Multiparametric assay using HepaRG cells for predicting drug-induced liver injury

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HIGHLIGHTS
- Drug-induced liver injury (DILI) is a major safety concern in drug development.
- The utility of HepaRG cells as a model for DILI risk assessment was investigated.
- HepaRG cells exhibited a 67% sensitivity and 73% specificity at 100-fold $C_{\text{max}}$.
- HepaRG cells distinguished relatively safe drugs from their high-risk analogs.
- HepaRG cells may be useful for DILI risk assessment.

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ABSTRACT
The utility of HepaRG cells as an in vitro cell-based assay system for assessing drug-induced liver injury (DILI) risk was investigated. Seventeen DILI-positive and 15 DILI-negative drugs were selected for the assay. HepaRG cells were treated with each drug for 24 h at concentrations that were 1.6-, 6.3-, 25-, and 100-fold the therapeutic maximum plasma concentration ($C_{\text{max}}$). After treatment, the cell viability, glutathione content, caspase 3/7 activity, lipid accumulation, leakage of lactate dehydrogenase, and albumin secretion were measured. The sensitivity and specificity were calculated to assess the ability of the assay to predict DILI. Our multiparametric assay using HepaRG cells exhibited a 67% sensitivity and 73% specificity at a 100-fold $C_{\text{max}}$ cut-off. When a 25-fold $C_{\text{max}}$ cut-off was applied, approximately 70% of drugs exhibiting positive responses were classified into the high DILI risk category. HepaRG cells distinguished relatively safe drugs from their high-risk analogs. Our study indicates that HepaRG cells may be of use to (1) prioritize drug analogs, (2) analyze the mechanism of DILI, and (3) assess the risk for DILI in the early drug discovery stage.

1. Introduction
Drug-induced liver injury (DILI) is the primary cause of the termination of clinical drug development programs and withdrawal of approved drugs from the market (Watkins, 2011). Thus, DILI has become one of the most important concerns in drug research and development. Many preclinical studies have been conducted to predict DILI in humans. However, the prediction of DILI in humans is difficult because of low concordance with preclinical animal studies (Olson et al., 2000).

Despite the difficulty in DILI prediction, various liver-derived in vitro model systems (e.g., liver tissue slices, isolated microsomes, perfused liver, immortalized cell lines, and primary hepatocytes) have been developed to enable the investigation of the potential adverse effects of chemicals and drugs (Soldatow et al., 2013). Immortalized cell lines and primary isolated liver cells are currently the most widely used in vitro models for liver toxicity.

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testing (Soldatow et al., 2013). The HepG2 cell line has been widely used to predict DILI in vitro. However, the HepG2 cell line is devoid of a substantial and variable set of liver-specific functions, particularly the major cytochrome P450s (CYPs) that are involved in xenobiotic metabolism (Guguen-Guillouzo and Guillouzo, 2010). Alternatively, human primary hepatocytes are the gold standard for xenobiotic metabolism and toxicity studies (Hewitt et al., 2007; Lecluse, 2001). However, they are phenotypically unstable, have a limited life span, and exhibit large inter-donor variability (Guguen-Guillouzo and Guillouzo, 2010).

More recently, HepaRG cells, a human cell line derived from hepatocellular carcinoma, have been developed (Gripon et al., 2002). HepaRG cells can differentiate into both the biliary and hepatocyte lineage (Cerec et al., 2007; Gripon et al., 2002). Unlike other immortalized cell lines (e.g., HepG2), HepaRG cells maintain many liver-specific functions as well as many CYPs, nuclear receptors, membrane transporters, and phase II enzymes (Aminat et al., 2006; Guillouzo et al., 2007). Therefore, HepaRG cells are expected to represent a suitable alternative to human primary hepatocytes for drug metabolism and toxicity studies.

HepaRG cells have been used to assess the toxicity of some hepatotoxic drugs (Aninat et al., 2011, 2012, 2013; McGill et al., 2011; Rodrigues et al., 2013). However, they did not evaluate the performance for predicting DILI risk in HepaRG cells. Indeed, there are no studies assessing the sensitivity and specificity for predicting DILI risk in HepaRG cells (Gómez-Lechón et al., 2014).

