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Original Article

Utility of human hepatocyte spheroids for evaluation of hepatotoxicity

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ABSTRACT — Drug-induced hepatotoxicity is a common reason for discontinuing the development of candidate clinical drugs. In the present study, we investigated the utility of three-dimensionally cultured human hepatocytes (spheroids) for prediction of hepatotoxicity, using a panel of model drugs: acetaminophen, benzbromarone, chlorpromazine, cyclosporin A, diclofenac, fialuridine, flutamide, imipramine, isoniazid, ticlopidine and troglitazone. Cultured spheroids showed a significant increase of albumin secretion from 2 to 7 days; the secretion started to decrease at 14 days, but continued from 14 days to 21 days. The morphology of the spheroids was well maintained for 21 days. Long-term exposure of spheroids to hepatotoxic drugs resulted in concentration-dependent depression of albumin secretion and elevation of aspartate aminotransferase (AST) leakage. The estimated 50% effective concentration (IC_{50}) values for decrease of albumin secretion changed from 7 days to 14 days, but similar values were obtained at 14 and 21 days, except for diclofenac. Since the IC_{50} values and the values of drug concentration inducing 1.2-fold elevation of AST leakage (F1.2) were similar at 14 and 21 days, an incubation period of 14 days was

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considered sufficient. The coefficient of determination (R^2) between IC_{50} values and F1.2 values of all drugs was 0.335. When cyclosporine A and fialuridine were excluded, the value of R^2 became 0.887. The results indicate that the proposed human hepatocyte spheroid assay should be helpful in the evaluation of hepatotoxicity during the early development stage of clinical drug candidates.

Key words: Hepatotoxicity, Human hepatocytes, Spheroid culture, Three-dimensional culture

INTRODUCTION

Drug-induced liver toxicity is a common reason for withdrawal of approved drugs from the market (Lasser *et al*., 2002). At present, *in vivo* animal studies and *in vitro* studies using primary-cultured human hepatocytes are performed to evaluate the hepatotoxicity of drug candidates. However, around 50% of the drugs found to be responsible for liver injury during clinical trials did not cause any liver damage in animals, due to species differences in hepatotoxic responses (Olson *et al*., 2000). Although conventional primary cultures of human hepatocytes are increasingly used to screen for hepatotoxic effects as early as possible in the process of drug development, this approach can identify only about 50% of hepatotoxic drugs and has low sensitivity for detection of drug-induced hepatotoxicity (Xu *et al*., 2008).

Drug-induced toxicity may be caused not only by the parent compounds, but also by their metabolites and intermediates. However, the expression of many metabolic enzymes decreases during the initial cultivation of primary human hepatocytes, which exhibit a concomitant decline in metabolic capacity (Guillouzo and Guguen-Guillouzo, 2008; Hewitt *et al*., 2007). Therefore, primary cultures of hepatocytes may not be suitable for long-term exposure studies of drug candidates to evaluate hepatotoxicity. Instead, the use of three-dimensionally (3D) cultured hepatocytes (hepatocyte spheroids) that better maintain liver-specific functions has been proposed (Meng, 2010).

We recently showed that human hepatocyte spheroids are suitable for long-term metabolic assays (Ohkura *et al*., 2014), and we identified sequential metabolic reactions of diclofenac, midazolam and propranolol by Phase I and Phase II enzymes. Moreover, lamotrigine and salbutamol were metabolized to lamotrigine-N-glucuronide and salbutamol 4-O-sulfate, respectively. These metabolites, which are human-specific, have previously been found in clinical studies, but not in conventional hepatocyte culture systems. These results indicated that human hepatocyte spheroids would be more effective than conventional culture systems to predict hepatotoxicity. In the present study, we investigated the utility of this assay using a panel of drugs for which metabolic enzymes are known to play an important role in the hepatotoxicity. We also included drugs whose hepatotoxicity had not been detected in pre-clinical animal studies, such as troglitazone and fialuridine. The aim of this work was to evaluate the utility of human hepatocyte spheroids for prediction of hepatotoxicity in humans.

