



Original Article

Usefulness of HepaRG cells in the mitochondrial function assay for the estimation of potential risk of idiosyncratic drug-induced liver injury

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ABSTRACT — Drug-induced liver injury (DILI) is one of the serious and frequent drug-related adverse events and is classified into intrinsic and idiosyncratic types. Almost all of DILI caused in humans is known to be idiosyncratic type. The estimation of the potential risk for a drug candidate to induce idiosyncratic DILI is important to facilitate the development of new drugs, however, the estimation is difficult from the results of non-clinical toxicity studies using animals. We have previously reported the *in vitro* combination assay of mitochondrial function and apoptosis using human primary hepatocytes as a useful model for estimation of the risk of idiosyncratic DILI. In this study, to improve the *in vitro* assay for estimation of the risk of idiosyncratic DILI, we evaluate the usefulness of HepaRG cells for the mitochondrial function assay. We measured the oxygen consumption rate (OCR) as an endpoint of mitochondrial function in HepaRG cells treated with some compounds causing idiosyncratic DILI (troglitazone, leflunomide, ranitidine and diclofenac) and others known not to cause idiosyncratic DILI (acetaminophen and ethanol) and compared the results in HepaRG cells and with those for human primary hepatocytes as previously reported. As the results, HepaRG cells showed comparable or even higher sensitivity for detecting mitochondrial dysfunction than human primary hepatocytes, in all tested compounds. Taking into account these results and many other useful properties of the cells, HepaRG cells are considered to be much more suitable for this mitochondrial function assay than the human primary hepatocytes.

Key words: Idiosyncratic Drug-induced liver injury, HepaRG cells, Mitochondrial function assay

INTRODUCTION

Drug-induced liver injury (DILI) is one of the most serious and frequent drug-related adverse events in humans and 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, and causing 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 using animals and many novel *in vitro* approaches are being developed to predict idiosyncratic DILI (Elaut *et al.*, 2006; Gomez-Lechon *et al.*, 2010; Greer *et al.*, 2010; Hewitt *et al.*, 2007; McKim, 2010; Sahi *et al.*, 2010; Vinken *et al.*, 2012).

We have previously reported the *in vitro* combination

assay of mitochondrial function and apoptosis in human primary hepatocytes as a useful tool for estimation of risk of idiosyncratic DILI (Goda *et al.*, 2016). Including our *in vitro* assay, the use of human primary hepatocytes is considered to be a gold standard for the estimation of the potential risk of idiosyncratic DILI in human because they contain natural enzyme clusters, co-substrates and drug transporters (Guillouzo *et al.*, 2007). However, there are several important limitations for the use of human primary hepatocytes such as unpredictable availability, large inter-donor variation, difficulty in handling and high cost. In our *in vitro* combination assay, these limitations sometimes became a problem.

HepaRG cells are derived from a hepatocholangiocarcinoma of a female patient and have the ability to differentiate into both the biliary and hepatocyte lineage when treated with dimethyl sulfoxide (DMSO) (Cerec *et al.*, 2007; Gripon *et al.*, 2002). Differentiated HepaRG cells contain hepatocyte-like and biliary-like epithelium cells with a hepatocyte-like population of approximately 50-55% and maintain hepatic functions with relevant gene expressions for various liver-specific proteins, including cytochrome P450s (CYPs), transporters and enzymes of Phase II metabolism (Aninat *et al.*, 2006; Kanebratt and Andersson, 2008). In addition, their inter-lot and inter-vial variations are considered much smaller than those of human primary hepatocytes.

In this study, to improve the *in vitro* assay for estimation of risk of idiosyncratic DILI, we evaluated the usefulness of HepaRG cells for the mitochondrial function assay. We measured the oxygen consumption rate (OCR) as an endpoint of mitochondrial function in HepaRG cells treated with some compounds causing idiosyncratic DILI (troglitazone, leflunomide, ranitidine and diclofenac) and others not causing idiosyncratic DILI (APAP and ethanol) and compared the results in HepaRG cells with those for the human primary hepatocytes as previously reported (Goda *et al.*, 2016).