Conventional cytotoxicity assays have exhibited poor sensitivity because some of these in vitro assays were developed to measure a single endpoint or lethal events in the late stages of toxicity to predict hepatotoxicity (O’Brien et al., 2006; Xu et al., 2004). Because many mechanisms are involved in DILI, an in vitro model that predicts DILI should be assessed with different parameters. Recently, multiparametric in vitro assays have been developed to predict DILI (Garside et al., 2014; Khetani et al., 2013; O’Brien et al., 2006; Persson et al., 2013; Porceddu et al., 2012; Tolosa et al., 2012; Xu et al., 2008).

The present study aimed to investigate the utility of HepaRG cells as an in vitro cell-based assay system to assess DILI risk. The cytotoxicity was assessed with multiple parameters. To assess the ability of the HepaRG cell-based toxicity assay to predict DILI, data were obtained for 32 drugs, 17 of which were DILI-positive, and 15 of which were DILI-negative.

2. Materials and methods

2.1. Cells, culture medium, and plate

The HepaRG cell line is derived from a liver tumor of a female patient suffering from hepatocarcinoma (Gripon et al., 2002). Differentiated cryopreserved HepaRG cells are commercially available (Life Technologies Japan, Tokyo, Japan). William’s E Medium, HepaRG Thaw, Plate and General Purpose Medium Supplement, HepaRG Tox Medium Supplement, GlutaMAX Supplement, and Collagen 1-coated 48-well plates were purchased from Life Technologies Japan. The composition of these supplements is not disclosed by the manufacturer.

2.2. Drugs

Acetaminophen, amiodarone hydrochloride, ascorbic acid, benz bromarone, flutamide, furosemide, miconazole nitrate, olanzapine, simvastatin, and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO). Acetylsalicylic acid, caffeine, chlorpromazine hydrochloride, clozapine, cyclosporin A, dexamethasone, diclofenac sodium, donepezil hydrochloride, famotidine, ibuprofen, isoniazid, ketoc nazole, losartan potassium, rosiglitazone, sodium valproate, tetracycline hydrochloride, theophylline, ticlopidine hydrochloride, troglitazone, valsartan, and warfarin sodium were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bromfenac sodium was purchased from AvaChem Scientific (San Antonio, TX). All drugs were of the highest commercially available grade.

2.3. Drug classification

The drugs were classified into 2 categories, DILI-positive and DILI-negative, according to a previous report (Xu et al., 2008). DILI-positive drugs met at least one of the following criteria: withdrawn from the market mainly due to hepatotoxicity, not marketed in the United States due to hepatotoxicity, received black box warnings from the Food and Drug Administration (FDA) due to hepatotoxicity, marketed with hepatotoxicity warnings in their labels, or had well-known associations with liver injury and a significant number (>10) of independent clinical reports of serious hepatotoxicity that meet the criteria of Hy’s Law. DILI-negative drugs did not meet any of the above-mentioned positive criteria. The DILI-positive category included the following 17 drugs: acetaminophen, amiodarone hydrochloride, benz bromarone, bromfenac sodium, chlorpromazine hydrochloride, clozapine, cyclosporin A, diclofenac sodium, flutamide, furosemide, isoniazid, ketocnazole, sodium valproate, tamoxifen, tetracycline hydrochloride, ticlopidine hydrochloride, and troglitazone. The DILI-negative category included the following 15 drugs: acetylsalicylic acid, ascorbic acid, caffeine, dexamethasone, donepezil hydrochloride, famotidine, ibuprofen, losartan potassium, miconazole nitrate, olanzapine, rosiglitazone, simvastatin, theophylline, valsartan, and warfarin sodium.

