Evaluation of 23 Lots of Commercially Available Cryopreserved Hepatocytes for Induction Assays of Human Cytochromes P450

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ABSTRACT

Due to the importance of in vitro cytochrome P450 (P450) induction assay to assess the possible drug-drug interaction events, the recent US Food and Drug Administration draft guidance and European Medicines Agency guideline recommend to assess P450 induction using fresh or cryopreserved hepatocytes at mRNA level and/or enzyme activity level. Although cryopreserved hepatocytes are commercially available for P450 induction assays, feasibility and practicability of these hepatocytes have not been fully investigated. In this study, a total of 23 lots of human cryopreserved hepatocytes were treated with three typical inducers (omeprazole, phenobarbital, and rifampicin), and induction of CYP1A2, CYP2B6, and CYP3A4 enzyme activity was measured. In 8 of these 23 hepatocyte lots, induction of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 mRNA was also analyzed. The results revealed that CYP1A2, CYP2B6, and CYP3A4 were induced (>2-fold) by omeprazole, phenobarbital, and rifampicin, respectively, in all the hepatocyte lots tested at enzyme activity level (23 lots) and mRNA level (8 lots). In contrast, of the 8 hepatocyte lots treated with rifampicin, CYP2C8 and CYP2C9 mRNA were not induced in 5 and 2 hepatocyte lots, respectively, and CYP2C19 mRNA was not induced in any of the 8 hepatocyte lots tested. These results suggest that induction of CYP1A2, CYP2B6, and CYP3A4 can be readily assessed, but evaluation for CYP2C mRNA induction might not be feasible, using commercially available human cryopreserved hepatocytes.

Introduction

In vitro P450 induction assays are one of the important experiments in drug discovery and development to assess the potential for drug-drug interaction. The recent Food and Drug Administration (FDA) draft guidance recommends to evaluate induction of CYP1A2, CYP2B6, CYP2C, and CYP3A4 using fresh or cryopreserved hepatocytes by measuring mRNA and/or enzyme activity level (https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf). A similar recommendation has also been documented in European Medicines Agency (EMA) guidelines (https://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf). One of the major changes from the previous FDA draft guidance is the use of P450 mRNA levels as an endpoint for evaluation of induction. P450 induction has been assessed by measuring enzyme activity; however, mRNA data are also essential to reliably detect test compounds as inducers, which are also mechanism-based inactivators.

The FDA draft guidance indicates the use of fresh or cryopreserved hepatocytes in induction assays. Induction of various P450 mRNAs, including CYP1A2, CYP2B6, CYP2C, and CYP3A4 mRNA, has been largely demonstrated mostly using fresh hepatocytes (Raucy et al., 2002; Madan et al., 2003). In contrast, only a limited number of papers has reported such induction results using cryopreserved hepatocytes (Rojmans et al., 2005; Hewitt et al., 2007). For P450 induction assays at pharmaceutical companies, the cryopreserved hepatocytes are often used after selection by evaluating a large number of the hepatocyte lots for P450 inducibility prior to the experiments. Therefore, it is essential to assess feasibility of the assays using commercially available human cryopreserved hepatocytes.

Another major difference in the latest FDA draft guidance from the previous version is inclusion of CYP2C in induction assays. Human CYP2C enzymes, including CYP2C8, CYP2C9, and CYP2C19, are important because CYP2Cs exhibit approximately 20% of total P450 content in human livers (Shimada et al., 1994). Moreover, CYP2Cs are responsible for metabolizing approximately 20% of all prescribed drugs, including tolbutamide, phenytoin, warfarin, and ibuprofen (Goldstein, 2001). Despite the importance of CYP2Cs for drug metabolism, only a limited number of papers has described induction of human CYP2C8, CYP2C9, and CYP2C19 using fresh hepatocytes (Gerbal-Chaloin et al., 2001; Raucy et al., 2002), or induction of CYP2C9 using cryopreserved hepatocytes (Rojmans et al., 2005). Moreover, the reports on induction of CYP2Cs are contradictory. For example, induction of CYP2C9 and CYP2C19 by rifampicin in human hepatocytes has been documented by several groups (Morel et al., 1990; Chang et al., 1997; Gerbal-Chaloin et al., 2001; Raucy et al., 2002), but others failed to show such induction events (Li et al., 1997; Runge et al., 2000). Therefore, induction of human CYP2Cs needs to be investigated using cryopreserved hepatocytes.

