI are necessary in the early phase of drug discovery and development for the pharmaceutical industry. Although safety assessments are conducted in rodent and non-rodent dosing toxicological studies in the pre-clinical stages of drug discovery, the discrepancies in toxicity results between human and animal models exist because of the species differences. Accordingly, in vitro human hepatic cells are useful human models for toxicity testing in risk assessment of DILI.

Human hepatocytes are considered the gold standard of human hepatic models for toxicological studies. Although fresh primary hepatocytes are difficult to obtain, cryopreserved human hepatocytes (CHHs) are currently commercially available. However, several disadvantages exist with the use of CHHs. In addition to their limited availability and high-cost, CHHs show large inter-individual differences especially in CYP activities and exhibit considerable between lot differences. In some cases, the activity of particular CYP isozymes is deficient or at an extremely low level. The lot-to-lot differences in liver function could lead to differences in results of toxicity testing. In toxicity testing during the early phase of drug discovery, stable testing systems are needed to estimate the risk of compounds.
Accordingly, hepatic cellular models that have stable liver-function and minimal lot-to-lot variations are needed. The human hepatic cell line is a useful tool that can be easily applied as a hepatotoxicity model. The HepG2 cell line has been most widely used as a human hepatic cell model for toxicity studies. However, HepG2 cells are known to show extremely low activity of numerous xenobiotic metabolizing enzymes,\(^4\)\(^-\)\(^6\) which could provide misleading results in toxicity tests of compounds that require biotransformation.

HepaRG cells are a newly established hepatoma cell line obtained from a human hepatocellular carcinoma.\(^7\) HepaRG cells can be differentiated into both hepatocytes and a biliary lineage by treatment with dimethyl sulfoxide (DMSO). Differentiated HepaRG cell cultures are typically a mixture of both hepatocyte- (approximately 50–55%) and biliary-like epithelial cells.\(^7\)\(^,\)\(^8\) Differentiated HepaRG cells have been shown to maintain liver-specific functions such as drug metabolizing enzymes and transporters.\(^7\)\(^-\)\(^9\)

These reports suggest that differentiated HepaRG cells could be a useful model for in vitro studies of drug metabolism and hepatotoxicity and as surrogate models of CHHs. In drug metabolism studies, gene expression of drug metabolizing enzymes and enzyme activities are investigated in HepaRG cells.\(^10\)\(^,\)\(^11\) HepaRG cells have also been useful for investigating CYP induction and inhibition.\(^12\)\(^-\)\(^15\) Furthermore, HepaRG cells have been shown to be quantitatively comparable to human hepatocytes for predicting the clearance of CYP drug substrates and as alternative in vitro tools.\(^16\)
Toxicological studies have reported the usefulness of HepaRG cells as in vitro hepatic models.\textsuperscript{17,18} Several cytotoxicity and toxicogenomic studies have shown that HepaRG cells could be a more accurate human hepatocyte-like model than other hepatic cell lines and would be useful for toxicological studies.\textsuperscript{9,17-19}

In these studies, the expression of drug metabolizing enzymes and activities in differentiated HepaRG cells were determined and compared with those of human hepatocytes and HepG2 cells.\textsuperscript{7,10,11} The sensitivity and cytotoxicity of hepatotoxicants in HepaRG cells was examined and compared with that in human hepatocytes and HepG2 cells.\textsuperscript{9,18} According to the results of these studies, drug metabolizing enzyme expression levels and activities in HepaRG cells more closely simulate those in human hepatocytes than HepG2 cells do, and the sensitivity of HepaRG cells to the cytotoxicity of hepatotoxicants is higher than that of HepG2 cells and more closely related to the human hepatocytes because they express drug metabolizing enzymes. As mentioned above, previous reports\textsuperscript{9} have shown that HepaRG cells are useful hepatic cell models, which have moderate drug metabolizing enzyme activities, although the relationship between cytotoxicity and CYP activities have not been elucidated fully.

In this study, we focused on toxicity via metabolic activation by drug metabolizing enzymes and further investigated the relationship between CYP-mediated activation and the test compound-induced cytotoxicity. Lot-to-lot differences in differentiated HepaRG have not been reported yet. Therefore, we determined the enzyme activity of six CYP isoforms.
(CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4) and two phase II enzymes (uridine diphosphate glucuronosyltransferase [UGT] and sulfotransferase [SULT]) in three different lots of HepaRG cells. Furthermore, we compared these enzyme activities with those in HepG2 and CHHs (three donors). We also assessed the cytotoxicity of five well-known hepatotoxictants and compared their sensitivities in CHHs. We investigated the effects of CYP inhibition on the cytotoxicity of hepatotoxicants. Furthermore, toxicogenomics analyses were conducted in HepaRG and HepG2, which are apparently distinguishable based on drug metabolizing enzyme activities, using two hepatotoxicants. One was aflatoxin B1 (AFB1), while the other was cyclophosphamide (CPA). Both are well known hepatotoxins activated by CYP-mediated oxidation.

Finally, we demonstrated that HepaRG cells are a useful human hepatic cellular model for toxicity testing where toxicity induced by metabolic activation could be detected. Furthermore, these cells are a suitable alternative tool to CHHs in drug discovery research.