2.4. Culture of HepaRG cells

Differentiated HepaRG cells were cultured according to the manufacturer’s instructions. The cells were thawed and plated at a density of approximately 225,000 cells/well in 48-well collagen I-coated plates using William’s E Medium supplemented with HepaRG Thaw, Plate and General Purpose Medium Supplement, and GlutaMAX Supplement. After 24 h, the medium was replaced with William’s E Medium supplemented with HepaRG Tox Medium Supplement and GlutaMAX Supplement. The medium was renewed every 3 days. Seven days after plating, the cells were highly differentiated. The cells were maintained at 37 °C and 5% CO2 with a water reservoir for humidity control.

2.5. Drug exposure

The drugs were tested at concentrations that were 1.6-, 6.3-, 25-, and 100-fold the therapeutic maximum plasma concentration (Cmax) (Table 1). The drugs were initially dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries) and diluted in the culture medium to obtain the desired final concentrations. The final DMSO concentration in the culture medium was 0.5% (vol/vol), and control cultures were treated with the same amount of solvent. The drugs that were insoluble in DMSO (bromfenac sodium, caffeine, sodium valproate, tetracycline hydrochloride, and theophylline) were prepared in the culture medium, and DMSO was added to the culture medium to a final concentration of 0.5% (vol/vol). The 100-fold Cmax concentrations of bromfenac sodium and tetracycline hydrochloride were not tested because they were insoluble in the culture medium. Seven days after plating, the cells were treated with each drug for 24 h.

2.6. Multiparametric assay

The cell viability, intracellular glutathione (GSH) content, caspase 3/7 activity, intracellular lipid accumulation, leakage of...
lactate dehydrogenase (LDH) into the culture medium, and albumin secretion into the culture medium were measured after drug treatment. The cell viability was assayed by using the WST-1 reagent (Roche Diagnostic GmbH, Mannheim, Germany). The intracellular GSH content was assayed using the GSH-Glo Assay Kit (Promega Corporation). The intracellular lipid accumulation was assayed using the Steatosis Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI). The leakage of LDH into the culture medium was assayed using the LDH Cytotoxicity Assay Kit (Promega Corporation). The intracellular caspase 3/7 activity was assayed using the Caspase-Glo 3/7 Assay Kit (Promega Corporation). The intracellular GSH content was assayed using the GSH-Glo Assay Kit (Roche Diagnostic GmbH, Mannheim, Germany). The total score of each parameter was calculated.

2.9. Assessment of predictivity

The sensitivity was calculated as the proportion of DILI-positive drugs that tested positive, defined as TP/(TP + FN), where TP is the number of DILI-positive drugs that tested positive, and FN is the number of DILI-positive drugs that tested negative. The specificity was calculated as the proportion of DILI-negative drugs that tested negative, defined as TN/(TN + FP), where TN is the number of DILI-negative drugs that tested negative, and FP is the number of DILI-negative drugs that tested positive. The sensitivity and specificity were calculated for each parameter. In addition, the sensitivity and specificity were calculated for a combination of 6 parameters and for a combination of 4 parameters (GSH content, caspase 3/7 activity, leakage of LDH, and albumin secretion).

3. Results

3.1. Representative data from the multiparametric assay

The cytotoxic effects of troglitazone and rosiglitazone are shown in Fig. 1. Troglitazone decreased the GSH content at concentrations ≥40 μM (25-fold C_{max}) and decreased albumin secretion at concentrations ≥160 μM (25-fold C_{max}). In addition, troglitazone decreased the cell viability and increased the leakage of LDH at a concentration of 640 μM (100-fold C_{max}). Troglitazone did not meet the positive cut-off value in the caspase 3/7 activity and lipid accumulation. Conversely, rosiglitazone decreased the GSH content at concentrations ≥26 μM (25-fold C_{max}). Rosiglitazone did not meet the positive cut-off value in the other parameters.
3.2. MPC and DILI concern category