MATERIALS AND METHODS

Materials

Acetaminophen, benzbromarone, chlorpromazine, cyclosporine A, diclofenac, flutamide, imipramine, isoniazid, ticlopidine, troglitazone, Dulbecco's modified Eagle's medium (D-MEM) and penicillin (10,000 unit/mL)-streptomycin (10,000 μg/mL) were purchased from Sigma Aldrich (St. Louis, MO, USA). Fialuridine was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Cryopreserved human hepatocytes were purchased from Becton Dickinson (Lot No. 228, Tokyo, Japan). ISOM's medium was purchased from Becton Dickinson. A 3D culture system, Cell-able® with 96-well plates, and RM101 medium were purchased from Toyo Gosei (Tokyo, Japan). 3T3 Swiss Albino cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All other reagents and solvents were commercial products of analytical grade.

3D culture of human hepatocytes and toxicity assays

Feeder cells (3T3 Swiss Albino) were cultured in D-MEM with penicillin (100 unit/mL)-streptomycin (100 μg/mL) and 10% FBS. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Feeder cells were plated at a density of 8×10^3 cells/well on a 96-well Cell-able®. After 3 days, cryopreserved human hepatocytes were seeded. Briefly, they were stored in liquid nitrogen until use, then immediately immersed in water prewarmed to 37°C. After dissolution, they were decanted into ISOM's medium and centrifuged at 50 × g for 5 min. The hepatocytes were resuspended in ISOM's medium containing 10% FBS. Cell viability was assessed

by trypan blue exclusion, and only suspensions with viability of over 80% were used. These were seeded at a density of 2.0×10^4 cells/well on the Cell-able® and maintained for 2 days in RM101 medium (containing 1% FBS) for spheroid formation before assays. In toxicity assays, the hepatocyte spheroids were cultured in RM101 medium containing the test compounds for 21 days. Medium with test compounds was replaced at 0, 2, 5, 7, 9, 12, 14, 16, 19, and 21 days. Human albumin concentration in the collected culture medium was measured using the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) according to the manufacturer's protocol. Aspartate aminotransferase (AST) activity in the collected medium was measured using the Hitachi 7180 Automatic Analyzer system (Hitachi High-Technologies Corporation, Tokyo, Japan). γ-Glutamyl transpeptidase and lactate dehydrogenase were also measured, but these enzymes were secreted by feeder cells, so they were not suitable for evaluation of toxicity. Representative results at days 7, 14 and 21 days are presented.

Data analysis

The 50% effective concentration (IC_{50}) of drugs for decrease of albumin secretion was calculated using JMP® 10 software (SAS Institute Inc., Cary, NC, USA). Drug concentration causing 1.2-fold elevation of AST leakage (F1.2) was calculated by means of the following equations (1, 2):

$$(\text{AST leakage}) = (\text{AST leakage at 0 } \mu\text{M test drug}) \times \exp(A \times C) \quad (1)$$

$$F1.2 = \ln 1.2 / A \quad (2)$$

where A is an exponential constant, and C is the drug concentration.

RESULTS

Characterization of spheroid-cultured primary human hepatocytes

Albumin secretion by spheroids increased significantly from 2 to 7 days after the start of culture (Fig. 1A). The secretion was decreased at 14 days, but continued from 14 days to 21 days. AST leakage in the medium was highest on day 2, and then decreased, remaining stable from day 7 to day 14 (Fig. 1A). The morphology of the spheroids was maintained for 21 days (Fig. 1B).

