Usefulness of *in vitro* combination assays of mitochondrial dysfunction and apoptosis for the estimation of potential risk of idiosyncratic drug induced liver injury

Keisuke Goda, Tadakazu Takahashi, Akio Kobayashi, Toshiyuki Shoda, Hideyuki Kuno and Shoichiro Sugai

Toxicology Research Lab., Central Pharmaceutical Research Institute, JAPAN TOBACCO INC.,
23 Naganuki Hadano, Kanagawa 257-0024, Japan

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**ABSTRACT** — Drug-induced liver injury (DILI) is one of the serious and frequent drug-related adverse events. This adverse event is a main reason for regulatory action pertaining to drugs, including restrictions in clinical indications and withdrawal from clinical trials or the marketplace. Idiosyncratic DILI especially has become a major clinical concern because of its unpredictable nature, frequent hospitalization, need for liver transplantation and high mortality. The estimation of the potential for compounds to induce idiosyncratic DILI is very difficult in non-clinical studies because the precise mechanism of idiosyncratic DILI is still unknown. Recently, many *in vitro* assays which indicate a possibility of the prediction of the idiosyncratic DILI have been reported. Among these, some *in vitro* assays focus on the effects of compounds on mitochondrial function and the apopotic effects of compounds on human hepatocytes. In this study, we measured oxygen consumption rate (OCR) and caspase-3/7 activity as an endpoint of mitochondrial dysfunction and apoptosis, respectively, with human hepatocytes after treatment with compounds causing idiosyncratic DILI (troglitazone, leflunomide, ranitidine and diclofenac). Troglitazone and leflunomide decreased the OCR but did not affect caspase-3/7 activity. Ranitidine increased caspase-3/7 activity but did not affect the OCR. Diclofenac decreased the OCR and increased caspase-3/7 activity. Acetaminophen and ethanol, which are also hepatotoxicants but do not induce idiosyncratic DILI, did not affect the OCR or caspase-3/7 activity. These results indicate that a combination assay of mitochondrial dysfunction and apoptosis is useful for the estimation of potential risk of compounds to induce idiosyncratic DILI.

**Key words:** Idiosyncratic DILI, *In vitro*, Apoptosis, Mitochondrial dysfunction

**INTRODUCTION**

Drug-induced liver injury (DILI) is one of the serious and frequent drug-related adverse events. This adverse event is a main reason for regulatory action pertaining to drugs, including restrictions in clinical indications and withdrawal from clinical trials or the marketplace (Lee, 2003; Smith and Schmid, 2006). DILI is classified into intrinsic and idiosyncratic types. Intrinsic DILI is limited to a few cases in clinical practice, such as acetaminophen (APAP) hepatotoxicity (Russmann et al., 2009), while idiosyncratic DILI has become a major clinical concern because of its unpredictable nature, frequent hospitalization, need for liver transplantation and high mortality. The estimation of the potential for compounds to induce idiosyncratic DILI is very difficult in non-clinical studies because the precise mechanism of idiosyncratic DILI is still unknown.

One of the possible mechanisms of idiosyncratic DILI is inflammation of the liver due to inflammatory cytokines from the macrophages or induction of Th17 which produces IL-17 (Yano et al., 2012). Based on this possible mechanism, there are some *in vivo* non-clinical animal models designed for the estimation of the idiosyncratic DILI. Lipopolysaccharide (LPS) co-treated rats have been reported to be one of the *in vivo* models.
to estimate the potential of idiosyncratic DILI for some drugs, including chlorpromazine (Buchweitz et al., 2002), diclofenac (Deng et al., 2006), ranitidine (Luyendyk et al., 2003), sulindac (Zou et al., 2009) and trovafloxacin (Waring et al., 2006). However, the utility of this model for the estimation of idiosyncratic DILI remains controversial because treatment with LPS intentionally destroys the immune system in normal rats.