A summary of the cytotoxic effects of DILI-positive and -negative drugs is shown in Table 2, which indicates the MPCs in the multiparametric assay. Nine drugs (benzbromarone, bromfenac sodium, troglitazone, ketoconazole, sodium valproate, ticlopidine hydrochloride, tetracycline hydrochloride, ibuprofen, rosiglitazone) were positive at concentrations of ≤25-fold $C_{\text{max}}$ in the multiparametric assay. Of these 9 drugs, 6 drugs were classified as being of most concern for DILI in the Liver Toxicity Knowledge Base (LTKB) (Chen et al., 2011), 7 drugs were classified as severe or high DILI in the DILI category (Gustafsson et al., 2014), and 6 drugs were withdrawn from the market because of hepatotoxicity or boxed warning labels about hepatotoxicity.

3.3. Toxicity score and DILI concern category

The toxicity scores of DILI-positive and -negative drugs in the multiparametric assay are shown in Table 2. The top 12 drugs (score ≥3) were benzbromarone (score 10), sodium valproate (score 9), ibuprofen (score 8), troglitazone (score 7), ketoconazole (score 6), ticlopidine hydrochloride (score 6), diclofenac sodium (score 6), tetracycline hydrochloride (score 6), bromfenac sodium (score 4), acetaminophen (score 4), amiodarone hydrochloride (score 3), and cyclosporin A (score 3). Of the top 12 drugs, 11 drugs were DILI-positive. Eight drugs were classified as being of most concern for DILI in the LTKB annotation (Chen et al., 2011), 8 drugs were classified as severe or high DILI concern (Gustafsson et al., 2014), and 8 drugs were withdrawn from the market because of hepatotoxicity or boxed warning labels about hepatotoxicity.

3.4. ROC curve analysis

An ROC curve analysis was performed to determine the optimal positive cut-off value of each parameter (Fig. 2 and Table 3). According to this analysis, the optimal positive cut-off values of each parameter were as follows: <70% for cell viability (sensitivity, 41%; specificity, 93%; Youden’s index, 0.345), <60% for GSH content (sensitivity, 59%; specificity, 80%; Youden’s index, 0.388), >4.9-fold for caspase 3/7 activity (sensitivity, 59%; specificity, 100%; Youden’s index, 0.588), >2.8-fold for lipid accumulation (sensitivity, 18%; specificity, 100%; Youden’s index, 0.177), >1.9-fold for leakage of LDH (sensitivity, 65%; specificity, 87%; Youden’s index, 0.514), and <40% for albumin secretion (sensitivity, 47%; specificity, 93%; Youden’s index, 0.399). The caspase 3/7 activity yielded the highest area under the ROC curve (AUC) (0.867), followed by albumin secretion (0.767), leakage of LDH (0.733), GSH content (0.624), cell viability (0.494), and lipid accumulation (0.455). The positive cut-off values of each parameter are also indicated as dashed lines in Fig. 1.

3.5. Ability of the multiparametric assay to predict DILI

The ability of the multiparametric assay to predict DILI is shown in Table 4. Compared with the other parameters, the GSH content, caspase 3/7 activity, and leakage of LDH were most sensitive (60%) at a concentration 100-fold of $C_{\text{max}}$ and the GSH was most sensitive (29%) at a concentration 25-fold of $C_{\text{max}}$. According to the combination of 6 parameters, the sensitivity for the detection of DILI-positive drugs was 0%, 12%, 41%, and 67% at concentrations of 1.6-, 6.3-, 25-, and 100-fold of $C_{\text{max}}$, respectively. The specificity for the detection of DILI-negative drugs was 100%, 100%, 87%, and 73% at concentrations of 1.6-, 6.3-, 25-, and 100-fold of $C_{\text{max}}$, respectively. In addition, the combination of 4 parameters (GSH content, caspase 3/7 activity, leakage of LDH, and albumin...
secretion) showed equivalent sensitivity and specificity as compared with the combination of 6 parameters. Compared with a single parameter, the combination of parameters improved the sensitivity. However, the cell viability and lipid accumulation did not improve the sensitivity and specificity.