Materials and Methods

Chemicals

Acetaminophen (APAP), cyclophosphamide (CPA), 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide, diclofenac, caffeine, niflumic acid, and 1-aminobenzotriazole (ABT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aflatoxin B1 (AFB1), tamoxifen
(TAM), phenacetin, midazolam, acetonitrile, methanol and DMSO were purchased from Wako Pure Chemical Industries (Osaka, Japan). The 4ʹ-hydroxy diclofenac, 1ʹ-hydroxyl midazolam, and 7-hydroxycoumarin sulfate were purchased from Corning Gentest (Corning, NY, USA). The 4ʹ-hydroxy mephenytoin, hydroxyl bupropion, and bupropion were purchased from Toronto Research Chemicals (North York, ON, Canada). Bufuralol and 1ʹ-hydroxybufuralol were purchased from Sumika Chemical Analysis Service, Ltd., (Osaka, Japan). S-Mephenytoin and troglitazone (TGZ) were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

**Cell culture**

HepaRG cells were purchased from Biopredic International (Rennes, France) and the cryopreserved human hepatocytes were obtained from BioreclamationIVT (Westbury, NY, USA). The CP Medium® and Torpedo Antibiotic Mix® were purchased from BioreclamationIVT while the Medium 670, Medium 620, and Medium 640 were purchased from Biopredic International. These hepatocytes were cultured according to each manufacturer’s instructions. The human hepatocellular carcinoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HepG2 cells were grown in minimum essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA, USA). The cells were grown at 37°C in a humidified
5% CO2 atmosphere in a T75 or T225 flask to ~80% confluence and passaged using a 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich).

**Measurement of drug-metabolizing enzyme activity**

Phenacetin O-deethylase (CYP1A2), bupropion hydroxylase (CYP2B6), diclofenac 4'-hydroxylase (CYP2C9), S-mephenytoin 4'-hydroxylase (CYP2C19), bufuralol 1'-hydroxylase (CYP2D6), midazolam 1'-hydroxylase (CYP3A4), 7-hydroxycoumarin glucuronidation (UGT), and 7-hydroxycoumarin sulfation (SULT) activities were measured in each hepatic cellular model. HepaRG cells (7.2 × 10⁴ cells/0.1 mL per well) were seeded in a 96-well collagen type I-coated plate and cultured in 0.1 mL/well Medium 670. After an overnight incubation, the medium was replaced with Medium 620, and after a 6-day culture, the Medium 620 was exchanged every 1–3 days for differentiation, and the assay was conducted using Medium 640. The CHHs (3.5 × 10⁴ cells/0.1 mL per well) were seeded on a 96-well collagen type I-coated plate in CP Medium® with Torpedo Antibiotic Mix® (1:45, v/v). After an overnight incubation, the assay was performed. The HepG2 cells (7 × 10⁴ cells/0.1 mL per well) were seeded into 96-well plates 1 day prior to the enzyme activity assay. In all cell models, drug-metabolizing enzyme activities were measured at the initial time point of test compound exposure in the cytotoxicity assays described below.
At the predetermined assay points of each cell models, the culture medium was replaced with fresh medium containing the cocktail substrates, i.e., 40 μM phenacetin, 50 μM bupropion, 5 μM diclofenac, 100 μM S-mephenytoin, 5 μM bufuralol, 5 μM midazolam, and 100 μM 7-hydroxycoumarin, followed by a 60-min incubation. The following media were used for the various cell models: medium 640 for HepaRG cells, CP Medium® with Torpedo Antibiotic Mix® for CHHs, and the culture medium described above for the HepG2 cells. After incubation at 37°C, aliquots of the various media (100 μL) were collected from each well, and the reaction was stopped by adding a half volume of acetonitrile containing 2 μM caffeine (internal standard for the positive ion mode) with ice chilling. Then, 1 μM niflumic acid (internal standard for the negative ion mode) was added to the samples and then methanol was added to the samples for protein precipitation, followed by drying and reconstitution in a mixture of water/acetonitrile/methanol (9/5/1, v/v/v). The concentrations of metabolites of APAP, hydroxybupropion, 4ʹ'-hydroxymephenytoin, 1ʹ'-hydroxybufuralol, 1ʹ'-hydroxymidazolam, 7-hydroxycoumarin-glucuronide, and 7-hydroxycoumarin-sulfate were determined using LC-MS/MS [Waters ACQUITY UPLC (Waters, Milford, MA, USA) interfaced with an API4000 (Sciex)] using an electrospray ion source. The mobile phase consisted of 0.1% (v/v) acetic acid in water (A) and acetonitrile (B). The separation was performed using a UPLC ACQUITY HSS T3 column (2.1 × 50 mm, 1.8-μm; Waters) at a flow rate of 0.4 mL/min with a column temperature of 40°C and the gradient conditions were
as follows; A/B = 99/1, 99/1, 10/90, 2/98, 2/98, 99/1, and 99/1 (at 0, 0.4, 2.0, 2.2, 2.6, 2.61, and 4.0 min, respectively). For the LC-MS/MS analysis, the Q1/Q3 (m/z) transition for the positive ion mode was 152/110 for APAP, 256/238 for hydroxybupropion, 312/231 for 4’-hydroxydiclofenac, 235/150 for 4’-hydroxymephenytoin, 278/186 for 1’-hydroxybufuralol, 342/324 for 1’-hydroxymidazolam, 339/163 for 7-hydroxycoumarin-glucuronide, and 195/138 for caffeine. Furthermore, the Q1/Q3 (m/z) transition for the negative ion mode was 241/161 for 7-hydroxycoumarin-sulfate and 281/237 for niflumic acid. After removal of the media from each well for analysis, 100 μL of lysis buffer (CelLytic™ M, Sigma-Aldrich) was added to the well and the cell lysate was collected. The protein concentrations in the cell lysates were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol of the manufacturer. The absorbance was measured using a spectrophotometer, SpectraMax Plus (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cytotoxicity assay

The HepaRG cells and CHHs for the cytotoxicity assay were cultured under the same conditions used in the enzyme activity assay described above. After differentiation of the HepaRG cells, the medium was replaced with Medium 640 containing the test compounds with or without ABT. The next day after the seeding of the CHHs, the medium was replaced with fresh medium containing the test compounds with or
without ABT. Stock solutions of test compounds were prepared in DMSO and diluted 200-fold with the medium (0.5% DMSO, v/v) except for CPA. The stock solution of CPA was prepared in distilled water and diluted 200-fold with the medium. After exposure to the test compounds for 48 h, the cell viability was examined by analyzing the intracellular ATP contents using the CellTiter-Glo™ luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the protocol of the manufacturer. The luminescence was measured using a Multilabel Counter (1420 ARVO SX, PerkinElmer, Waltham, MA, USA). IC_{50} values were calculated using SOFTMax Pro (Molecular Devices).