Many in vitro assays which estimate the potential of the idiosyncratic DILI have been also reported and one of their advantages is that human hepatocytes can be used (Elaut et al., 2006; Gomez-Lechon et al., 2007; McKim, 2010; Sahi et al., 2010). Among these, some in vitro assays focus on the effects of compounds on mitochondrial function in the human hepatocytes. Mitochondria play a crucial role in cell death (both necrosis and apoptosis) in the hepatocytes and are considered to be one of the main factors determining the idiosyncrasy of human liver (Boelsterli and Lim, 2007). Many drugs which cause idiosyncratic DILI can impair mitochondrial function as assessed experimentally (Wang, 2014; Dykens et al., 2008; Boelsterli and Lee, 2014). Mitochondrial dysfunction includes uncoupling of oxidative phosphorylation, inhibition of complex I/III, an increase in mitochondrial oxidant stress, an increase in cellular calcium ion and activation of c-Jun N-terminal kinase (JNK), some of which induce stress, an increase in cellular calcium ion and activation of complex I/III, an increase in mitochondrial oxidant stress, some of which induce apoptosis (Armstrong, 2006). Idiosyncratic DILI is also known to be related to apoptosis of the hepatocytes. Trovafloxacin, which causes idiosyncratic DILI, induces hepatocellular apoptosis in vitro when tumor necrosis factor-α (TNF-α) is co-treated. TNF-α activates caspase cascade and leads to apoptotic cell death (Beggs et al., 2014).

In order to establish an in vitro assay for the estimation of the potential risk of idiosyncratic DILI, we focused on mitochondrial dysfunction and apoptosis induced by compounds. We investigated the usefulness of combination assays measuring the reduction of oxygen consumption rate (OCR) and induction of caspase-3/7 activity which are endpoints of mitochondrial dysfunction and apoptosis, respectively, using cryopreserved human primary hepatocytes of the same lot (Caucasian, female, aged 68 years). Reproducibility of the results was confirmed using another lot of the hepatocytes.

Cryopreserved hepatocytes were thawed in a 37°C-water bath and transferred into Cryopreserved Hepatoocytes Recovery Medium (Invitrogen). The cell suspension was centrifuged at 750 rpm for 10 min at room temperature and the supernatant was removed. The pelleted cells were suspended in plating medium (William’s medium E (Invitrogen) containing Hepatocytes Plating Supplement Pack (Invitrogen)) and part of the cell suspension was stained with 0.4% Trypan blue (Invitrogen) and the number of cells was counted microscopically using a cell counting chamber. Aliquots of the hepatocytes suspension (6 x 10⁴ cells/100 μL/well) were added to the collagen-coated culture plate (percentage of live cells was more than 90%). A 96-well white cell culture plate (clear bottom) was used for the measurements of cell viability and caspase-3/7 activity and a 24-well cell culture plate designed for XF24 Extracellular Flux Analyzer (Seahorse bioscience, Inc., North Billerica, MA, USA) was used for the measurements of OCR. The medium was replaced with 200 μL incubation medium (William’s medium E containing Hepatocytes Maintenance Supplement Pack (Invitrogen)) after 4 to 6 hr of plating. Stationary culturing was carried out at 37°C in a humidified (100%) atmosphere containing 5% CO₂. In all the assays, 3 to 4 wells were used per dose.

**Measurements of cell viability and caspase-3/7 activity**

Treatment with compounds was conducted on the next day of cell plating and the hepatocytes were treated with compounds for 24 hr. The formulations of compounds were prepared with incubation medium (including 1% dimethyl sulfoxide (DMSO)). The activities of live cell protease and caspase-3/7 were measured as indices of cell viability and apoptosis, respectively, using the commercial ApoLive-Glo™ Multiplex Assay (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer’s instructions. A microplate reader (Infiniti M200Pro, Tecan Group Ltd. (Zürich, Switzerland)) and

**Materials and Methods**

**Materials**

APAP, leflunomide and diclofenac were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Troglitazone and ranitidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other reagents were the highest grade available.