3.6. Pharmacological and structural analogs

The cytotoxic effects of pharmacological and structural analogs are shown in Table 5. Nonsteroidal anti-inflammatory drugs, azole antifungal drugs, and thiazolidinedione antidiabetic drugs that yielded a lower MPC/C_{\text{max}} value than their analogs were withdrawn from the market because of hepatotoxicity or boxed warning labels about hepatotoxicity, except for ibuprofen. High-risk drugs (bromfenac sodium, ketoconazole, and troglitazone) could be distinguished from their lower-risk analogs based on the MPC/C_{\text{max}} Value.

### 4. Discussion

Drug development studies are often terminated and approved drugs are often withdrawn from the market because of DILI. In vitro cell-based assays that predict DILI have been developed to improve the success rate of drug development. In the present study, we investigated the utility of HepaRG cells as an in vitro cell-based assay system to assess DILI risk. HepaRG cells maintain many liver-specific functions as well as CYPs, nuclear receptors, membrane transporters, and phase II enzymes at levels comparable to those of human primary hepatocytes (Aninat et al., 2006; Guillouzo et al., 2007). Although human primary hepatocytes are phenotypically unstable and exhibit large inter-donor variability (Guguen-Guillouzo and Guillouzo, 2010), HepaRG cells maintain a drug metabolism capacity comparable to that of primary human hepatocytes (Guillouzo, 2010) and therefore exhibit large inter-donor variability (Guguen-Guillouzo and Guillouzo, 2010). HepaRG cells therefore maintain a drug metabolism capacity comparable to that of primary human hepatocytes.
observed with time in culture (Lambert et al., 2009). Alternatively, the HepG2 cell line also has been widely used but is devoid of a substantial and variable set of liver-specific functions, particularly the major CYPs involved in xenobiotic metabolism (Guguen-Guillouzo and Guillouzo, 2010). Therefore, HepaRG cells appear to be more suitable than the HepG2 cell line for DILI risk assessment based on the liver-specific functions.

We selected specific toxicity parameters to establish a toxicity assay using HepaRG cells. Conventional cytotoxicity assays have exhibited poor sensitivity because they measure lethal events in the late stages of toxicity as well as only 1 endpoint (O’Brien et al., 2006; Xu et al., 2004). However, DILI is a complex phenomenon that includes many mechanisms (e.g., bioactivation, mitochondria

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive cut-off value</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC</th>
<th>Youden’s index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell viability</td>
<td>&lt;70%</td>
<td>41</td>
<td>93</td>
<td>0.494</td>
<td>0.345</td>
</tr>
<tr>
<td>GSH content</td>
<td>&lt;60%</td>
<td>59</td>
<td>80</td>
<td>0.624</td>
<td>0.388</td>
</tr>
<tr>
<td>Caspase 3/7 activity</td>
<td>&gt;4.9-fold</td>
<td>59</td>
<td>100</td>
<td>0.867</td>
<td>0.588</td>
</tr>
<tr>
<td>Lipid accumulation</td>
<td>&gt;2.8-fold</td>
<td>18</td>
<td>100</td>
<td>0.455</td>
<td>0.177</td>
</tr>
<tr>
<td>Leakage of LDH</td>
<td>&gt;1.9-fold</td>
<td>65</td>
<td>87</td>
<td>0.733</td>
<td>0.514</td>
</tr>
<tr>
<td>Albumin secretion</td>
<td>&lt;40%</td>
<td>47</td>
<td>93</td>
<td>0.767</td>
<td>0.399</td>
</tr>
</tbody>
</table>

AUC: the area under the receiver operating characteristic curve.