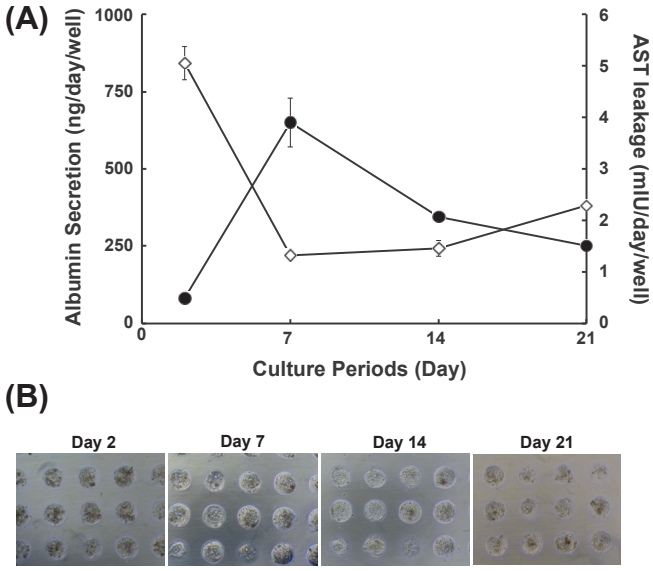

Fig. 1. Characterization of human hepatocytes in spheroid culture. (A) Albumin concentration and AST leakage in spheroid culture medium were measured. Closed circles and open diamonds represent albumin secretion and AST leakage, respectively. Each point represents the mean ± S.E.M. (n = 3). (B) Morphology of human hepatocyte spheroids. Numbers indicate the day from the start (day 0) of the exposure period. Original magnification, × 100.

Detection of drug-induced hepatotoxicity in human hepatocytes spheroids

Spheroids were exposed to a panel of well-known hepatotoxic drugs. AST leakage and albumin secretion in the presence of acetaminophen, fialuridine, and cyclosporine A as representative compounds are shown in Fig. 2. Concentration-dependent depression of albumin secretion and elevation of AST leakage were observed. The estimated IC_{50} values for albumin secretion and the F1.2 values for AST leakage are listed in Tables 1 and 2, respectively. The IC_{50} values for albumin secretion changed from 7 to 14 days, but similar values were observed at 14 and 21 days, except for diclofenac. The IC_{50} values of most drugs were within twenty times the reported maximum plasma concentration (C_{max}), except in the case of imipramine. The correlation between IC_{50} values for albumin secretion and F1.2 values for AST leakage is shown in Fig. 3. The coefficient of determination (R^2) among IC_{50} values and F1.2 values of all drugs in Fig. 3 was 0.338. When cyclosporine A and fialuridine were excluded, the value of the coefficient became 0.887.