**Cell culture**

Commercial cryopreserved human primary hepatocytes obtained from Gibco™ (Invitrogen, Carlsbad, CA, USA) were used. This study was conducted using the hepatocytes of the same lot (Caucasian, female, aged 68 years). Reproducibility of the results was confirmed using another lot of the hepatocytes.
analysis software (Magellan V7.2, Tecan Group Ltd.) were used for the measurements of fluorescence and luminescence. The cell viability and caspase-3/7 activity were represented as relative fluorescence units (RFU) and relative luminescence units (RLU), respectively.

**Measurements of OCR**

Assay was conducted on the next day of cell plating. Assay medium (Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, without glucose, L-glutamine, phenol red, sodium pyruvate and sodium bicarbonate, powder) containing 11 mM glucose, 4 mM sodium pyruvate and Glutamax (Invitrogen)) was prepared and pH was adjusted to 7.4 at 37°C by adding NaOH immediately before use. The formulations of compounds were prepared with assay medium (including 10% DMSO (final concentration: 1%)). All compounds were treated at the concentration of one-tenth of that for the measurements of cell viability and caspase-3/7 activity because measurements of OCR should be conducted at the non-cytotoxic concentration of compounds for accurate evaluation of mitochondrial function. The OCR was measured using the XF24 Extracellular Flux Analyzer (Fluxanalyzer, Seahorse Bioscience, Inc.). From the day before measurement, the XF assay cartridge (Seahorse Bioscience, Inc.) was hydrated with 1 mL Calibrant solution (Seahorse Bioscience, Inc.) in a 37°C non-CO₂ incubator. The incubation medium was replaced with 675 μL of assay medium and the culture plate was incubated for 1 hr in the 37°C non-CO₂ incubator. The compounds or vehicle solution (75 μL) and the carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) solution (83.3 μL) were loaded automatically in the XF24 Extracellular Flux Analyzer. After cell incubation for 1 hr, measurement of the OCR was conducted. The measurements were conducted using the following procedure: baseline data of the OCR were collected in the 1st loop (mix-wait-measurement: 4-2-2 min, 3 times). After injection of the compounds or vehicle solution, the 2nd loop (mix-wait-measurement: 4-2-2 min, 4 times) was conducted and the OCR were measured. After injection of FCCP solution (final concentration: 0.03 μM), the 3rd loop (mix-wait-measurement: 4-2-2 min, 4 times) was conducted and the OCR were measured.

Data were analyzed using the XF24 Analyzer Software v1.8.0.14 (Seahorse Bioscience, Inc.). For the calculation of the area under the curve (AUC) of the OCR before and after injection of FCCP, the OCR values were corrected with the OCR value immediately before injection of the compounds or FCCP, respectively, and used for the calculation of the AUC. The image of the calculation of the AUC is shown in Fig. 1.

**Statistical analysis**

Values are expressed as mean ± S.D. Statistical analysis was conducted for comparison of AUC of the OCR before and after treatment with FCCP between the control and treatment groups. The data were analyzed by a Dunnett test or One-Way ANOVA. The levels of significance were set at 5% and 1% (two-tailed).

**RESULTS**

**Troglitazone**

The AUC of the OCR before treatment with FCCP was decreased dose-dependently at 12.5 μM and above (Fig. 2A). The AUC of the OCR after treatment with FCCP was decreased dose-dependently from the lowest dose level (Fig. 2B). The cell viability was markedly decreased at 125 μM and above with an approximate IC₅₀ value ranged between 62.5 and 125 μM (Table 1). Although a very slight increase in caspase-3/7 activity was observed at 125 μM (Fig. 3), an increase in caspase-3/7 activity was not observed at any dose level in another lot of the hepatocytes (data not shown). Therefore, this finding was not reproducible and was considered not to be meaningful.