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

Commercially available HepaRG cells obtained from

Gibco™ (Invitrogen, Carlsbad, CA, USA) were used. This study was conducted using two lots (Lot. Nos. 1728239 and 1781094).

Cryopreserved HepaRG cells were thawed in a 37°C-water bath and transferred into plating medium (William's medium E (Invitrogen) containing HepaRG™ Maintenance/Metabolism Medium Supplement (Invitrogen) and GlutaMax™ (Invitrogen)). The cell suspension was centrifuged at 375 g for 2 min at room temperature and the supernatant was removed. The pelleted cells were suspended in plating medium 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 hepatocyte suspension (1×10^5 cells/100 μ L/well) were added to a collagen-coated culture plate. A 24 well cell culture plate designed for XF24 Extracellular Flux Analyzer (Seahorse bioscience, Inc., North Billerica, MA, USA) was used for the assay of the mitochondrial function. The medium was replaced with 200 μ L incubation medium (William's medium E containing HepaRG™ Tox Medium Supplement (Invitrogen) and GlutaMax™) according to the User Guide. 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 Oxygen Consumption Rate (OCR) and Extra-Cellular Acidification Rate (ECAR)

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 the pH was adjusted to 7.4 at 37°C by adding NaOH immediately before use. The formulations of the compounds were prepared with assay medium (including 10% DMSO (final concentration: 1%)). The OCR was measured using the XF24 Extracellular Flux Analyzer (Flux analyzer, 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 μ L of assay medium and the culture plate was incubated for 1 hr in the 37°C non-CO₂ incubator. The compounds or the vehicle solution (75 μ L) and the carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) solution (83.3 μ L) were loaded automatically in the XF24 Extracellular Flux Analyzer. After cell incubation for 1 hr, the

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OCR was measured. 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 the FCCP solution (final concentration: 1 μM), the 3rd loop (mix-wait-measurement: 4-2-2 min, 4 times) was conducted and the OCR was measured. The ECAR was measured by the XF24 Extracellular Flux Analyzer simultaneously for the OCR when diclofenac was treated in HepaRG cells.

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 and ECAR before and after injection of FCCP, the OCR and ECAR values were corrected with the value immediately before injection of the compounds or FCCP, respectively. In this assay, the AUC of the OCR before treatment with FCCP (AUC of the Basal OCR) is an index for the basal mitochondrial function and the AUC of the OCR after treatment with FCCP (AUC of the Maximum OCR) is an index for the maximum mitochondrial function.

Statistical analysis

All numerical data are shown as mean + standard deviation. The differences in the data were determined by one-way analysis of variance (ANOVA), followed by pairwise comparisons (Dunnett's test) using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). The levels of significance were set at 5%, 1% and 0.1% (two-tailed).

RESULTS

Troglitazone

In Lot. 1728239 of HepaRG cells, the AUC of the basal and maximum OCR was decreased dose-dependently from the lowest dose level, 6.25 μM (Fig. 1A and 1B). In Lot. 1781094 of HepaRG cells, the AUC of the basal OCR was decreased dose-dependently at 25 μM and above (Fig. 1C). The AUC of the maximum OCR was decreased dose-dependently from the lowest dose level, 6.25 μM (Fig. 1D).

Leflunomide

In Lot. 1728239 of HepaRG cells, the AUC of the basal OCR was decreased dose-dependently at 12.5 μM and above (Fig. 2A). The AUC of the maximum OCR