In this study, a total of 23 lots of human cryopreserved hepatocytes was assessed using three typical inducers (omeprazole, phenobarbital, and rifampicin) for P450 induction. First, 15 cryopreserved hepatocyte lots were evaluated for induction of CYP1A2, CYP2B6, and CYP3A4 by measuring enzyme activities. Second, 8 cryopreserved hepatocyte lots were evaluated for induction of CYP1A2, CYP2B6, and CYP3A4 by determining mRNA and enzyme activity levels. Finally, in the same 8 cryopreserved hepatocyte lots, induction of CYP2C8, CYP2C9, and CYP2C19 was assessed using three typical inducers (omeprazole, phenobarbital, and rifampicin).

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ABBREVIATIONS: DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P450, cytochrome P450; PCR, polymerase chain reaction; qPCR, quantitative PCR; RT, reverse transcription.
CYP2C9, and CYP2C19 mRNA was also analyzed by quantitative polymerase chain reaction (qPCR). We report in this study that HepaRG cells could represent individual cryopreserved hepatocytes commercially available for P450 induction study in drug development.

Materials and Methods

Ethoxyresorufin, midazolam, omeprazole, phenobarbital, and rifampicin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bupropion and S-mephenytoin were purchased from Corning (Tewksbury, MA) and Sigma-Aldrich (St. Louis, MO), respectively. Human cryopreserved hepatocytes were purchased from Biopredic International (Rennes, France). Celsis in Vitro Technologies (Baltimore, MD), Corning, In Vitro ADME Laboratories (Columbia, MD), Invitrogen (Carlsbad, CA), and Xenotech (Lenexa, KS). HepaRG cells were purchased from Biopredic International. Clinical characteristics of hepatocyte donors are shown in Supplemental Table 1. The 23 cryopreserved hepatocyte and 4 HepaRG cell lots were supplied by six commercial vendors (A–F) and one commercial vendor (G), respectively, and were anonymously named alphabetical and numeral codes (from A1 to G4) in this study. William’s E medium was purchased from Biopredic International. All other reagents were purchased from Sigma-Aldrich or Wako Pure Chemical Industries, unless otherwise specified.

Cell Culture for Induction Analysis. Human cryopreserved hepatocytes and HepaRG cells were cultured as recommended by the suppliers in 48-well plates. The hepatocytes (0.7 × 10^5, 0.8 × 10^5, or 1.0 × 10^5 cells/well depending on the supplier) were treated for 48 or 72 hours with the P450 inducers, omeprazole (50 μM), phenobarbital (1000 μM), or rifampicin (10 μM), which were dissolved in 0.1% dimethylsulfoxide (DMSO) (v/v). The cell treated with 0.1% DMSO (v/v) was used as a control. Total RNA was extracted from the hepatocytes using RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol.

qPCR Analysis. Reverse transcription (RT)-polymerase chain reaction (PCR) was performed using total RNA extracted from the hepatocytes. Briefly, the first-strand cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Invitrogen) with random primers and total RNA (250 or 300 ng from cryopreserved hepatocytes, 1.5 μg from HepaRG) at 42°C for 1 hour and was used for the subsequent PCR. The amplification was carried out in a total volume of 20 μl using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) with the ABI PRISM 7500 Fast sequence detection system (Applied Biosystems) following the manufacturer’s protocol. CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were measured with real-time RT-PCR using TaqMan gene expression assays (Applied Biosystems), including CYP1A2 (assay ID: Hs00703747_m1), CYP2B6 (assay ID: Hs04183483_g1), CYP2C8 (assay ID: Hs02383390_s1), CYP2C9 (assay ID: Hs02460376_m1), CYP2C19 (assay ID: Hs00426380_m1), CYP3A4 (assay ID: Hs01546612_m1), and GAPDH (assay ID: Hs99999905_m1) mRNA. The experiments were performed in duplicate, and the relative expression level was determined by normalizing the raw data to the GAPDH RNA level.