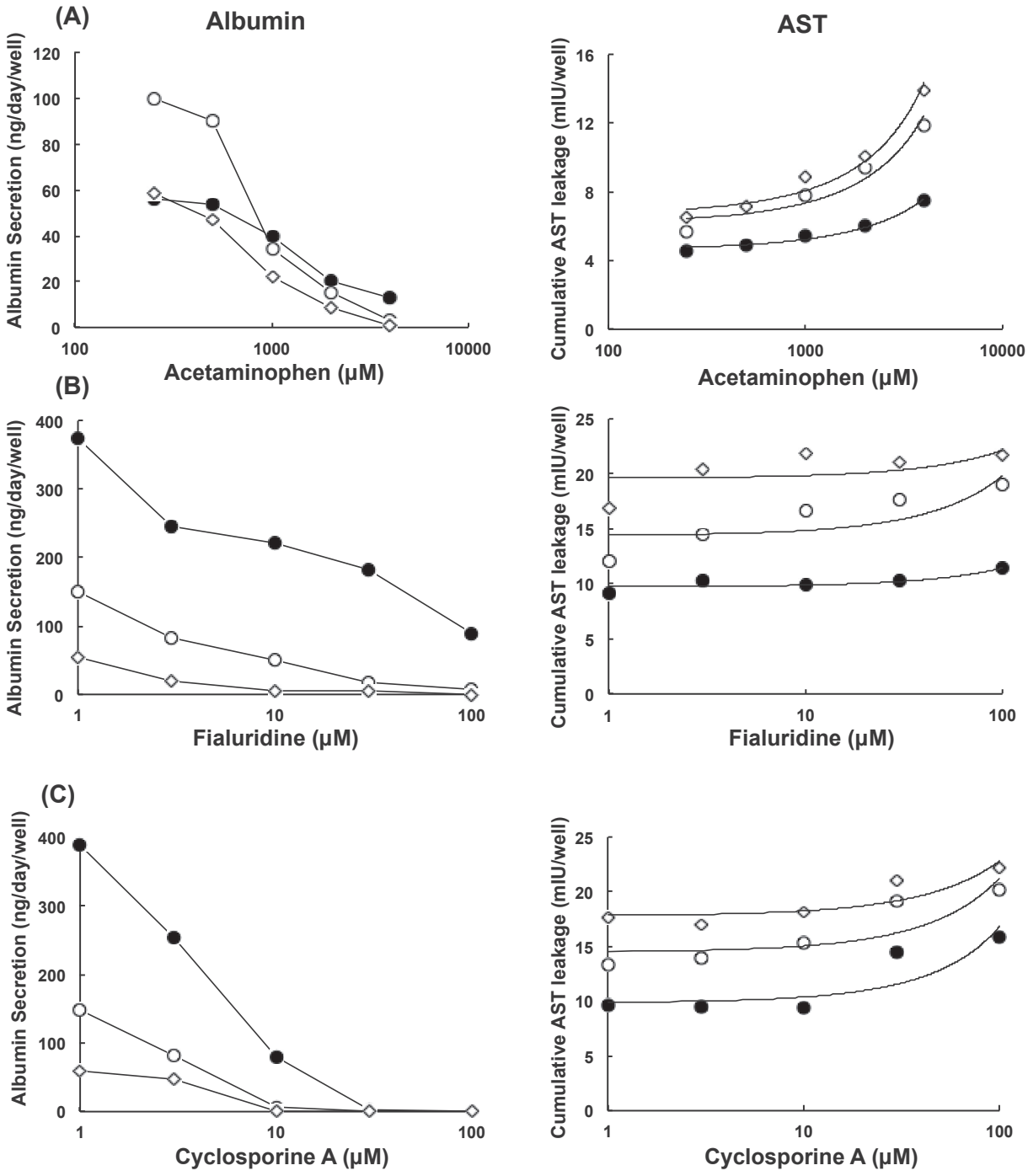

Fig. 2. Representative results of spheroid-cultured primary human hepatocyte assay for detection of drug-induced hepatotoxicity. Spheroids were treated for 21 days with (A) acetaminophen, (B) fialuridine, and (C) cyclosporin A. Albumin concentration (left side) and cumulative AST leakage (right side) in the culture medium were measured as described in Materials and Methods. Closed circles, open circles and open diamonds represent incubation periods of 7, 14, and 21 days, respectively. Each point represents the mean of two incubation wells.

Table 1. IC_{50} values of test compounds for albumin secretion in human primary hepatocyte spheroid assay.

Compound	Albumin secretion IC_{50} (μM)			Reported IC_{50} (μM) of cytotoxicity assays	Clinical C_{max} (μM)	References for Reported IC_{50}	References for Clinical C_{max}
	Day 7	Day 14	Day 21				
Acetaminophen	1295.2	809.3	772.4	28,200 (HH) 29,755 (HepG2)	139	(Jemnitz *et al*., 2008; Wang *et al*., 2002)	(Sevilla-Tirado *et al*., 2003)
Benzbromarone	48.8	< 20	22.2	> 40 (HepG2) *	4.3	(Iwamura *et al*., 2011)	(Ferber *et al*., 1981)
Chlorpromazine	10.3	11.7	6.1	1.73 – 18.3 (HH) 42.9 – 62.6 (HepG2)	1.41	(Gerets *et al*., 2012)	(Xu *et al*., 2008)
Cyclosporine A	3.9	2.7	2.0	24.4 – 56.8 (HH) > 100 (HepG2)	0.78	(Gerets *et al*., 2012)	(Baraldo *et al*., 2001)
Diclofenac	98.4	103.3	104.6	331 (HH) 763 (HepG2)	8.1	(Bort *et al*., 1999)	(Xu *et al*., 2008)
Fialuridine	18.1	3.4	0.9	> 400 (HepG2)	0.64	(Donato *et al*., 2009)	(Bowsher *et al*., 1994)
Flutamide	21.0	60.4	46.5	6.29 – > 100 (HH) > 100 (HepG2)	4.16	(Gerets *et al*., 2012)	(Regenthal *et al*., 1999)
Imipramine	37.0	4.1	16.8	37 (HepG2)	0.14	(O'Brien *et al*., 2006)	(Abernethy *et al*., 1985)
Isoniazid	> 1000	254.1	336.2	> 10,000 (HepG2) *	76.6	(Wang *et al*., 2002)	(Xu *et al*., 2008)
Ticlopidine	55.8	23.9	28.1	Not reported	7.1		(Desager, 1994)
Troglitazone	42.0	46.6	21.5	> 50 (HH)* 30 (HepG2)	6.4	(Kostrubsky *et al*. 2000; Yamamoto *et al*., 2002)	(Loi *et al*., 1999)