**Table 1. Results of the combination assays.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Idiosyncratic DILI</th>
<th>Mitochondrial dysfunction</th>
<th>Apoptosis</th>
<th>Approximate IC₅₀* (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troglitazone</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>62.5 - 125</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>250 - 500</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2,500 - 5,000</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>250 - 500</td>
</tr>
<tr>
<td>APAP</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>5,000 - 10,000</td>
</tr>
<tr>
<td>Ethanol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&gt; 10,000</td>
</tr>
</tbody>
</table>

+: Positive, -: Negative, *: Cell viability is shown as an approximate IC₅₀ value.
Leflunomide

The AUC of the OCR before treatment with FCCP was decreased dose-dependently at 12.5 μM and above (Fig. 4A). The AUC of the OCR after treatment with FCCP did not change at any dose level (Fig. 4B). The cell viability was decreased dose-dependently after treatment with leflunomide at 62.5 μM and above with an approximate IC50 value ranged between 250 and 500 μM.

Fig. 1. The image of the calculation of the data obtained using Fluxanalyzer. (A) Calculation of the AUC of the OCR before treatment with FCCP, (B) Calculation of the AUC of the OCR after treatment with FCCP.

Fig. 2. AUC of the OCR in the human hepatocytes treated with troglitazone. (A) AUC of the OCR before treatment with FCCP, (B) AUC of the OCR after treatment with FCCP. Each bar represents mean ± S.D. with 3-4 determinations. Significantly different from control (Dunnett test or One-Way ANOVA): ** P < 0.01.
A marked decrease in caspase-3/7 activity was observed from the lowest dose level (Fig. 5).

**Ranitidine**

The AUC of the OCR before treatment with FCCP did not change at any dose level (Fig. 6A). Although there were some statistically significant differences from the control in the AUC of the OCR after treatment with FCCP, these findings were slight and not dose-dependent indicating that there were no effects of ranitidine on the AUC of the OCR after treatment with FCCP (Fig. 6B). The cell viability was markedly decreased after treatment at 2,500 μM and above with an approximate IC₅₀ value ranged between 2,500 and 5,000 μM (Table 1). An increase in caspase-3/7 activity was observed at 10,000 μM (Fig. 7).

**Diclofenac**

The AUC of the OCR before treatment with FCCP did not change at any dose level (Fig. 8A). The AUC of the OCR after treatment with FCCP was decreased dose-dependently at 25 μM and above (Fig. 8B). The cell viability was markedly decreased after treatment with diclofenac at 250 μM and above with an approximate IC₅₀ value ranged between 2,500 and 5,000 μM (Table 1). An increase in caspase-3/7 activity was observed at 10,000 μM (Fig. 7).
between 250 and 500 μM (Table 1). An increase in caspase-3/7 activity was observed at 250, 500 and 1,000 μM (Fig. 9).

### Acetaminophen and ethanol

The AUC of the OCR before and after treatment with FCCP did not change at up to 1,000 μM (Fig. 10). The cell viability was slightly decreased after treatment with APAP at 2,500 μM and above with an approximate IC₅₀ value ranging between 5,000 and 10,000 μM (Table 1) and did not change after treatment with ethanol at up to 10,000 μM with an approximate IC₅₀ value greater than 10,000 μM (Table 1). Caspase-3/7 activity did not change at any dose level after treatment with APAP at up to 10,000 μM. A slight increase in caspase-3/7 activity was observed at 1,250 μM and above after treatment with ethanol (Fig. 11). Since this increase in caspase-3/7 activity was very slight and correlated with the number of hepatocytes, this finding was considered not to be due to an induction of apoptosis.

### DISCUSSION

In order to predict idiosyncratic DILI, many novel in vitro and in vivo approaches are being developed. In vitro approaches have many advantages; human hepatocytes...
cytes can be used and many compounds can be assessed in a screening assay with small amounts of compound. It has been reported that idiosyncratic DILI is closely related to mitochondrial dysfunction (Wang, 2014; Dykens et al., 2008; Boelsterli and Lee, 2014) and apoptosis of the hepatocytes (Beggs et al., 2014). In the present study, in order to establish an \textit{in vitro} assay for the estimation of the potential risk of the idiosyncratic DILI, we employed a combination assay of two endpoints, mitochondrial dysfunction and apoptosis, using cryopreserved human hepatocytes as a new approach.