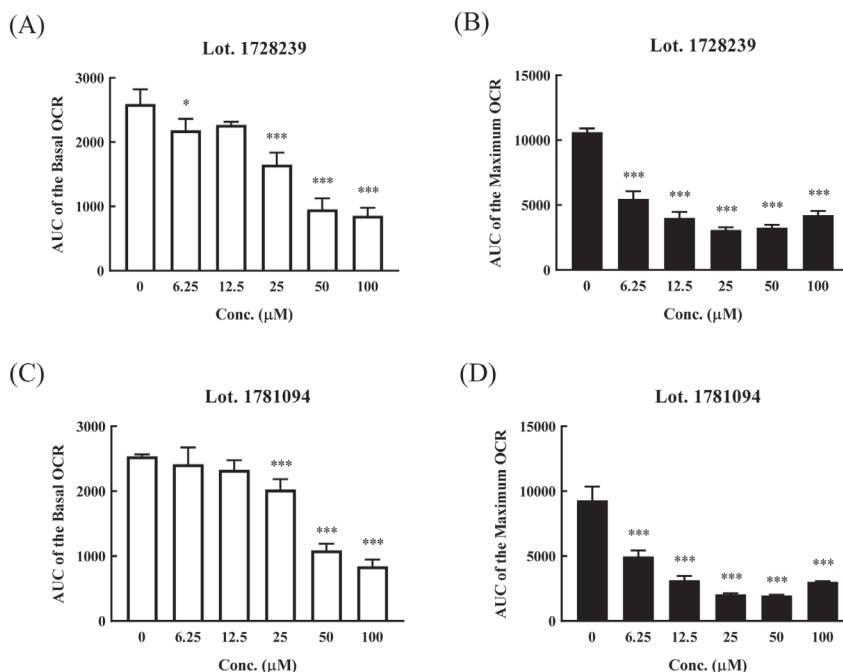


Fig. 1. AUC of the OCR in the HepaRG cells treated with troglitazone. (A) AUC of the basal OCR in Lot. 1728239, (B) AUC of the maximum OCR in Lot. 1728239. (C) AUC of the basal OCR in Lot. 1781094, (D) AUC of the maximum OCR in Lot. 1781094. Each bar represents mean + S.D. with 4 determinations. Significantly different from control (Dunnett's test): * $P < 0.05$, *** $P < 0.001$

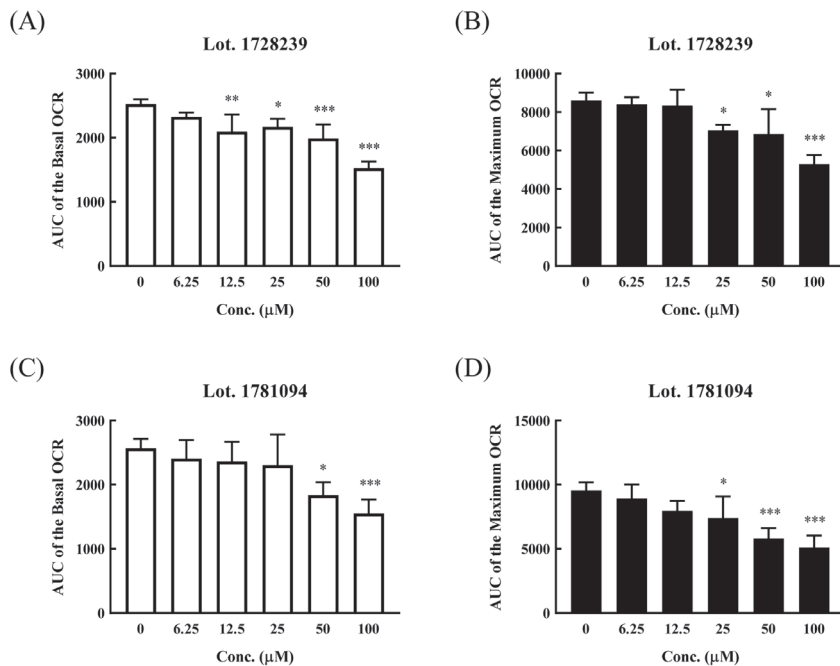


Fig. 2. AUC of the OCR in the HepaRG cells treated with leflunomide. (A) AUC of the basal OCR in Lot. 1728239, (B) AUC of the maximum OCR in Lot. 1728239. (C) AUC of the basal OCR in Lot. 1781094, (D) AUC of the maximum OCR in Lot. 1781094. Each bar represents mean + S.D. with 4 determinations. Significantly different from control (Dunnett's test): * P < 0.05, ** P < 0.01, *** P < 0.001.