Each value represents the mean of two incubation wells. The incubation period was 7, 14, and 21 days. Reported IC_{50} values in cytotoxicity assays are listed.
An asterisk (*) indicates that IC_{50} values were estimated from data taken from the cited reference. HH: 2D culture of human hepatocytes.

Table 2. F1.2 values of test compounds for AST leakage in human primary hepatocyte spheroid assay.

Compound	AST leakage F1.2 (μM)		
	Day 7	Day 14	Day 21
Acetaminophen	1823	912	912
Benzbromarone	-	-	-
Chlorpromazine	4.6	8.2	2.4
Cyclosporine A	36.5	48.0	72.9
Diclofenac	79.3	91.2	-
Fialuridine	114.0	57.0	151.9
Flutamide	19.8	26.4	82.9
Imipramine	15.9	1.8	1.8
Isoniazid	-	-	-
Ticlopidine	37.2	46.7	70.1
Troglitazone	-	-	26.6

Each value represents the mean of two incubation wells. The incubation period was 7, 14, and 21 days . " - " means that F1.2 could not be calculated using the equations in Materials and Methods.

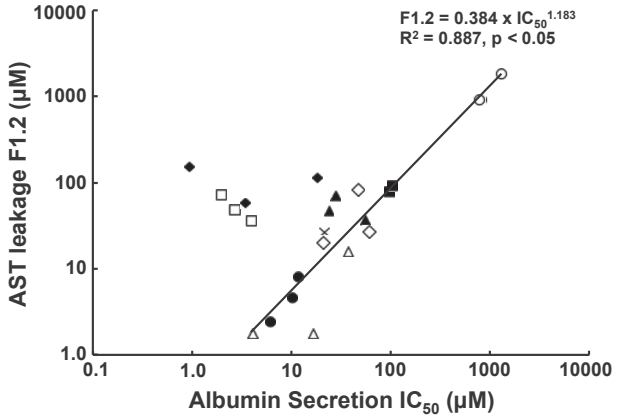

Fig. 3. Correlation between IC_{50} values for albumin secretion and F1.2 values for AST leakage. IC_{50} values for albumin secretion and F1.2 values for AST leakage were plotted for acetaminophen (○), chlorpromazine (●), cyclosporine A (□), diclofenac (■), fialuridine (◆), flutamide (◇), imipramine (△), ticlopidine (▲), and troglitazone (×). The results of plural measurements of each compound are shown. The correlation between IC_{50} and F1.2 was calculated after exclusion of cyclosporine A and fialuridine.

DISCUSSION

In this study, we evaluated the utility of human hepatocyte spheroids as an assay system for predicting drug-induced hepatotoxicity. The spheroids remained active for at least 21 days, as indicated by continued secretion of albumin, stabilization of AST leakage level and unchanged morphology (Fig. 1). Conventional primary cultures of human hepatocytes remain useful for only a few days. In contrast, metabolic activity towards several drugs in our spheroid system was found to be stable from day 7 to day 21 in our previous study. Therefore, the spheroid assay system is considered to be suitable for detection of toxicity associated with sequential metabolic reactions and human-specific metabolites that are difficult to identify by means of conventional assays (Ohkura *et al*., 2014).

Cell viability and ATP depletion assays are also used to measure *in vitro* hepatotoxicity, but these assays are not suitable for clinical studies (Gomez-Lechon *et al*., 2001). In the present work, we used AST leakage and albumin secretion as markers of hepatotoxicity. Since an AST change of 150% could not be estimated by interpolation, we chose F1.2 as an index.