### Table 4

<table>
<thead>
<tr>
<th>Drug concentration</th>
<th>Cell viability</th>
<th>GSH content</th>
<th>Caspase 3/7 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.16 × Cmax</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.63 × Cmax</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2.5 × Cmax</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>100 × Cmax</td>
<td>47</td>
<td>93</td>
<td>60</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<th>Drug</th>
<th>DILI Cmax (μM)</th>
<th>MPC (μM)</th>
<th>MPC/Cmax</th>
<th>WDN/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsteroidal anti-inflammatory</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromfenac sodium</td>
<td>26</td>
<td>650</td>
<td>&gt;25</td>
<td>WDN</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>7.4</td>
<td>740</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>5.5</td>
<td>&gt;550</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>241</td>
<td>6000</td>
<td>25</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### Table 6

<table>
<thead>
<tr>
<th>Drug</th>
<th>DILI Cmax (μM)</th>
<th>MPC (μM)</th>
<th>MPC/Cmax</th>
<th>WDN/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thienobenzodiazepine atypical antipsychotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td>0.95</td>
<td>&gt;95</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>0.18</td>
<td>&gt;18</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>Azole antifungal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>3.2</td>
<td>80</td>
<td>&gt;25</td>
<td>BW</td>
</tr>
<tr>
<td>Miconazole nitrate</td>
<td>0.021</td>
<td>&gt;2.1</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>Thiazolidinedione antidiabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trogilitazone</td>
<td>6.4</td>
<td>40</td>
<td>6.3</td>
<td>WDN</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>1.04</td>
<td>26</td>
<td>&gt;25</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Sen: sensitivity; Spe: specificity. Sensitivity (percentage of DILI-positive drugs that meet the positive cut-off value) and specificity (percentage of DILI-negative drugs that do not meet the positive cut-off value) are presented for each parameter and for the combination of 6 and 4 parameters.

The combination of 4 parameters includes the GSH content, caspase 3/7 activity, leakage of LDH, and albumin secretion.

Fig. 2. Receiver operating characteristic (ROC) curve analysis for 6 parameters in the 17 DILI-positive and 15 DILI-negative drugs. The true and false positive fractions indicate the sensitivity and specificity of each index, respectively.

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**Guillouzo and Guillouzo, 2010.** Therefore, HepaRG cells appear to be more suitable than the HepG2 cell line for DILI risk assessment based on the liver-specific functions.

We selected specific toxicity parameters to establish a toxicity assay using HepaRG cells. Conventional cytotoxicity assays have exhibited poor sensitivity because they measure lethal events in the late stages of toxicity as well as only 1 endpoint (O’Brien et al., 2006; Xu et al., 2004). However, DILI is a complex phenomenon that includes many mechanisms (e.g., bioactivation, mitochondria.
impairment, oxidative stress, steatosis, phospholipidosis, cholestasis, and apoptosis (Gómez-Lechón et al., 2010). In the present study, we selected 6 parameters associated with the mechanisms of DILI according to several previously developed in vitro systems (Anthérieu et al., 2011; Khetani et al., 2013; O’Brien et al., 2006): the cell viability to evaluate the overall vitality of cells, the GSH content to assess the cellular redox state, the caspase 3/7 activity to estimate cell apoptosis, the lipid accumulation to evaluate the abnormal retention of lipids within cells, the leakage of LDH to assess the membrane disruption of cells, and the albumin secretion to determine the liver function.

Rodríguez et al. (2013) investigated the basal cytotoxicity of 10 chemicals using differentiated HepaRG cells by the neutral red uptake assay as a single parameter. In the study by Rodríguez et al. (2013), valproic acid did not show any toxic effect on differentiated HepaRG cells for detoxification. In contrast, valproic acid (sodium valproate) showed positive responses in the cell viability, GSH contents, caspase 3/7 activity, lipid accumulation and albumin secretion in the present study. Therefore, this result suggests that the multiparametric assay may be useful to assess the DILI risk.

Lin and Will (2012) demonstrated that the prediction of hepatotoxicity can be significantly improved when human Cmax values are incorporated in the cell-based assay. In addition, a dose-response study found that the 100-fold Cmax scaling factor represented a reasonable threshold to differentiate safe vs. toxic drugs using human primary hepatocytes (Xu et al., 2008). Xu et al. (2008) also reported a high specificity (95–100%) when cells were treated for 24h with drugs at 100-fold therapeutic Cmax concentrations. Thus, we selected the 100-fold Cmax as the maximum concentration and 24h as the treatment period.