Measurement of Drug-Metabolizing Activity. Ethoxyresorufin O-deethylase (CYP1A1), bupropion hydroxylase (CYP2B2), S-mephenytoin 4′-hydroxylation (CYP2C19), and midazolam 1′-hydroxylation (CYP3A4) activities were measured in intact cultured hepatocytes. After a 48- or 72-hour exposure to the inducers, the culture media were replaced with William’s E medium (200 μl) containing 10 μM ethoxyresorufin, 100 μM bupropion, 100 μM S-mephenytoin, and 50 μM midazolam. Aliquots of the medium (200 μl) were removed, and the formation of each metabolite was measured as described previously with a slight modification (Fukazawa et al., 2008). The mobile phases were as follows: A, 0.02 M phosphate buffer/trifluoroacetic acid (87/13, v/v) for the assay of ethoxyresorufin metabolite; B, 0.1% formic acid solution; C, 0.1% formic acid in acetonitrile for all other assays. Ethoxyresorufin metabolite was eluted by isocratic elution, and other metabolites were eluted by gradient elution. The determination of ethoxyresorufin metabolite was performed by fluorescence high-performance liquid chromatography. For liquid chromatography coupled with tandem mass spectrometry analysis, the Q1/Q3 (m/z) transition of other metabolites was 256/139 for hydroxybupropion, 235/150 for 4′-hydroxymephenytoin, and 342/203 for 1′-hydroxymidazolam, and the Q1/Q3 (m/z) transition of internal standard was 262/139 for 6′-hydroxybupropion, 238/150 for 4′-hydroxymephenytoin-d3, and 345/206 for 1′-hydroxymidazolam-[13C3].

Correlation Analysis. Linear regression analyses with 95% confidence intervals between fold induction in enzyme activity and mRNA were analyzed with Prism (GraphPad Software, San Diego, CA).

Results

Induction of CYP1A2, CYP2B6, and CYP3A4 Enzyme Activity. By enzyme assays, we first assessed CYP1A2, CYP2B6, and CYP3A4 induction in 15 human cryopreserved hepatocyte lots (from five commercial vendors). The hepatocytes were cultured and treated with omeprazole (CYP1A2), phenobarbital (CYP2B6), or rifampicin (CYP3A4) for 48 hours. Hepatocytes treated with...
solvent were used as the control. Induction (>2.0-fold) of P450 enzyme activity was observed in all the 15 hepatocyte lots tested (Fig. 1, Table 1), but induction levels were substantially varied in the 15 hepatocyte lots for CYP1A2 (5.5- to 64.4-fold), CYP2B6 (4.7- to 32.2-fold), and CYP3A4 (2.7- to 66.7-fold) with omeprazole, phenobarbital, and rifampicin, respectively (Table 1). Induction (>2.0-fold) of CYP1A2, CYP2B6, and CYP3A4 enzyme activity was also observed in 3 HepaRG lots (Fig. 1, Table 1), and the induction level was much less variable among these HepaRG lots than the cryopreserved hepatocytes (Table 1). These results revealed induction (>2.0-fold) of P450 (CYP1A2, CYP2B6, and CYP3A4) enzyme activities in all the 15 cryopreserved hepatocyte lots tested, but induction levels were substantially varied among these hepatocyte lots.