**RNA isolation**

HepG2 cells were seeded into 24 well plates at a density of 1 × 10^5 cells/1 mL per well one day prior to drug exposure. The cells were incubated with 1 mL of the test compounds solutions for 24 h. At the end of the exposure, total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the protocol of the manufacturer.

Terminally differentiated human hepatocellular carcinoma HepaRG cells (approximately 0.45 × 10^6 cells/cm^2) seeded in a 24-well plate were purchased. The cells were subsequently cultured in 0.5 mL/well medium 630 for 2 days at 37°C in a humidified 5% CO_2 atmosphere. Then, the medium was replaced with Medium 620. After a 5-day culture, the medium was replaced with medium 630 containing AFB1. HepaRG cells (48 × 10^4 cells/0.5 mL per well)
were seeded in a 24-well plate and cultured in 0.5 mL/well Medium 670. After a 1-day incubation, the medium was replaced with Medium 620, and after a 6-day culture, the medium was replaced with Medium 640 containing CPA. After a 24-h treatment with AFB1 or CPA, total RNA was isolated using the RNeasy Mini Kit according to the protocol of the manufacturer.

The concentration at which the total RNA yield was approximately 60% was chosen for the transcriptomic analysis, which was conducted as N = 1.

**DNA microarray analysis**

Quality of the total RNA was assessed based on the ratio of the absorbance at 260 and 280 nm (A260/A280) using a NanoDrop ND-1000 spectrophotometer (Asahi Glass Co., Ltd., Tokyo, Japan) and the integrity of the RNA was monitored by the RNA integrity number (RIN) generated using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA samples that exceeded the criteria of 1.8 and 6 for A260/A280 and RIN, respectively, were used in the subsequent experiments. Biotin-labeled cRNA was synthesized using a GeneChip® HT One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA) and GeneChip® HT IVT Labeling Kit (Affymetrix) or GeneChip® 3’ IVT Express Kit (Affymetrix) according to the protocols of the manufacturers. The GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix), which is a whole human genome expression
array was used for the transcriptomic analysis. The hybridization, washing, and staining were performed using the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix) using Fluidics Station 450 (Affymetrix). Hybridization signal was detected using the GeneChip® Scanner 3000 (Affymetrix). The scanned images were processed using a gene expression analysis software, Expression Console Ver.1.1 (Affymetrix). The 3ʹ/5ʹ signal ratios of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were confirmed by the quality control criteria that no gene was expected to have a value > 3. The GeneChip data were normalized using the MAS 5.0 algorithm with the GeneSpring GX (Agilent Technologies). The fold-change relative to the vehicle control group was calculated for the treatment groups based on the gene expression data after normalization. The thresholds of fold changes to determine upregulated and downregulated genes were set at ≥ 2 and < 0.5, respectively. Hierarchical clustering was performed using the Spotfire DecisionSite 9.1.1 (TIBCO, Palo Alto, CA, USA) using the UPGMA method.

**Statistical analysis**

The data are presented as the means ± standard deviation (S.D.). Each value corresponds to a different cell culture experiment. Student's t-tests were conducted at the same concentration of test compound with or without ABT for Figure 3.
Results

Drug metabolizing enzyme activities

The basal drug metabolizing enzyme activities were measured in HepaRG cells, CHHs, and HepG2 cells. The activities of six phase I and two phase II enzymes, namely phenacetin O-deethylation, bupropion hydroxylation, diclofenac 4′-hydroxylation, mephenytoin 4′-hydroxylation, bufuralol 1′-hydroxylation, midazolam 1′-hydroxylation, 7-hydroxycoumarin-glucuronidation, and 7-hydroxycoumarin sulfation were analyzed in each cell line (Figure 1 and Table 1).

For phenacetin-O-deethylation activity, which is catalyzed by CYP1A2, the values were 7.65 to 10.2 pmol·min⁻¹·mg protein⁻¹ in HepaRG cells and 1.94 to 4.34 pmol·min⁻¹·mg protein⁻¹ in the three different CHH cultures.

For bupropion hydroxylation activity, which is catalyzed by CYP2B6, the values were 10.6 to 50.7 pmol·min⁻¹·mg protein⁻¹ in HepaRG cells. The values of the two different CHH cultures were 1.66 and 2.61 pmol·min⁻¹·mg protein⁻¹. In lot SHM, the activity was not detectable. Although the CHHs exhibited high inter-donor variations, the bupropion hydroxylation activity tended to be higher in HepaRG cells than it was in the three different CHH lots.

For diclofenac 4′-hydroxylation activity, which is catalyzed by CYP2C9, the values were
3.79 to 19.4 pmol·min\(^{-1}\)·mg protein\(^{-1}\) in HepaRG cell while those of the three different CHHs were 1.81 to 3.72 pmol·min\(^{-1}\)·mg protein\(^{-1}\).

For S-mephenytoin hydroxylation activity (CYP2C19), the values were 2.73 to 10.3 pmol·min\(^{-1}\)·mg protein\(^{-1}\) in HepaRG cells while those in the two different CHHs were 0.788 and 2.49 pmol·min\(^{-1}\)·mg protein\(^{-1}\). In lot SHM, S-mephenytoin hydroxylation activity was not detectable while the CHHs exhibited high inter-donor variations.

For bufuralol 1′-hydroxylation activity (CYP2D6), the values were 2.21 to 3.34 pmol·min\(^{-1}\)·mg protein\(^{-1}\) in HepaRG cells while those in the three different CHH lots were 0.178 to 0.908 pmol·min\(^{-1}\)·mg protein\(^{-1}\). Bufuralol 1′-hydroxylation activity tended to be higher in HepaRG cells than it was in the three different CHH lots.

For midazolam 1′-hydroxylation activity (CYP3A4), the values were 69.6 to 131 pmol·min\(^{-1}\)·mg protein\(^{-1}\) in HepaRG cells. The values in three different CHH lots were 0.349 to 72.6 pmol·min\(^{-1}\)·mg protein\(^{-1}\), and they exhibited high inter-donor variations.

