Studies on evaluation of bilirubin glucuronidation activity using hepatoma cell lines

September 2020

平野　隆之

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

ADME	absorption, distribution, metabolism and excretion
Arg	arginine
BDG	bilirubin di-glucuronide
BMG	bilirubin mono-glucuronide
BSA	bovine serum albumin
CO_2	carbon dioxide
DLM	dog liver microsome
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
Gly	glycine
h	hour
HLM	human liver microsome
HPLC	high-performance liquid chromatography
IS	internal standard
LC/MS	liquid chromatography mass spectrometry
$MgCl_2$	magnesium chloride
min	minute
mM	millimolar
MRP	multidrug-resistance protein
UCB	unconjugated bilirubin
UDPGA	uridine-diphosphate glucuronic acid
UGT1A1	UDP-glucuronosyltransferases 1A1
°C	Celsius

µg/ml	microgram/milliliter
µM	micromolar
2D	2-dimentional
3D	3-dimentional

1. INTRODUCTION

1.1. Introduction

Bilirubin is the toxic end product of heme catabolism. In circulating blood, unconjugated bilirubin bound to albumin is transported to the liver where it is removed from the plasma. Subsequently, within the cytoplasm of hepatocytes, bilirubin is bound to ligandin and transported to the endoplasmic reticulum where conjugation with glucuronic acid takes place (Sticova and Jirsa 2013). The conjugation is primarily catalyzed by a conjugating enzyme, UDP-glucuronosyltransferases (UGT) 1A1 (Kadakol, et al. 2000). The conjugated bilirubin is excreted into bile and finally eliminated from the body.

Several kinds of genetic polymorphism of UGT1A1 have been reported in humans, and they show defective bilirubin conjugation in varying degrees, leading to a decrease in bilirubin clearance (Erlinger, et al. 2014). For example, UGT1A1*6, a single nucleotide substitution causing a missense mutation (Gly71Arg), and UGT1A1*28, insertion of two extra bases (TA) in the TATAA element of the promoter region of *UGT1A1* gene (A(TA)$_7$TAA instead of the normal A(TA)$_6$TAA), are common polymorphism and the both cause a decreased bilirubin clearance (Aono, et al. 1995; Bosma, et al. 1995; Erlinger, et al. 2014). In addition to genetic polymorphism that impairs bilirubin glucuronidation, species difference in bilirubin glucuronidation activity have been identified between humans and dogs using liver microsomes and recombinant UGT1A1, in which dogs have lower activity than humans (Soars, et al. 2001; Troberg, et al. 2015). In general, non-clinical evaluations of toxicity for the new pharmaceutical compound are performed using rodents, usually rats, and non-rodents such as dogs. However, species differences of metabolism, as the case of bilirubin, seem to affect the appropriate interpretation of non-clinical toxicity information. Therefore, it is very important to understand the differences of metabolic properties between humans and animals to extrapolate the results of

non-clinical experiments to humans. An appropriate and detailed evaluation of pharmaceutical compounds, including species differences, can lead an appropriate explanation of its toxicological information.

As popular *in vitro* models, hepatocytes or liver microsomes are commonly used to study drug metabolism (Brandon, et al. 2003). The hepatocytes can demonstrate the most realistic system in point of the complement of drug metabolic enzymes in a more physiological cellular environment (Dalvie, et al. 2009). Regarding bilirubin glucuronidation activity, some papers demonstrated the in vitro evaluation of bilirubin elimination using microsomes or recombinant UGT1A1 (Ma, et al. 2014; Wang, et al. 2015), but little information is available concerning the in vitro evaluation of bilirubin glucuronidation activity in hepatocytes. Although primary hepatocytes are useful and frequently used to study drug metabolism, the drug metabolic activities of hepatocytes cultured in conventional two-dimensional (2D) conditions may differ from that of *in vivo* condition due to changes in phenotypes including expression of drug-metabolizing enzymes or morphology (Hewitt, et al. 2007; Ohkura, et al. 2014). The difference from *in vivo* condition is generally still more evident in liver cell lines such as HepG2, which have lower expression levels of most drug metabolic enzymes compared to primary hepatocytes (Brandon, et al. 2003).