When levels of fold induction were compared in the 15 hepatocyte lots, the differences of CYP1A2, CYP2B6, and CYP3A4 enzyme activities were 11.7- to 14.0-fold, and 24.7-fold, respectively. In the 15 hepatocyte lots, CYP1A2, CYP2B6, and CYP3A4 enzyme activities were variable in the control and induced hepatocytes, partly accounting for the variability of these P450 enzyme activities in fold induction. In contrast, the variability was much less for HepaRG cells; the differences of CYP1A2, CYP2B6, and CYP3A4 enzyme activities in the 3 HepaRG lots ranged from 1.1- to 1.5-fold in the control cells, the inducer-treated cells, and levels of fold induction.

Induction of CYP1A2, CYP2B6, and CYP3A4 mRNA. Next, by qPCR analysis, we assessed induction of CYP1A2, CYP2B6, and CYP3A4 mRNA in eight additional human cryopreserved hepatocyte lots treated with omeprazole, phenobarbital, and rifampicin for 72 hours, respectively. For these hepatocyte lots, CYP1A2, CYP2B6, and CYP3A4 enzyme activities were analyzed. A comparison between control hepatocytes and the inducer-treated hepatocytes revealed that induction (>2.0-fold) of CYP1A2, CYP2B6, and CYP3A4 mRNA was observed in all the hepatocyte lots using omeprazole, phenobarbital, and rifampicin, respectively (Fig. 2). Fold induction in enzyme activity mediated by CYP3A4 significantly correlated with those in mRNA ($r = 0.82, P = 0.007$; Fig. 2) under the present conditions. Fold induction in CYP1A2 enzyme activity also correlated with those in CYP1A2 mRNA ($r = 0.66, P = 0.05$; Fig. 2). Induction (>2.0-fold) of CYP2B6 enzyme activities was observed in these hepatocyte lots, although induction levels of these P450s are not always comparable to those observed at mRNA levels (Fig. 2), presumably because of further conjugation of primary metabolite, hydroxypiperadion, under the present conditions. The extent of CYP1A2, CYP2B6, and CYP3A4 mRNA induction was varied; 8.1- to 34-fold, 4.4- to 20-fold, and 3.1- to 33-fold, respectively. Induction of CYP1A2, CYP2B6, and CYP3A4 was also observed in HepaRG (one lot) at mRNA and enzyme activity levels, and induction levels were much less variable than the cryopreserved hepatocytes (Fig. 2).

These results indicated that induction of P450 (CYP1A2, CYP2B6, and CYP3A4) mRNA was observed in all the eight human cryopreserved hepatocyte lots tested, but induction levels were substantially varied depending on the hepatocyte lot.

**Induction of CYP2C8, CYP2C9, and CYP2C19 mRNA.** Induction of human CYP2C8, CYP2C9, and CYP2C19 mRNA was evaluated using 8 lots of cryopreserved hepatocytes after 72 hours of treatment with rifampicin. Although induction of CYP3A4 mRNA was observed with rifampicin in all eight hepatocyte lots, as described earlier (Fig. 2), induction (>2.0-fold) of CYP2C8 and CYP2C9 mRNA was observed in 3 and six hepatocyte lots, respectively, whereas induction (>2.0-fold) of CYP2C19 mRNA was not observed in any of the eight hepatocyte lots tested (Table 2). Induction of CYP2C8, CYP2C9, and CYP2C19 mRNA was also tested in three of the 15 cryopreserved hepatocyte lots supplied by five commercial vendors (A- E) and 3 HepaRG lots supplied by one commercial vendor (G) were cultured; treated with omeprazole, phenobarbital, or rifampicin for 48 h; and used for enzyme assay to measure CYP1A2, CYP2B6, and CYP3A4 enzyme activity, as described in Materials and Methods. Values are shown as mean of duplicate.

**TABLE 1**

<table>
<thead>
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<th>P450</th>
<th>Cryopreserved Hepatocytes</th>
<th>HepaRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot No.</td>
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<td>A2</td>
</tr>
<tr>
<td>CYP1A2</td>
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</tr>
<tr>
<td>CYP3A4</td>
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</table>

The 15 cryopreserved hepatocyte lots supplied by five commercial vendors (A-E) and 3 HepaRG lots supplied by one commercial vendor (G) were cultured; treated with omeprazole, phenobarbital, or rifampicin for 72 hours; and used for enzyme assay to measure CYP1A2, CYP2B6, and CYP3A4 enzyme activity, as described in Materials and Methods. Values are shown as mean of duplicate.