For the two phase II enzyme activities, HepaRG cells showed 7-hydroxycoumarin-glucuronidation activity (UGT) and 7-hydroxycoumarin sulfation activity (SULT) at 1644 to 2254 and 20.5 to 61.8 pmol·min\(^{-1}\)·mg protein\(^{-1}\), respectively. In CHHs, the UGT and SULT activity values were 255 to 391 and 6.24 to 22.7 pmol·min\(^{-1}\)·mg protein\(^{-1}\), respectively. CHHs exhibited low inter-donor variations in UGT activity. In both phase II enzyme activities, the HepaRG cells showed higher activities than the CHHs did.
In HepG2 cells, almost no basal drug metabolizing enzyme activities were detected, and the levels were lower than those of the HepaRG cells and CHHs except for SULT (20.8 pmol·min⁻¹·mg protein⁻¹). CYP2D6, CYP3A4, and UGT activities were detectable in HepG2 cells at 0.0150, 0.225, and 33.7 pmol·min⁻¹·mg protein⁻¹, respectively.

**Cytotoxicity of hepatotoxicants**

The toxicity of five hepatotoxicants, APAP, AFB1, CPA, TAM, and TGZ, was examined in the HepaRG cells and CHHs after a 48-h exposure to the test compounds using an ATP assay (Figure 2). All the compounds except for TGZ showed cytotoxicity against HepaRG cells that was comparable to that against the three different CHH lots (Table 2). Based on the results of the drug metabolizing enzyme activity assay, a voluntary lot of HepaRG cells (lot. HPR116150) was chosen for the subsequent cytotoxicity assays.

Treatment with AFB1, TGZ, and TAM (IC₅₀ values of 5.0, 80, and 15.8 μM, respectively) was cytotoxic against the HepaRG cells (Figure 2 and Table 2). For the CPA-treated cells, the IC₅₀ value was not calculated, but the cell viability was 56.7% of the control at the highest dose of 25,000 μM after a 48-h exposure of HepaRG cells. The viability of the APAP-treated HepaRG cells was 75.4% of the control at the highest dose of 10,000 μM.

The CHHs showed cytotoxicity following treatment with AFB1 (IC₅₀, 4.7 to approximately
20 μM) and TAM (IC_{50}, 40.3 to 60.9 μM; Figure 2 and Table 2). In the sensitivity of the CPA-treated cells to the cytotoxic effects differed between lots, and the cell viability values were 50.4 to 91.2% of control at the highest concentration of 25,000 μM in the three different CHH lots. The viabilities of the APAP- and TGZ-treated cells were > 90% of the control at the highest concentration of each compound, while no cytotoxicity was observed in any of the CHH lots.

**Effects of CYP activity on cytotoxicity**

The previous experiments performed in this study (Figure 2) showed that the cytotoxicities of AFB1 and CPA on HepaRG cells at the tested concentrations were comparable to those on the CHHs. AFB1- and CPA-induced toxicity is well known to be attributable to the covalent binding of reactive metabolites, which are formed by CYPs.\textsuperscript{20,21} Therefore, the AFB1 and CPA were chosen for the further investigations.

To examine the effects of these CYP-mediated metabolites on cytotoxicity in HepaRG cells and CHHs, the cytotoxicity of AFB1 and CPA was examined following co-treatment with ABT, a broad CYP inhibitor. Cell viability was determined using an ATP assay (Figure 3).

The AFB1-induced cytotoxicity was markedly decreased by co-treatment with 100 μM ABT in HepaRG cells. Although the cell viability was 10.5% without ABT following 20 μM AFB1 treatment for 48 h, co-treatment with ABT inhibited cell death (cell viability, 64.8%).
In the case of CHHs, cytotoxicity was observed in all lots after AFB1 treatment for 48 h, but the cell death was almost completely inhibited by 500 \( \mu \text{M} \) ABT. Furthermore, CPA induced cell death of HepaRG cells after exposure for 48 h (cell viability, 56.7% at 25,000 \( \mu \text{M} \)). The cell death was decreased by co-treatment with 100 \( \mu \text{M} \) ABT (cell viability, 84.7% at 25,000 \( \mu \text{M} \)).

In the CHHs, ABT inhibited CPA-induced cell death only in lot YEM, which was the most vulnerable to CPA exposure. CPA induced cell death of lot YEM cells following exposure to 25,000 \( \mu \text{M} \) for 48 h (cell viability, 50.4%). The cell death was decreased by co-treatment with 500 \( \mu \text{M} \) ABT (cell viability, 80.5%). However, ABT did not have inhibitory effects against CPA-induced cytotoxicity in lot SHM and CPQ hepatocytes.

**Gene expression analysis**

The toxicogenomic analyses were performed in HepG2 and HepaRG cells after exposure to AFB1 and CPA for 24 h. Effects on gene expression were analyzed globally using the Human GeneChip® Human Genome U133 plus 2.0 array cartridge. A total of 54675 probe sets were present on each array. Following AFB1 and CPA treatment, a larger number of genes in the HepaRG cells were up- or downregulated more than those in the HepG2 cells (Supplemental Table). AFB1 affected 2329 (upregulated)/1403 (downregulated) probe sets at 5.63 \( \mu \text{M} \) with a threshold of fold-change \( \geq 2.0 \) or \(< 0.5 \) after 24-h treatment of HepaRG cells. In the HepG2
cells, AFB1 affected 320 (upregulated)/319 (downregulated) probe sets at 5.63 μM with a threshold of fold-change ≥ 2.0 or < 0.5 after 24 h treatment. CPA affected 4424 (upregulated)/2275 (downregulated) probe sets at 20 mM with a threshold of fold-change ≥ 2.0 or < 0.5 after 24 h treatment of HepaRG cells. CPA affected 429 (upregulated)/518 (downregulated) probe sets at 20 mM with a threshold of fold-change ≥ 2.0 or < 0.5 after 24 h treatment of HepG2 cells.