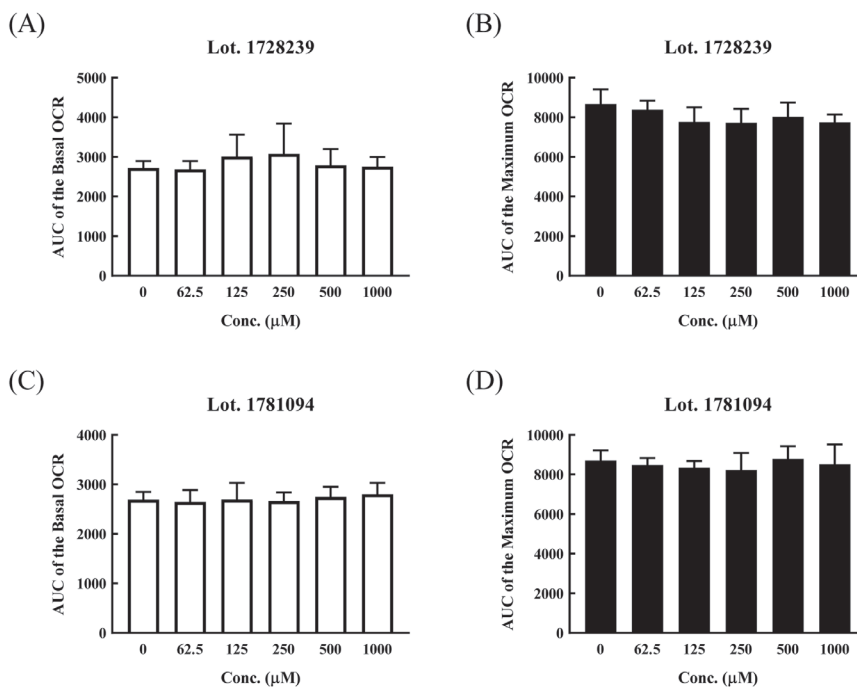


Fig. 3. AUC of the OCR in the HepaRG cells treated with ranitidine. (A) AUC of the basal OCR in Lot. 1728239, (B) AUC of the maximum OCR in Lot. 1728239. (C) AUC of the basal OCR in Lot. 1781094, (D) AUC of the maximum OCR in Lot. 1781094. Each bar represents mean + S.D. with 4 determinations. Not significantly different from control (Dunnett's test).

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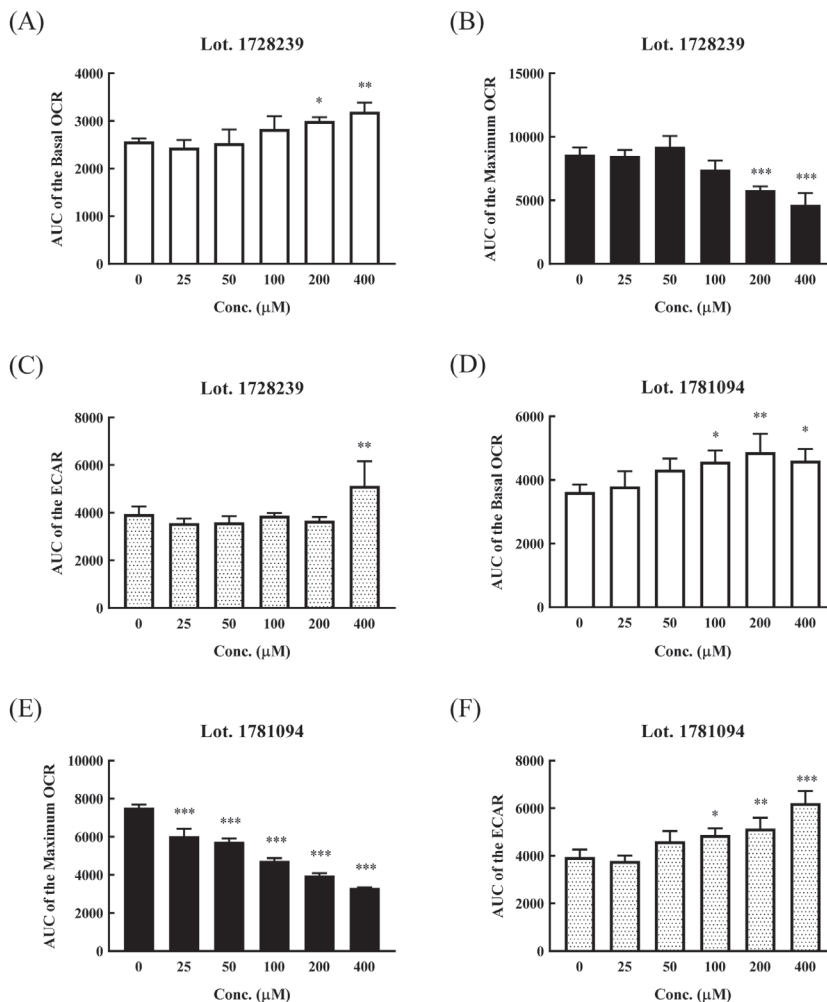