Induction of CYP1A2, CYP2B6, and CYP3A4 mRNA. Next, by qPCR analysis, we assessed induction of CYP1A2, CYP2B6, and CYP3A4 mRNA in eight additional human cryopreserved hepatocyte lots treated with omeprazole, phenobarbital, and rifampicin for 72 hours, respectively. For these hepatocyte lots, CYP1A2, CYP2B6, and CYP3A4 enzyme activities were analyzed. A comparison between control hepatocytes and the inducer-treated hepatocytes revealed that induction (>2.0-fold) of CYP1A2, CYP2B6, and CYP3A4 mRNA was observed in all the hepatocyte lots using omeprazole, phenobarbital, and rifampicin, respectively (Fig. 2). Fold induction in enzyme activity mediated by CYP3A4 significantly correlated with those in mRNA ($r = 0.82, P = 0.007$; Fig. 2) under the present conditions. Fold induction in CYP1A2 enzyme activity also correlated with those in CYP1A2 mRNA ($r = 0.66, P = 0.05$; Fig. 2). Induction (>2.0-fold) of CYP2B6 enzyme activities was observed in these hepatocyte lots, although induction levels of these P450s are not always comparable to those observed at mRNA levels (Fig. 2), presumably because of further conjugation of primary metabolite, hydroxypiperadion, under the present conditions. The extent of CYP1A2, CYP2B6, and CYP3A4 mRNA induction was varied; 8.1- to 34-fold, 4.4- to 20-fold, and 3.1- to 33-fold, respectively. Induction of CYP1A2, CYP2B6, and CYP3A4 was also observed in HepaRG (one lot) at mRNA and enzyme activity levels, and induction levels were much less variable than the cryopreserved hepatocytes (Fig. 2).
the eight cryopreserved hepatocyte lots after 72 hours of phenobarbital treatment. Again, although induction of CYP3A4 mRNA was observed with phenobarbital in all three hepatocyte lots, induction (>2.0-fold) of CYP2C8 and CYP2C9 mRNA was observed in one and two hepatocyte lots, respectively, whereas induction (>2.0-fold) of CYP2C19 mRNA was not observed in any of the three hepatocyte lots tested (Table 2, Supplemental Fig. 1). Similarly, although induction (>2.0-fold) of CYP3A4 mRNA was observed in HepaRG (one lot), induction (>2.0-fold) of CYP2C8, CYP2C9, and CYP2C19 mRNA was not observed by treatment with rifampicin. CYP2C9 and CYP2C19 mRNA, but not CYP2C8 mRNA, were induced (>2.0-fold) by phenobarbital. These results revealed induction of human CYP2C8 and CYP2C9 mRNA, but not CYP2C19 mRNA, in cryopreserved hepatocytes, depending on the hepatocyte lot.

Discussion

The recent FDA draft guidance recommends induction studies for human CYP1A2, CYP2B6, CYP2C, and CYP3A4 using fresh or cryopreserved hepatocytes at mRNA and/or enzyme activity levels. Evaluation of CYP1A2, CYP2B6, and CYP3A4 induction is routinely conducted during drug development; however, CYP2C induction might not always be investigated at pharmaceutical companies. Moreover, feasibility of commercially available human cryopreserved hepatocytes has not been fully investigated for P450 induction, especially CYP2C isoforms. In this study, 23 commercially available human cryopreserved hepatocyte lots were evaluated for induction of human CYP1A2, CYP2B6, and CYP3A4 enzyme activity, whereas 8 commercially available human cryopreserved hepatocyte lots were evaluated for induction of human CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 mRNA level. Substantial induction of CYP1A2, CYP2B6, and CYP3A4 mRNA/enzyme activity levels was observed in all the hepatocyte lots tested; induction levels were substantially varied among the hepatocyte lots (Figs. 1, 2, Table 1). The basal and induced levels of P450 enzyme activities were different among the hepatocyte lots, and this variability also partly depends on the commercial source of the hepatocyte lots, reflecting some variable values for fold induction of P450 by the typical P450 inducers. A large variability of P450 induction levels at mRNA/enzyme activity levels suggests that hepatocyte lots need to be evaluated for P450 basal expression levels and their induction levels prior to actual use in the induction assays.