**Effects of p53 signaling-related gene expression**

It is well known that the cytotoxicity of AFB1 and CPA is related to the cellular DNA damage induced by their reactive metabolites.20-23) The p53 molecule plays an important role as a tumor suppressor, and p53 signaling is related to the DNA damage response.24) Therefore, the gene expression profiles of the p53 signaling pathway were examined in HepG2 and HepaRG cells after exposure to AFB1 and CPA.

Gene expression changes related to p53 signaling in the HepG2 and HepaRG cells (control and treatment groups) are shown as a heat map [Figure. 4 (a) and (b)].

In the AFB1 treatment group [Figure. 4 (a)], a larger number of p53-related genes were upregulated in HepaRG cells after AFB1 exposure than in the HepG2 cells. CPA treatment of HepaRG cells induced considerable p53-related gene expression changes compared to the HepG2 [Figure. 4 (b)].
p53 signaling genes/pathway

The representative p53 signaling-related genes are listed, and the differences in their gene expression responses between HepaRG and HepG2 cells was compared after exposure to AFB1 and CPA (Table 3).

In both HepaRG and HepG2 cells, BTG2, CDKN1A (p21) and MDM2 were upregulated after AFB1 exposure. GADD45 and DDB2 were upregulated with a threshold of fold-change ≥ 2.0 in HepaRG cells following exposure to 1.41 μM AFB1, whereas these genes were upregulated in HepG2 cells only at the highest concentration of 11.25 μM. APAF1, BAX, FBXW7, MSH2, PCNA, RAD51, and XPC were upregulated only in HepaRG cells. Expression responses of these genes were not observed in HepG2 cells. Other significant genes such as ATR, GTSE1, MLH1, TP53, and WRN tended to be upregulated in HepaRG although the levels were not higher than 2-fold changes. CPA treatment upregulated BTG2, CDK1A, DDB2, GADD45, and XPC with a threshold of fold-change ≥ 2.0 only in HepaRG cells at 10 and 20 mM, but not in HepG2 cells.

Discussion

In this study, the feasibility of using HepaRG cells as a human hepatocyte model that expresses drug-metabolizing enzymes and has relevant characteristics for toxicity testing was examined using selected hepatotoxic compounds, whose toxicities are mediated by
metabolism. After differentiation with commercially supplied medium (Medium 620) for 6 days, the activities of six major CYPs and two phase II conjugating enzymes were determined in differentiated HepaRG cells. Furthermore, these activities were compared with those of CHHs and HepG2 cells, which are typical in vitro human hepatic models used for toxicity testing. The enzyme activities in HepaRG cells were confirmed with all isozymes. Furthermore, these activities were almost equal to or higher than those of CHHs and much higher than those of the HepG2 cells except for the 7-hydroxycoumarin sulfation activity.

In human hepatocytes, the drug-metabolizing enzyme activities exhibited a between lot variability derived from the inter-individual differences. For example, the midazolam 1’-hydroxylation activity (CYP3A4) of lot SHM of the human hepatocytes was approximately 200-fold lower than that of lot YEM. Furthermore, some isoforms exhibit low activity in human hepatocyte. In contrast, the three different lots of differentiated HepaRG cells examined exhibited a low between lot variation in drug-metabolizing enzyme activities. The largest difference in HepaRG cells was only about 5-fold in CYP2B6 activity. Furthermore, all the examined enzyme isoforms exhibited moderate activities in differentiated HepaRG cells. However, CYP3A4 activity in HepaRG cells of lot HPR116169 was lower than that of the other lots. HepaRG cells do have variation between lots, but it is only slight compared to that of human hepatocytes. CYP3A4 isoforms play important roles in the metabolism and toxicity of many drugs, so these lot differences in CYP3A4 activities
should be taken into consideration. In previous research studies, it was reported that HepaRG cells were derived from a CYP2D6 poor metabolizer and the metabolic capacity of these cells was slower than that of the wild-type.\textsuperscript{7,8} However, many drug metabolizing enzyme activities were confirmed, and the metabolites produced by CYP2D6 were detectable, and its activity in HepaRG cells was not lower than that of the CHHs in this study. These results support previous research, which demonstrated CYP2D6 activity in HepaRG cells.\textsuperscript{10} The UGT-dependent 7-hydroxycoumarin-glucuronidation activities of the HepaRG cells were higher than those of the CHHs. This might have affected the toxicity of the test compounds.

In HepG2 cells, which is a major human hepatic cell line that has been used in numerous toxicological studies, the CYP3A4 activity was approximately 200-fold lower than that in the differentiated HepaRG cells.

The cytotoxicity of the five hepatotoxic compounds, CPA, AFB1, APAP, TGZ, and TAM, were assessed in the differentiated HepaRG and CHHs. AFB1-induced cytotoxicity in CHHs differed between lots (IC\textsubscript{50}, 4.7–approximately 20 \(\mu\)M), and its cytotoxicity in HepaRG cells (IC\textsubscript{50}, 5 \(\mu\)M) was comparable to that observed in the CHHs. CPA toxicity in CHHs also exhibited between lot differences and was comparable to that in HepaRG cells. The toxicity assessments of TGZ in this study showed interesting results. In all the CHH lots, no toxicity was observed following treatment with 100 \(\mu\)M TGZ (cell viability, 96.4–99.1%). Nevertheless, 100 \(\mu\)M TGZ induced the death of differentiated HepaRG cells (cell viability,
0.3%). This result was reproducible in another HepaRG lot (data were not shown). There were differences in sensitivity to TGZ cytotoxicity between the CHHs and differentiated HepaRG cells.

We could not explain this difference completely, but it might have been related to the high SULT activity. It has been reported that the formation of the sulfo-conjugated metabolite of TGZ plays an important role in its toxicity because of its cytotoxicity and inhibition of the bile salt export pump (BSEP), which causes bile salt accumulation in hepatocytes.\(^{25,26}\)

The TAM-induced cytotoxicity in HepaRG cells tended to be slightly higher than that in the CHHs. This effect might have been associated with the differences in CYP2D6, 2C9, and 3A4 activities, which contribute to the production of 4-hydroxy-TAM, the toxic metabolite of TAM,\(^{27}\) in two cell models.