In the present study, we examined the 100-fold Cmax concentration. However, information on the human Cmax is not available in the early drug discovery stage. Physiologically based pharmacokinetic modeling can predict the Cmax with reasonable accuracy using in silico and in vitro data (Jones et al., 2006), although the Cmax tends to be underestimated (Poulin et al., 2011). Therefore, applying the prediction values of Cmax may be useful to assess the DILI risk in the early drug discovery stage.

The optimal positive cut-off value of each parameter should be determined by applying rational statistical methods. Xu et al. (2008) generated an ROC curve and rationally determined the threshold value for each parameter in a cell-based assay to predict the DILI. Therefore, we analyzed ROC curves to determine the optimal positive cut-off value of each parameter. Among the parameters, the caspase 3/7 activity yielded the highest AUC (0.867), suggesting that this parameter can predict DILI well in HepaRG cells. In contrast, the cell viability and lipid accumulation yielded low AUC values (0.494 and 0.455, respectively), suggesting that these parameters cannot accurately predict DILI in HepaRG cells. In addition, O’Brien et al. (2006) reported that conventional parameters (e.g., caspase 3 induction: 5% sensitivity and 95% specificity; GSH depletion: 19% sensitivity and 85% specificity; and cell viability: 10% sensitivity and 92% specificity) are not predictive of DILI in HepG2 cells. The ability (especially the sensitivity) of the caspase 3/7 activity, GSH content, and cell viability to predict DILI in the HepaRG cells of the present study was higher than that reported by O’Brien et al. (2006) for HepG2 cells. These results indicate that HepaRG cells may be superior to HepG2 cells as an in vitro model for DILI risk assessment, although the number of drugs tested in our study differs from that tested by O’Brien et al. (2006) (32 vs. 611 drugs). In the present study, the potential for DILI was detected with 67% sensitivity and 73% specificity at a concentration of 100-fold of Cmax. In multiparametric assays using human primary hepatocytes, Xu et al. (2008) reported a 50–60% sensitivity and 95–100% specificity using a high content analysis, and Khetani et al. (2013) reported a 66% sensitivity and 90% specificity using a biochemical assay when 100-fold Cmax was applied as the maximum concentration. These results suggest that HepaRG cells are equally sensitive but less specific than human primary hepatocytes when a 100-fold Cmax cut-off is applied, although the assessment parameters of the present study differ from those utilized by Xu et al. (2008) and Khetani et al. (2013).

The sensitivity was low (41%) when a 25-fold Cmax cut-off was applied; however, many drugs that showed positive responses were classified as follows: most DILI concern (6/9 drugs) in LTKB (Chen et al., 2011), severe or high DILI concern (7/9 drugs) in the DILI category (Gustafsson et al., 2014), and withdrawn from the market because of hepatotoxicity or boxed warning labels about hepatotoxicity (6/9 drugs). This result suggests that the drugs that show positive responses at a 25-fold Cmax concentration may pose a high DILI risk. Therefore, lowering the priority of drug candidates that show positive responses to a concentration of 25-fold Cmax may improve drug development efficiency in the early drug discovery stage.

To compare the degree of injury to HepG2 cells, damage scores were established according to difference from control cells (Tolosa et al., 2012). Tolosa et al. (2012) used this score system to compare the degree of injury and distinguish hepatotoxic compounds from non-hepatotoxic compounds with high specificity (92%). Thus, the score system appears to be useful to compare the degree of toxicity or prioritize drug candidates. In the present study, more than 90% of toxicity score ≥3 drugs were classified as DILI-positive drugs. This result suggests that toxicity score ≥3 drugs are likely to cause DILI. In addition, lowering the priority of drug candidates with high toxicity scores in the early drug discovery stage appears to improve the success rate of drug development.