In the eight cryopreserved hepatocyte lots treated with rifampicin, induction of CYP2C8 and CYP2C9 mRNA was not observed in some of the hepatocyte lots tested, and induction of CYP2C19 mRNA was not observed in any of the eight hepatocyte lots tested (Table 2). Similar induction profiles were observed for the three hepatocyte lots treated with phenobarbital (Table 2). Induction of CYP3A4 and CYP2B6 mRNA/enzyme activity levels was observed in all these hepatocyte lots using rifampicin and phenobarbital (Fig. 2), indicating that induction mechanisms via pregnane X receptor and constitutively active receptor, respectively, appear to be maintained in these hepatocytes. A previous study also reported the weaker induction levels of CYP2C8 and CYP2C9 mRNAs than CYP3A4 mRNA in the human fresh hepatocytes treated with rifampicin (Gerbal-Chaloin et al., 2001). However, another study reported the stronger induction of CYP2C8 mRNA than CYP3A4 mRNA in the human fresh hepatocytes treated with rifampicin (Raucy et al., 2002). The reason for this discrepancy is not clear but might be the variability of the hepatocyte lots used.

Induction of human CYP2C9 mRNA (Roymans et al., 2005), but not CYP2C8 and CYP2C19 mRNA, has been demonstrated using cryopreserved hepatocytes treated with rifampicin, although induction of all these CYP2C mRNAs by rifampicin using fresh hepatocytes has been reported (Gerbal-Chaloin et al., 2001; Raucy et al., 2002). In this study, induction (>2.0-fold) of CYP2C8 and CYP2C9 mRNA was observed in three and six of the eight hepatocyte lots treated with rifampicin, respectively (Table 2). These results indicate that induction of CYP2C8 and CYP2C9 mRNA could be evaluated using commercially available cryopreserved hepatocytes if the hepatocyte lots were tested for P450 induction prior to the studies. In contrast, induction of CYP2C19 mRNA was not observed in any of the eight hepatocyte lots tested (Table 2). These results raise the concern for the use of the commercially available cryopreserved hepatocytes for assessing CYP2C19 mRNA induction.

In conclusion, this study found that evaluating induction of human CYP1A2, CYP2B6, and CYP3A4 mRNA/enzyme activity was practical using commercially available cryopreserved hepatocytes. Pre-evaluation of hepatocyte lots in terms of basal expression levels for P450 mRNA is needed when using commercially available cryopreserved hepatocytes to assess induction of CYP2C8 and CYP2C9 mRNA. In contrast, induction of CYP2C19 mRNA using the cryopreserved hepatocytes might be difficult and might require pre-evaluation of a large number of hepatocyte lots prior to the induction studies to find suitable hepatocyte lots for the study. The present results also suggest that HepaRG cells are able to represent more than 20 lots of individual cryopreserved hepatocytes commercially available for P450 induction study in drug development.

Acknowledgments

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TABLE 2

<table>
<thead>
<tr>
<th>P450 mRNA</th>
<th>Inducer</th>
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<th>Phenobarbital</th>
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<tbody>
<tr>
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<tr>
<td>CYP3A4</td>
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</table>

The eight cryopreserved hepatocyte lots, supplied by three commercial vendors (A, C, and F), and HepaRG (G) were cultured, treated with rifampicin or phenobarbital for 72 h, and used for qPCR analysis, which was carried out as described in Materials and Methods. Values are shown as mean of duplicate.
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