The cytotoxicity of the hepatotoxicants is mediated not only by the parent drugs but also by their toxic metabolites, produced by CYP. To investigate the CYP-mediated toxicity of the selected hepatotoxicants, we assessed the effects of ABT, which is a nonspecific CYP inhibitor,\(^{28,29}\) on the AFB1- and CPA-induced cytotoxicity in CHHs and HepaRG cells. The concentrations of 100 \(\mu\)M and 500 \(\mu\)M were adopted in the CHH and HepaRG cell models, respectively. Five hundred micromolar of ABT was used for CHHs in a previous report.\(^{30}\) We also confirmed sufficient inhibitory effects of the same concentration of ABT on broad CYP activities in CHHs. Furthermore, we confirmed that 100 \(\mu\)M of ABT even sufficiently
inhibited broad CYP activities in HepaRG cells as with the case of CHHs (data not shown). The hepatotoxicity of AFB1 is mediated by its toxic epoxide form (AFB1-8,9-epoxide), which is produced by CYP1A2 and CYP3A4.\textsuperscript{31} This metabolite is well known to be toxic due to its cellular interactions.\textsuperscript{32} ABT treatment reduced the AFB1-induced cytotoxicity in CHHs. This result explains that the AFB1-induced toxicity in CHHs was mediated by CYP activation. Furthermore, co-treatment of HepaRG cells with ABT decreased the AFB1-induced cytotoxicity, which suggests that CYP activation was associated with the cytotoxicity of this compound also in HepaRG cells. CPA is an anticancer chemotherapeutic prodrug that is bioactivated to the therapeutically active, cytotoxic 4-hydroxylated CPA by specific human hepatic CYP enzymes including CYP2B6, 3A4, and 2C8/9.\textsuperscript{33,34} CPA-induced cytotoxicity was decreased by ABT treatment in both CHHs and HepaRG cells. These results suggest that CPA-induced cytotoxicity was mediated by CYP activation in both CHHs and HepaRG cells. According to these results, the cytotoxicity of hepatotoxicants mediated by CYP activation is detectable in HepaRG cells similar to CHHs.

From the viewpoint of the between lot variations in metabolic activity, lot No. YEM of the CHHs, which had the highest CYP3A activity, was the most sensitive to the cytotoxicity of AFB1 and CPA and its sensitivity was comparable to that of the HepaRG cells. The cytotoxic effects of AFB1 in the lot SHM CHHs were higher than those in the lot CPQ CHHs, whereas the CYP3A4 activity in lot SHM was very low compared to that of lot CPQ. The toxic
metabolite of AFB1 (AFB1-8,9-epoxide) is produced not only by CYP3A4 but also by CYP1A2. The cytotoxicity of AFB1 in CHHs is believed to be due to both CYP1A2 and CYP3A4 because CYP1A2 activity in lots SHM and CPQ were the same extent (2.06 and 1.94 pmol·min⁻¹·mg protein⁻¹, respectively). Furthermore, lot YEM, which had the highest CYP3A4 and CYP1A2 activities of the three lots, was the most susceptible to AFB1.

In this study, toxicogenomics analyses were conducted in HepaRG and HepG2 cells to determine the more useful cell line as a human hepatocyte model after hepatotoxicants exposure. The mechanistic analyses were focused on the gene expressions of p53 pathway genes induced by DNA damage\(^{24}\), which are related to the AFB1- and CPA-induced toxicities.

The analysis of the p53-related genes revealed that the number of upregulated genes in the HepaRG cells was considerably higher than that in the HepG2 cells. CDKN1A (p21), GADD45, and BTG2, which are involved in G1 arrest, were upregulated in both HepaRG and HepG2 cells. AFB1 treatment induced both BAX and APAF1 in only HepaRG but not HepG2 cells. These results suggest that the DNA damage response mediated by p53 signaling in HepaRG cells was upregulated, leading to apoptosis signal activation after AFB1 treatment. The mismatch repair genes, MSH2 and PCNA, were also induced only in HepaRG cells by AFB1, which suggests that the DNA damage and repair responses were upregulated. According to these results, p53-related DNA damage responses were activated more
sensitively in HepaRG cells than HepG2 cells. The DNA damage responses induced by AFB1 are thought to be related to its toxic reactive epoxide form (AFB1-8,9-epoxide), which is produced by CYP1A2 and CYP3A4. In this regard, these DNA damage responses could be more sensitively expressed in HepaRG cells due to the CYP activities. In the CPA treatment group, p53 signaling-related genes such as CDKN1A (p21), GADD45, and BTG2, which are involved in G1 arrest, were upregulated significantly only in HepaRG cells. The active metabolite of CPA, 4-hydroxylated CPA, is involved in the DNA damage response. The effects of the CPA metabolites, which are produced by CYP2B6, 3A4, and 2C8/9, were evident in the HepaRG cells. Actually, the activities of these CYPs in HepaRG cells were much higher than those in HepG2 cells.

According to these results, the gene expression profile of the HepaRG cells reflected the mechanism of toxicity of AFB1 and CPA considerably more than the HepG2 cells did. A recent study compared the baseline and compound [AFB1 and benzo(a)pyrene]-induced gene expression profiles of the HepG2, HepaRG, and human hepatocytes of 11–13 donors. In their study, they concluded that the baseline gene expression levels and AFB1-affected pathways observed in human hepatocytes were more similar to those of HepaRG cells than the HepG2 cells. Therefore, HepaRG cells would likely be a useful hepatic model for mechanistic studies such as toxicogenomics analysis.