Fig. 4. AUC of the OCR in the HepaRG cells treated with diclofenac. (A) AUC of the basal OCR in Lot. 1728239, (B) AUC of the maximum OCR in Lot. 1728239. (C) AUC of the ECAR before treatment with FCCP in Lot. 1728239. (D) AUC of the basal OCR in Lot. 1781094, (E) AUC of the maximum OCR in Lot. 1781094, (F) AUC of the ECAR before treatment with FCCP in Lot. 1781094. Each bar represents mean + S.D. with 3-4 determinations. Significantly different from control (Dunnett's test): * P < 0.05, ** P < 0.01, *** P < 0.001.

was decreased dose-dependently at 25 μM and above (Fig. 2B). In Lot. 1781094 of HepaRG cells, the AUC of the basal OCR was decreased dose-dependently at 50 μM and above (Fig. 2C). The AUC of the maximum OCR was decreased dose-dependently at 25 μM and above (Fig. 2D).

Ranitidine

In both lots, the AUC of the basal and maximum OCR did not change at any dose level (Fig. 3A to 3D).

Diclofenac

In Lot. 1728239 of HepaRG cells, the AUC of the basal OCR was increased dose-dependently at 200 μM and above (Fig. 4A). The AUC of the maximum OCR was decreased dose-dependently at 200 μM and above (Fig. 4B). To clarify whether this change indicated mitochondrial dysfunction or not, we evaluate the changes of ECAR (index of glycolysis) after the treatment with diclofenac. The AUC of the ECAR before treatment with FCCP was increased at 400 μM (Fig. 4C). In Lot. 1781094 of HepaRG cells, the AUC of the basal OCR was increased dose-dependently at 100 μM and above

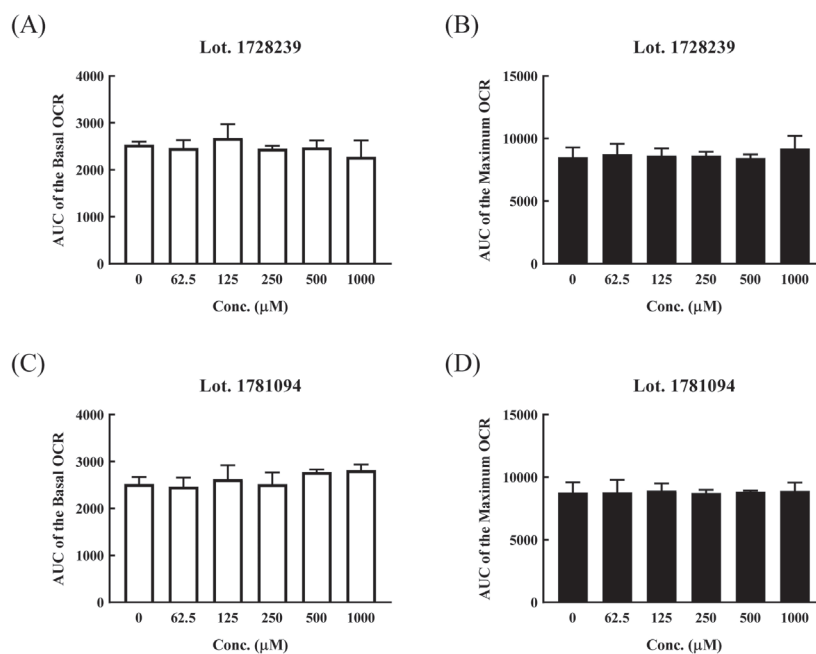


Fig. 5. AUC of the OCR in the HepaRG cells treated with APAP. (A) AUC of the basal OCR in Lot. 1728239, (B) AUC of the maximum OCR in Lot. 1728239. (C) AUC of the basal OCR in Lot. 1781094, (D) AUC of the maximum OCR in Lot. 1781094. Each bar represents mean + S.D. with 3-4 determinations. Not significantly different from control (Dunnett's test).