To overcome these limitations of hepatocytes cultured in 2D plates, three-dimensional (3D) culture systems have been developed in recent years. In the 3D culture system, hepatocytes form 3D aggregates, called spheroids. Spheroid culture enables hepatocytes to maintain the mature hepatic phenotypes and long-term stable hepatic functionality by mimicking a more natural physiological environment (Lauschke, et al. 2019). In fact, the usefulness of 3D hepatocyte culture systems has been revealed to study drug metabolism in primary hepatocytes and liver cell

lines including HepG2 or HepRG (Bell, et al. 2016; Ohkura, et al. 2014; Ramaiahgari, et al. 2014; Takahashi, et al. 2015).

1.2. Objectives of the Thesis

HepG2 is one of the most popular and the most widely used cell line for pharmacological and toxicological evaluations in pharmaceutical development research. In addition, HepG2 cells are generally easy to obtain and maintain in a usual culture system. Therefore, it is very significant to establish an evaluation system for bilirubin glucuronidation activity using HepG2, although HepG2 has lower activities of several drug-metabolizing enzymes, including UGT1A1. If the evaluation system is established, we can efficiently conduct a further and detailed study for bilirubin glucuronidation using the evaluation system.

Regarding the development of a pharmaceutical compound, species difference in toxicity is sometimes closely related to the ADME (absorption, distribution, metabolism, and excretion) properties of a compound. If we could reveal the mechanism of species difference in detail, for instance, at level of the gene or protein, the accuracy of extrapolation of animal data to humans would be improved. This improvement allows us to promote safer clinical development of pharmaceutical compounds.

In this study, first, we analyzed and compared the bilirubin glucuronidation activity of HepG2 cells cultured in conventional 2D plates and 3D culture systems, and demonstrated the efficiency of 3D-cultured hepatoma for evaluation of bilirubin metabolism properties. Furthermore, we tried to apply the 3D culture evaluation system to examination of species differences in bilirubin glucuronidation between humans and dogs.

2. LITERATURE REVIEW

2.1. Bilirubin Glucuronidation

In circulating blood, unconjugated bilirubin bound to albumin is transported to the liver where it is removed from the plasma. In the liver, bilirubin is conjugated with glucuronic acid. The conjugation is primarily catalyzed by a conjugating enzyme, UDP-glucuronosyltransferases (UGT) 1A1 (Kadakol, et al. 2000). Bilirubin glucuronidation step includes the conjugation of a glucuronyl moiety of the propionic acid chain of bilirubin (the C-8 and C-12 carbons of the central pyrrole rings), and then two types of bilirubin mono-glucuronide (BMG) are generated (BMG1 and BMG2). These BMGs can be further glucuronidated to produce bilirubin di-glucuronide (BDG), as shown in Figure 1 (Wang, et al. 2015).