Although the inter-individual differences of numerous drug metabolizing enzymes in CHHs
might be useful for examining how these inter-individual differences reflect the toxic effects, more uniform hepatic cell models with stable liver functions for screening assays in the early phase of drug discovery are required. It is thought that inter-individual variability could be overcome by using pooled human hepatocytes, which are currently widely available commercially. However, more stably available hepatic cell lines, such as HepaRG, could be more feasible for use in the early phase of drug discovery because of their applicability for high-throughput assays and reasonable costs. Recent studies also reported the utility of HepaRG cells for prediction of DILI in early drug development phase using the multiparametric assay method or high content analysis screening method.\(^{35-37}\)

In conclusion, this study showed that HepaRG could be used to detect the toxicity induced by metabolic activation of compounds and is a potentially useful tool for cytotoxicity testing and mechanistic studies. HepaRG cells could be an alternative valuable model for human hepatocytes, especially in the early phase of drug discovery.

**Conflict of interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.
References


18) Gerets HH, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, Atienzar FA. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the


Figure Legends

Figure 1. Comparison of drug metabolizing enzyme activities in HepG2 cells, cryopreserved human hepatocytes (CHHs), and HepaRG cells

Basal drug metabolizing enzyme activities using selective substrates, phenacetin O-deethylase (CYP1A2), bupropion hydroxylase (CYP2B6), diclofenac 4’-hydroxylase (CYP2C9), S-mephenytoin 4’-hydroxylase (CYP2C19), bufuralol 1’-hydroxylase (CYP2D6), midazolam 1’-hydroxylase (CYP3A4), 7-hydroxycoumarin glucuronidase (UGT), and 7-hydroxycoumarin sulfatase (SULT) activities were determined in HepG2 cells, cryopreserved human hepatocytes (CHHs), and HepaRG cells.

Three different lots of CHHs (Lot. SHM, CPQ, and YEM) and HepaRG cells (Lot. HPR116062, HPR116150, and HPR116169) were examined.

Results of HepG2 cell analysis: gray bars are means ± standard deviation (S.D., n = 8).

Results of CHHs: black bars are means ± S.D.; Lot. SHM: n = 6, CPQ: n = 3, YEM: n = 6.

Results of HepaRG cells: white bars are means ± S.D., Lot. HPR116062, n = 3; HPR116150, n = 6; HPR116169: n = 5. ND: Not detectable
Figure 2. Cytotoxicity of five hepatotoxicants in cryopreserved human hepatocytes (CHHs) and HepaRG cells

Comparative cytotoxic effects of aflatoxin B1 (AFB1), cyclophosphamide (CPA), acetaminophen (APAP), troglitazone (TGZ), and tamoxifen (TAM) on cryopreserved human hepatocytes (CHHs) and HepaRG cells. Results of CHHs: open symbols (white triangle: Lot. SHM, white diamond: Lot. CPQ, white square: Lot. YEM). Results of CHHs are closed symbols (black circle: Lot. HPR116062). CHHs and HepaRG cells were exposed to test compounds for 48 h. Cell viability was evaluated using CellTiter-Glo™ luminescent cell viability assay. Results are normalized to control cells levels and expressed as means ± standard deviation (S.D., n = 3 cultures).

Figure 3. Effects of broad CYP inhibitor on cytotoxicity of aflatoxin B1 (AFB1) and cyclophosphamide (CPA)

Cytotoxicity of AFB1 and CPA was examined in cryopreserved human hepatocytes (CHHs) and HepaRG cells with or without 1-aminobenzotriazole (ABT), a non-selective CYP inhibitor. Results are closed circle for with ABT and open circle for without ABT. CHHs (Lot. SHM, CPQ, and YEM) and HepaRG cells (Lot. HPR116150) were exposed to test compounds for 48 h with or without ABT. ABT concentrations were 500 μM for CHHs and
100 μM for HepaRG cells. Cell viability was evaluated using CellTiter-Glo™ luminescent cell viability assay. Results are normalized to control cells and expressed as means ± S.D. (n = 3 cultures). The data shown in Figure 3 (without ABT) and Figure 2 (a, b) are the same. Student's t-tests were conducted at the same concentration of test compound with or without ABT (*: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001).

**Figure 4. Gene expression profiles in p53 signaling pathway in HepG2 and HepaRG cells treated with (a) aflatoxin B1 (AFB1) or (b) cyclophosphamide (CPA)**

Gene expression was visualized as heatmap using hierarchal clustering with Spotfire DecisionSite using UPGMA method. Gene probes with detection call of “P” or “M” were used for analysis.
Figure 2

(a) Cell viability (% of control) vs. AFB1 (μM) for CHHs: Lot.SHM, CHHs: Lot.CPQ, CHHs: Lot.YEM, and HepaRG.

(b) Cell viability (% of control) vs. CPA (μM) for CHHs: Lot.SHM, CHHs: Lot.CPQ, CHHs: Lot.YEM, and HepaRG.

(c) Cell viability (% of control) vs. APAP (μM) for CHHs: Lot.SHM, CHHs: Lot.CPQ, CHHs: Lot.YEM, and HepaRG.

(d) Cell viability (% of control) vs. TGZ (μM) for CHHs: Lot.SHM, CHHs: Lot.CPQ, CHHs: Lot.YEM, and HepaRG.

(e) Cell viability (% of control) vs. TAM (μM) for CHHs: Lot.SHM, CHHs: Lot.CPQ, CHHs: Lot.YEM, and HepaRG.
Figure 3

(a) CHHs: Lot.SHM

(b) CHHs: Lot.SHM

(c) CHHs: Lot.CPQ

(d) CHHs: Lot.CPQ

(e) CHHs: Lot.YEM

(f) CHHs: Lot.YEM

(g) HepaRG

Cell viability (% of control) vs. AFB1 (μM) for CHHs: Lot.SHM, Lot.CPQ, Lot.YEM, and HepaRG.

Cell viability (% of control) vs. CPA (μM) for CHHs: Lot.SHM and Lot.CPQ.