Mitochondrial dysfunction leads to the release of cytochrome c and eventually apoptosis. There are a lot of mechanisms to induce mitochondrial dysfunction such as the accumulation of compounds in the mitochondria, the mutation of the mitochondrial DNA and suppression of mitochondrial respiration of course. Measurements of OCR are known as one of the most sensitive methods for the evaluation of mitochondrial function, namely the direct and rapid suppression of mitochondrial respiration caused by the compounds.

In the present study, we measured the OCR before treatment with an uncoupler, FCCP, and after treatment with FCCP. The change in OCR caused by compounds...
Fig. 9. Cell viability and caspase-3/7 activity in the human hepatocytes treated with diclofenac. Concentrations: 0, 250, 500, 1,000, 2,000 and 4,000 μM. Each points represents mean ± S.D. with 4 determinations.

Fig. 10. AUC of the OCR in the human hepatocytes treated with APAP and ethanol. (A) AUC of the OCR of APAP before treatment with FCCP, (B) AUC of the OCR of APAP after treatment with FCCP, (C) AUC of the OCR of ethanol before treatment with FCCP, (D) AUC of the OCR of ethanol after treatment with FCCP. Each bar represents mean ± S.D. with 3-4 determinations. Not significantly different from control (Dunnett test or One-Way ANOVA)
before treatment with FCCP indicates the effects of compounds on basal mitochondrial function and the change in OCR caused by compounds after treatment with FCCP indicates the effects of compounds on maximum mitochondrial function. We examined these values as the AUC of the OCR before and after treatment with FCCP corrected with the OCR values immediately before injection of compounds or FCCP, respectively (Fig. 1). We also measured caspase-3/7 activity at 24 hr after treatment with the compounds as an index of apoptosis. Although caspase-3/7 activity has been measured after short-term treatment with compounds in some reports (Gerets et al., 2009), we employed caspase-3/7 activity after 24 hr treatment with compounds to detect apoptosis induced by mitochondrial dysfunction through various mechanisms including not only direct and rapid suppression of the mitochondrial respiration but also other mechanisms.

The results of the multiple assays in this report are summarized in Table 1.

Troglitazone induced mitochondrial dysfunction (Fig. 2), but did not induce apoptosis (Fig. 3). The absence of apoptosis of the hepatocytes treated with troglitazone in our assay system was considered to be related to the metabolism of troglitazone. Troglitazone itself is known to have various cytotoxic effects including mitochondrial toxicity but the metabolites of troglitazone, including

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**Fig. 11.** Cell viability and caspase-3/7 activity in the human hepatocytes treated with APAP and ethanol. (A) Cell viability and caspase-3/7 activities of APAP, (B) Cell viability and caspase-3/7 activities of ethanol. Concentrations: 0, 625, 1,250, 2,500, 5,000 and 10,000 μM. Each points represents mean ± S.D. with 4 determinations.
troglitazone-sulfate, do not have such a cytotoxic effect (Masubuchi, 2006). After the 24 hr-treatment in hepatocytes, troglitazone may be metabolized into non-cytotoxic metabolites. Another possible explanation for the absence of apoptosis even at the concentration which was ten-fold higher than the concentration employed in mitochondrial toxicity assay is that the decrease in OCR is slight and cell viability was maintained by other energy pathways such as glycolytic system.

Leflunomide also induced mitochondrial dysfunction (Fig. 4), indicating that leflunomide itself has a potential to induce mitochondrial dysfunction as well as troglitazone. On the other hand, leflunomide decreased markedly caspase-3/7 activity from the lowest dose level, this was considered to be due to the effects of leflunomide on the Akt signal pathway which regulates the caspase pathway; leflunomide is known to inhibit the pro-apoptotic MAPK family member JNK1/2 and the pro-apoptotic protein Bel-2 (Vrenken et al., 2008). Cytotoxicity of leflunomide detected after a 24 hr treatment (an approximate IC50 value being 250 μM) was also strong as well as troglitazone, indicating that leflunomide induced cytotoxicity through mitochondrial dysfunction which is independent of apoptosis. The results obtained with troglitazone and leflunomide indicate that a combination of different endpoints is useful when one endpoint does not work due to metabolism of cytotoxic parent compounds or the pharmacological feature of compounds.