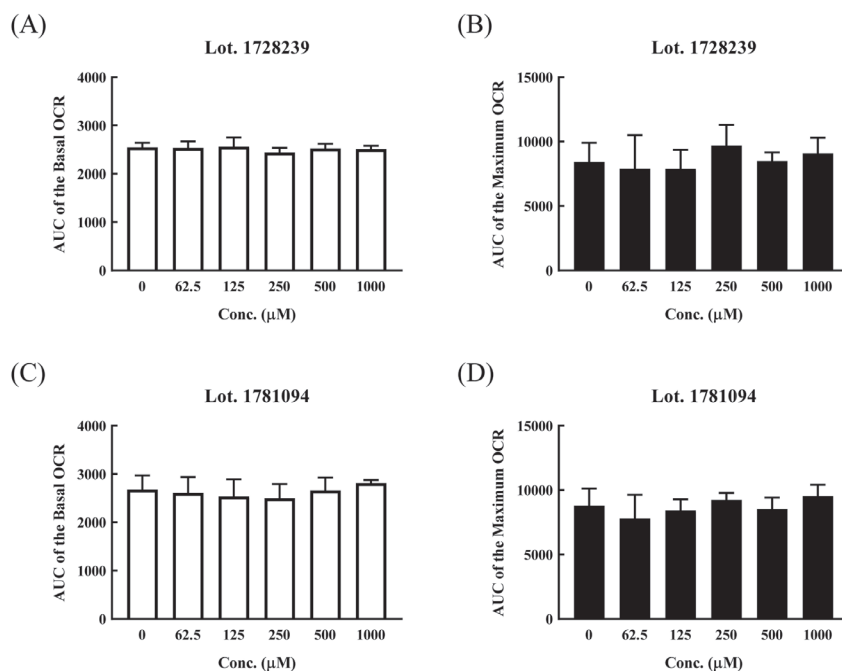


Fig. 6. AUC of the OCR in the HepaRG cells treated with ethanol. (A) AUC of the basal OCR in Lot. 1728239, (B) AUC of the maximum OCR in Lot. 1728239. (C) AUC of the basal OCR in Lot. 1781094, (D) AUC of the maximum OCR in Lot. 1781094. Each bar represents mean + S.D. with 3-4 determinations. Not significantly different from control (Dunnett's test).

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(Fig. 4D). The AUC of the maximum OCR was decreased dose-dependently from the lowest dose level, 25 μM (Fig. 4E). The AUC of the ECAR before treatment with FCCP was increased at 100 μM and above (Fig. 4F).

Acetaminophen and Ethanol

In both lots, The AUC of the basal and maximum OCR did not change at up to 1000 μM (Figs. 5 and 6).

DISCUSSION

The estimation of the potential risk for drug candidate to induce idiosyncratic DILI is important to facilitate the development of new drugs, however, the estimation is difficult from the results of non-clinical toxicity studies using animals (Goda *et al.*, 2019). *In vitro* approaches have many advantages; human hepatocytes can be used, and many compounds can be assessed in a screening assay with small amounts of compound. Although human primary hepatocytes are considered as the gold standard for the estimation of the potential risk of the idiosyncratic DILI *in vitro* (Guillouzo *et al.*, 2007), there are several important limitations for the use of human primary hepatocytes such as unpredictable availability, large inter-donor variation, difficulty in handling and high cost.

HepaRG cells are shown to maintain hepatic functions and to express genes for various liver-specific proteins, including CYPs, transporters and the enzymes of Phase II metabolism. Because their inter-lot and inter-vial variations are considered much smaller than those of human primary hepatocytes, *in vitro* assays using HepaRG cells are increasing year by year.