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* *** ***

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Table 1 Drug metabolizing enzyme activities determined in each cell model

<table>
<thead>
<tr>
<th>Cell</th>
<th>Lot No.</th>
<th>Drug metabolizing enzyme activity (pmol·min(^{-1})·mg protein(^{-1})) Mean ± SD</th>
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<tbody>
<tr>
<td></td>
<td>CYP1A2</td>
<td>CYP2B6</td>
</tr>
<tr>
<td>HepG2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CBHs</td>
<td>SHM</td>
<td>2.06 ± 1.33</td>
</tr>
<tr>
<td></td>
<td>CPQ</td>
<td>1.04 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>YEM</td>
<td>4.34 ± 1.05</td>
</tr>
<tr>
<td>HepaRG</td>
<td>HPR116002</td>
<td>6.65 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>HPR116150</td>
<td>8.35 ± 1.23</td>
</tr>
<tr>
<td></td>
<td>HPR116169</td>
<td>10.2 ± 1.9</td>
</tr>
</tbody>
</table>

CYP1A2: Phenacetin-O-deethylase activity
CYP2B6: Bupropion hydroxylase activity
CYP2C9: Diclofenac 4'-hydroxylase activity
CYP2C19: Mephenytoin 4'-hydroxylase activity
CYP2D6: Bufuralol 1'-hydroxylase activity
CYP3A4: Midazolam 1'-hydroxylase activity
UGT: 7-hydroxycoumarin glucuronidation activity
SULT: 7-hydroxycoumarin sulfation activity
ND: Not detectable
Table 2: IC$_{50}$ values in cytotoxicity assays of five hepatotoxicants in cryopreserved human hepatocytes (CHHs) and HepaRG cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM)</th>
<th>CHHs</th>
<th>HepaRG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lot. SHM</td>
<td>Lot. CPQ</td>
</tr>
<tr>
<td>AFB1</td>
<td>7.8</td>
<td>≧20</td>
<td>4.7</td>
</tr>
<tr>
<td>CPA</td>
<td>&gt;25,000</td>
<td>&gt;25,000</td>
<td>≧25,000</td>
</tr>
<tr>
<td>APAP</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>TGZ</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>TAM</td>
<td>40.3</td>
<td>54.4</td>
<td>60.9</td>
</tr>
</tbody>
</table>
Table 3: Gene expression changes in p53 signaling pathway after exposure of HepG2 and HepaRG cells to aflatoxin B1 (AFB1) or cyclophosphamide (CPA)

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Symbol</th>
<th>Fold-change HepG2</th>
<th>Fold-change HepaRG</th>
<th>Fold-change HepG2</th>
<th>Fold-change HepaRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>204859_s_at</td>
<td>APAF1</td>
<td>2.81 μM to 12.5 μM</td>
<td>1.60 to 1.75</td>
<td>1.65 to 2.22</td>
<td>1.20 to 1.11</td>
</tr>
<tr>
<td>209933_s_at</td>
<td>ATR</td>
<td>1.03 μM to 1.5 μM</td>
<td>0.89 to 0.93</td>
<td>1.60 to 1.49</td>
<td>1.03 to 0.96</td>
</tr>
<tr>
<td>208478_s_at</td>
<td>BAX</td>
<td>1.63 μM to 1.71 μM</td>
<td>1.45 to 1.71</td>
<td>2.34 to 2.60</td>
<td>0.97 to 0.81</td>
</tr>
<tr>
<td>201236_s_at</td>
<td>BTG2</td>
<td>2.37 μM to 2.58 μM</td>
<td>3.54 to 5.28</td>
<td>2.89 to 2.92</td>
<td>3.18 to NE</td>
</tr>
<tr>
<td>202284_s_at</td>
<td>CDKN1A</td>
<td>1.76 μM to 2.61 μM</td>
<td>1.95 to 2.61</td>
<td>2.58 to 2.63</td>
<td>3.28 to 1.42</td>
</tr>
<tr>
<td>229499_at</td>
<td>DDB2</td>
<td>1.77 μM to 2.27 μM</td>
<td>1.79 to 2.77</td>
<td>2.04 to 2.51</td>
<td>2.38 to 0.98</td>
</tr>
<tr>
<td>229415_at</td>
<td>E2F1</td>
<td>1.19 μM to 1.5 μM</td>
<td>1.28 to 1.62</td>
<td>2.26 to 2.71</td>
<td>3.34 to 1.19</td>
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<tr>
<td>201202_at</td>
<td>PCNA</td>
<td>0.95 μM to 0.81 μM</td>
<td>0.75 to 0.81</td>
<td>1.21 to 1.28</td>
<td>1.41 to 1.00</td>
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<tr>
<td>203020_at</td>
<td>P53</td>
<td>1.11 μM to 1.05 μM</td>
<td>1.05 to 1.08</td>
<td>1.52 to 1.46</td>
<td>1.42 to 0.99</td>
</tr>
<tr>
<td>205041_at</td>
<td>RAD51</td>
<td>1.06 μM to 1.36 μM</td>
<td>1.00 to 1.36</td>
<td>1.39 to 1.20</td>
<td>1.14 to 0.95</td>
</tr>
<tr>
<td>205024_s_at</td>
<td>RAD51</td>
<td>1.20 μM to 0.99 μM</td>
<td>1.03 to 0.99</td>
<td>2.36 to 2.19</td>
<td>2.54 to 1.22</td>
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<tr>
<td>201746_at</td>
<td>TP53</td>
<td>2.05 μM to 1.01 μM</td>
<td>1.01 to 0.99</td>
<td>1.67 to 1.63</td>
<td>1.57 to 0.97</td>
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<tr>
<td>209375_at</td>
<td>XPC</td>
<td>0.72 μM to 0.53 μM</td>
<td>0.82 to 1.26</td>
<td>1.01 to 1.26</td>
<td>0.98 to 1.30</td>
</tr>
</tbody>
</table>

Fold-change in gene expression related to p53 signaling pathway in treatment groups relative to control group is shown. Red and blue values represent fold-changes ≥ 2 and < 0.5, respectively. N.E., not evaluated, because detection call was “A” in one or more samples.