Ranitidine induced apoptosis (Fig. 6), but did not induce mitochondrial dysfunction (Fig. 7). As mentioned above, the mitochondrial dysfunction detected in this assay system is limited to the direct and rapid suppression of mitochondrial respiration by compounds. Ranitidine is considered to induce apoptosis through mechanisms other than direct and rapid suppression of mitochondrial respiration.

Diclofenac induced both mitochondrial dysfunction and apoptosis (Fig. 8 and Fig. 9). These results indicate that diclofenac induces apoptosis through inhibition of mitochondrial respiration. The results obtained in our assay system for diclofenac are reasonable taking into account the evidence that both diclofenac itself and its metabolites, including 5-hydroxydiclofenac, have cytotoxic effects (Bort et al., 1999).

In the analyses in the Fluxanalyzer, the AUC of the OCR before treatment with FCCP is an index for the basal mitochondrial function and the AUC of the OCR after treatment with FCCP is an index for the maximum mitochondrial function. Since adenosine triphosphate is produced by the basal mitochondrial function, the effect on the basal mitochondrial function is more important than that on the maximum mitochondrial function. Troglitazone affected both basal and maximum mitochondrial function, leflunomide affected only basal mitochondrial function and diclofenac affected only maximum mitochondrial function. Therefore, the magnitude of the potential of mitochondrial toxicity of these compounds is considered to be graded as troglitazone, leflunomide and diclofenac in descending order. This order is consistent with the order of drug label for DILI (troglitazone: withdrawn, leflunomide: boxed warning, diclofenac: warning and precautions) (Chen et al., 2011).

Based on the results obtained for troglitazone and diclofenac, our combination assay is considered to be useful for the estimation of the potential risk of idiosyncratic DILI for not only compounds for which the parent compounds themselves take part in the induction of idiosyncratic DILI but also compounds for which their metabolites take part in the induction of idiosyncratic DILI.

APAP and ethanol are also hepatotoxicant, but do not induce idiosyncratic DILI (Table 1). These compounds did not induce mitochondrial dysfunction or apoptosis in our assay systems at up to the highest concentration of compounds inducing idiosyncratic DILI (Fig. 10 and Fig. 11). An approximate IC50 values were greater than 5,000 μM for these compounds (Table 1).

APAP is known to induce hepatotoxicity through its reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) (Dahlin et al., 1984). APAP is usually metabolized by uridine diphosphate glucuronosyltransferase and sulfotransferase and NAPQI is not produced unless glucuronidation and sulfate conjugation of APAP is saturated. The absence of mitochondrial dysfunction in our assay system supports the position that APAP itself does not cause mitochondrial toxicity. Under the condition of our apoptosis assay, we consider that APAP was metabolized by glucuronidation and sulfate conjugation or NAPQI was detoxified by glutathione and these metabolic detoxification of APAP resulted in the absence of apoptosis.

Hepatotoxicity induced by ethanol is known to be related to the direct cytotoxicity of acetaldehyde, the metabolite of ethanol, or hepatic steatosis through inhibition of the beta-oxidation of fatty acids due to excessive production of NADH in the process of metabolism of ethanol and acetaldehyde after treatment with ethanol with extremely high dose levels and/or for a long time (Liu et al., 2005). The absence of mitochondrial dysfunction and apoptosis in our assay system is reasonable taking into account these mechanisms of hepatotoxicity induced by ethanol.

In conclusion, the combination assay of mitochondrial
dysfunction and apoptotic effect caused by compounds is considered to be useful for estimation of potential risk of compounds to induce idiosyncratic DILI.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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