In the present study, we evaluate the usefulness of HepaRG cells for the mitochondrial function assay to improve the *in vitro* assay for estimation of risk of idio-

syncratic DILI. We measured the OCR before treatment with an uncoupler, FCCP, and after treatment with FCCP. In the analyses of mitochondrial dysfunction, the AUC of the OCR before treatment with FCCP is used as 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.

The results of the assays of mitochondrial function in HepaRG cells and those in human primary hepatocytes are summarized in Table 1.

Troglitazone affected both basal and maximum mitochondrial function. In both the basal and maximum mitochondrial function, the sensitivity for detecting mitochondrial dysfunction indicated in the HepaRG cells was comparable to that in human primary hepatocytes previously reported. There were no marked differences in the sensitivity for detecting mitochondrial dysfunction between two lots of HepaRG cells. Leflunomide affected both the basal and maximum mitochondrial function. In basal mitochondrial function, the sensitivity for detecting mitochondrial dysfunction indicated in the HepaRG cells was comparable to that in human primary hepatocytes. In the maximum mitochondrial function, the sensitivity for detecting mitochondrial dysfunction indicated in HepaRG cells was higher than that in human primary hepatocytes because a decrease in that parameter was observed only in the HepaRG cells (observed in both lots). Ranitidine did not affect either the basal or the maximum mitochondrial function. These results reproduced the results in human primary hepatocytes. Diclofenac increased in the AUC of the OCR before treatment with FCCP and in the AUC of the ECAR before treatment with

Table 1. The concentrations observed with mitochondrial dysfunction in HepaRG cells and human primary hepatocytes.

Index	Cell	Tro (μM)	Lef (μM)	Ran (μM)	Dic (μM)	APAP (μM)	Eth (μM)
Basal mitochondrial function	Primary HepaRG	> 12.5	> 12.5	–	–	–	–
	Lot. 1728239 HepaRG	> 6.25	> 12.5	–	> 200*	–	–
	Lot. 1781094 HepaRG	> 25	> 50	–	> 100*	–	–
Maximum mitochondrial function	Primary HepaRG	> 6.25	–	–	> 25	–	–
	Lot. 1728239 HepaRG	> 6.25	> 25	–	> 200	–	–
	Lot. 1781094 HepaRG	> 6.25	> 25	–	> 25	–	–

–: No change, Tro: troglitazone, Lef: leflunomide, Ran: ranitidine, Dic: diclofenac, APAP: acetaminophen, Eth: ethanol, Primary: human primary hepatocytes. *: uncoupled effect.

The data for human primary hepatocytes was referenced by Goda *et al.*, 2016.

FCCP, indicating that diclofenac has an uncoupling effect. Interestingly, these results were observed only in HepaRG cells (observed in both lots) Since there are some reports indicating diclofenac has an uncoupling effect (Moreno-Sanchez *et al.*, 1999; Masubuchi *et al.*, 1999), the sensitivity to detect the mitochondrial dysfunction was considered to be much higher in HepaRG cells than that in human primary hepatocytes. In the maximum mitochondrial function, the sensitivity for detecting mitochondrial dysfunction indicated in HepaRG cells was comparable to that in human primary hepatocytes.

APAP and ethanol are also known to be hepatotoxicants, but do not induce idiosyncratic DILI. These compounds did not induce mitochondrial dysfunction in either lot of HepaRG cells at up to the highest concentration of the compounds inducing idiosyncratic DILI. These results reproduced the results in human primary hepatocytes.

In conclusion, the sensitivity detecting the mitochondrial dysfunction using HepaRG cells was comparable or even higher than that using human primary hepatocytes. The higher sensitivity detecting the mitochondrial dysfunction of HepaRG cells than that of cryopreserved primary human hepatocytes is considered to be due to the higher bioactivity of the cell, especially mitochondria in HepaRG cells. Taking into account the results of the present study and many other useful properties of the cells, HepaRG cells is considered to be much more suitable for the mitochondrial function assay than the human primary hepatocytes.

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

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