HepaRG cells have been used to study the mechanisms of some hepatotoxic drugs, namely acetaminophen (McGill et al., 2011), amiodarone and tetracycline (Anthérieu et al., 2011, 2012), and chlorpromazine (Anthérieu et al., 2013). In the present study, the GSH contents decreased at lower concentrations (<25-fold Cmax) of troglitazone, rosiglitazone, ketoconazole, and sodium valproate than the other parameters. These results suggest that the decreasing GSH content plays an important role in mechanisms of DILI in these drugs.

The central role played by apoptosis in the toxicity of many xenobiotics and P450-generated metabolites is now recognized (Gómez-Lechón et al., 2008; Russmann et al., 2009). In the present study, approximately 60% (10/17) of DILI-positive drugs induced caspase 3/7 (pro-apoptotic caspase) activity. In contrast, caspase 3/7 activity was not detected in response to treatment with DILI-negative drugs. These results indicate that apoptosis plays an important role in one of the mechanisms of DILI.

We compared the cytotoxic effects of pharmacological and structural analogs in the present study. Table 5 shows that the multiparametric assay using HepaRG cells can distinguish relatively safe drugs from their high-risk analogs, except for ibuprofen. These results indicate that HepaRG cells might be useful as a tool to prioritize drug candidates in the early drug discovery stage.

Ibuprofen and rosiglitazone were identified as DILI-negative drugs. However, positive responses were observed at concentrations of ≥25-fold Cmax (Table 2). Some DILI-positive cases have been reported for both ibuprofen (Alam et al., 1996; Javier Rodríguez-González et al., 2002; Sternlieb and Robinson, 1978) and rosiglitazone (Al-Salman et al., 2000; Floyd et al., 2009). In addition, ibuprofen is classified as being of less concern in LTKB (Chen et al., 2011) and the DILI reports in the DILI category (Gustafsson et al., 2014). Similarly, rosiglitazone is classified as being of high concern in the DILI category (Gustafsson et al., 2014).
Thus, ibuprofen and rosiglitazone may be controversial drugs in the DILI risk category. Of the DILI-positive drugs, clozapine, flutamide, furosemide, isoniazid, and tamoxifen did not elicit positive responses in the present study. Similarly, flutamide, furosemide, isoniazid, and tamoxifen did not elicit positive responses in the multiparametric assay using human primary hepatocytes (Xu et al., 2008). In the study by Xu et al. (2008), the cells were treated with drugs for 24 h at 100-fold Cmax as in the present study. Therefore, future studies should be conducted to detect the positive responses of these drugs by developing the proper parameters.

Of the 6 parameters, lipid accumulation was least sensitive in the present study. Tetracycline and amiodarone are well known to induce liver steatosis (Fromenty and Pessayre, 1995; Frénaux et al., 1988). However, lipid accumulation was not observed for either tetracycline or amiodarone in the present study. A dose-dependent induction of triglycerides was observed in HepaRG cells after repeated exposure (14 days) to either amiodarone or tetracycline (Anthérieu et al., 2011, 2012). Therefore, a long-term period may improve the sensitivity of lipid accumulation in HepaRG cells.

Several limitations in the present study in the prediction of DILI risk should be noted. The six parameters cannot sufficiently illustrate the detailed mechanisms of DILI. Further studies should be developed to select the proper parameters to attain highly specific and sensitive DILI predictions.

5. Conclusions

The utility of HepaRG cells as an in vitro cell-based assay system for assessing DILI risk was investigated. A multiparametric assay using HepaRG cells showed a 67% sensitivity and 73% specificity at a 100-fold of Cmax concentration and a 41% sensitivity and 87% specificity at a 25-fold of Cmax concentration. When a 25-fold Cmax cut-off was applied, approximately 70% of drugs showing positive responses were classified into the high DILI risk category. In addition, HepaRG cells distinguished relatively safe drugs from their high-risk analogs. Our study indicates that HepaRG cells may be of use to (1) prioritize drug analogs, (2) analyze the mechanism of DILI, and (3) assess the risk for DILI in the early drug discovery stage.

Conflict of interest

The authors declare that there are no conflicts of interest.

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