Long-term exposure may be necessary to induce hepatotoxicity in the cases of drugs or their metabolites that form irreversible adducts with DNA and proteins. Therefore, we exposed spheroids to test drugs for 21 days to mimic repeated-dose clinical use. Changes of the IC_{50} values for albumin secretion were observed, except in the case of diclofenac. In particular, the IC_{50} of fialuridine, a nucleoside analogue, changed dramatically from 18.1 μM at 7 days to 0.9 μM at 21 days; the latter value is close to the reported plasma concentration of fialuridine in clinical studies, 0.64 μM (Bowsher *et al*., 1994). Fialuridine or its metabolite is incorporated into mitochondrial DNA, leading to a decrease of mitochondrial function. The accumulation of adducts from day 7 to day 21 may account for

the changes in IC_{50} at 7, 14 and 21 days. On the other hand, the IC_{50} value of diclofenac for albumin secretion was not influenced by the culture period. The reported mechanism of diclofenac hepatotoxicity involves immunologic and metabolic processes. There are no immune cells in the spheroid assay. Also, our previous study showed that diclofenac was metabolized to the extent of 79% at 2 days and was converted to hydroxyl and glucuronated hydroxyl forms (Ohkura *et al*., 2014). Thus, the metabolic activity of spheroids towards diclofenac may be higher than towards the other tested compounds.

Most of the tested compounds showed a good correlation between IC_{50} for albumin secretion and F1.2 for AST leakage. This suggests that the F1.2 value can be considered as a useful index of drug-induced hepatotoxicity. Since AST leakage reflects damage to hepatocytes, while albumin secretion reflects metabolic activity of hepatocytes, the two processes could reflect different aspects of hepatotoxicity. On the other hand, no correlation was observed between the IC_{50} and F1.2 values of cyclosporine A and fialuridine. Since AST is localized in cytoplasm and mitochondria, two possibilities can be considered, i.e., a protective effect against mitochondrial dysfunction, and a decrease of AST production. Cyclosporine A is known to inhibit opening of the mitochondrial permeability transition pore (Crompton *et al*., 1988), thereby protecting cells against mitochondrial dysfunction and death. On the other hand, fialuridine and troglitazone are reported to induce mitochondrial dysfunction (Tujios and Fontana, 2011; Rachek *et al*., 2009). In particular, fialuridine decreases the number of mitochondrial cristae (McKenzie *et al*., 1995). These changes may result in reduced leakage or production of AST in mitochondria.

As shown in Table 1, the IC_{50} values of most drugs were within twenty times the reported C_{max}, except for imipramine. However, in the case of imipramine, it has been reported that a chemically reactive intermediate generated by CYP2D is involved in the hepatotoxicity (Masubuchi *et al*., 1999). Overall, these results suggest that a desirable clinical C_{max} would be less than one-twentieth of IC_{50} in this assay system. Hepatotoxicity of most of the tested drugs was detected using the spheroid assay with an incubation period of 14 days, during which time the activity of metabolic enzymes and the morphology remained stable. Furthermore, since the values of albumin and AST were similar at 14 and 21 days, an incubation period of 14 days is reasonable.

Conventional 2D primary human hepatocytes and HepG2 are frequently used for evaluation of *in vitro* hepatotoxicity (O'Brien *et al*., 2006). In Table 1, IC_{50} values of albumin secretion are compared with reported IC_{50} values in cytotoxicity assays using primary 2D human hepatocytes and/or HepG2 cells. Compared with the IC_{50} values in conventional assays, the IC_{50} values of most tested drugs in the present spheroid assay system were closer to reported clinical C_{max} values.

In conclusion, our human hepatocyte spheroids secreted albumin for up to 21 days, during which period their morphology remained stable. Calculated IC_{50} values were within twenty times the reported maximum clinical concentration of hepatotoxic drugs, except in the case of imipramine. Measurement of albumin secretion may be preferable for *in vitro* hepatotoxicity screening compared with AST, because AST leakage could be affected by mitochondrial injury. Our present results suggest that the proposed assay with an incubation period of 14 days should prove useful for prediction of hepatotoxicity during the early development stages of drug candidates.

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

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