Figure 1: **The structure of unconjugated bilirubin (UCB), bilirubin mono-glucuronides (BMGs), and bilirubin di-glucuronide (BDG) and the cascade of bilirubin glucuronidation reaction.**
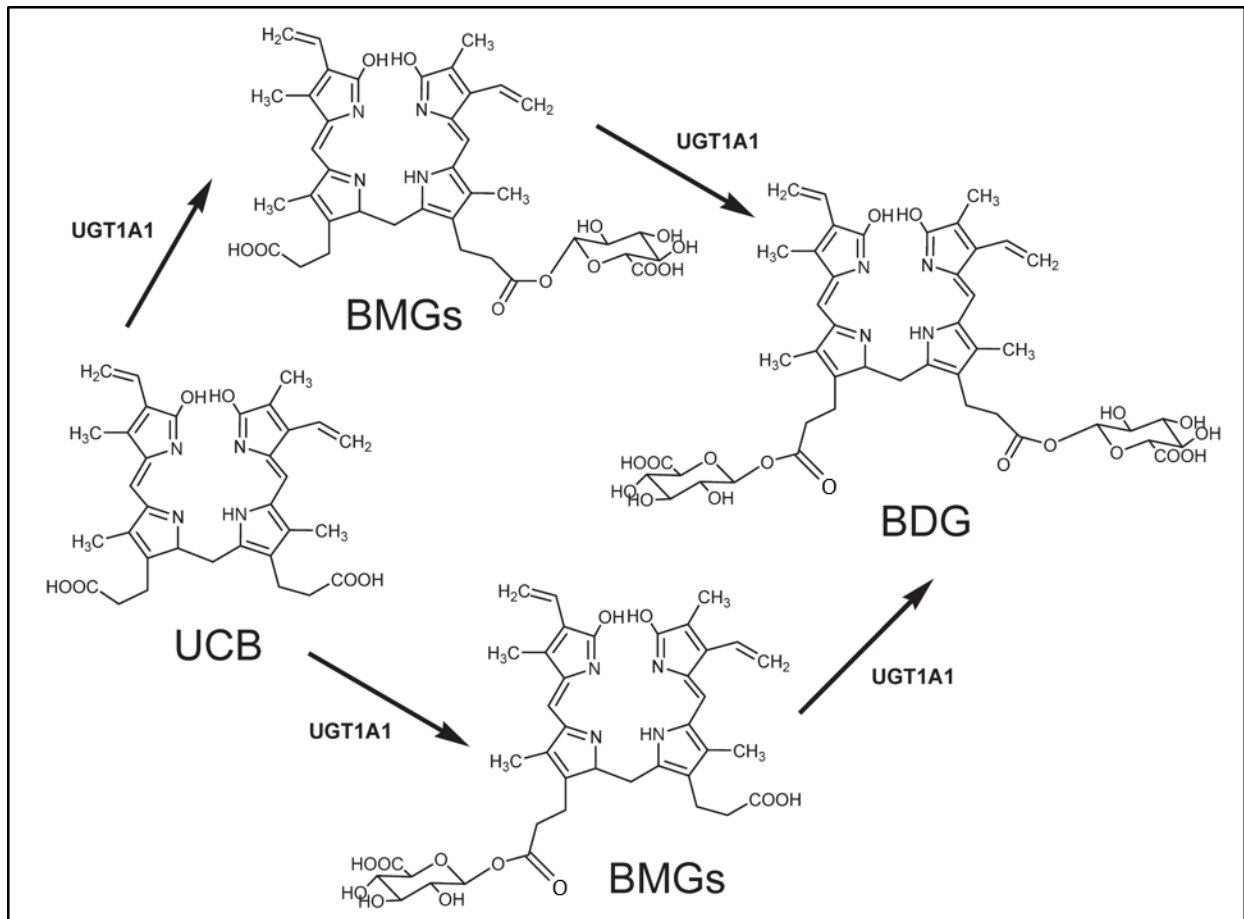
2.2. Drug-induced hyperbilirubinemia increased blood bilirubin

Generally, circulating bilirubin is transported into the liver by uptake transporters such as organic anion-transporting polypeptides OATP1B1 and OATP1B3 (Chang, et al. 2013). Subsequently, bilirubin is conjugated with glucuronic acid catalyzed by UGT1A1, and glucuronide metabolites are excreted into the bile via multidrug resistance-associated protein MRP2, Figure 2 (Chang, et al. 2013).

Figure 2: **Schematic illustration of bilirubin elimination way in the liver**
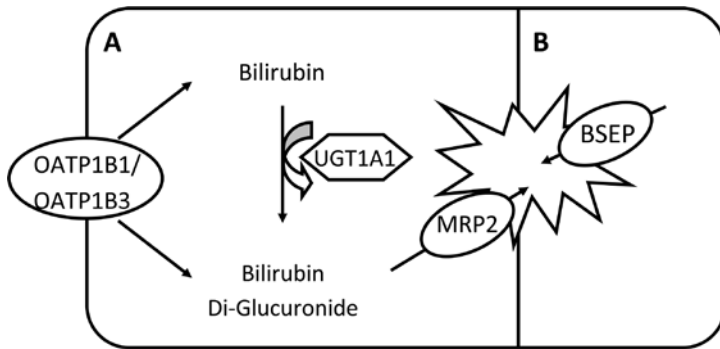
Drugs can induce hyperbilirubinemia. Blood bilirubin level increases when the drug interrupts bilirubin clearance via inhibition of uptake transporter to hepatocyte (OATP1B1 and OATP1B3), glucuronidation enzyme for bilirubin (UGT1A1), and excretion transporter into bile (MRP2). These hyperbilirubinemia are recognized as adverse effects (toxicity) of drugs. Some of the protease inhibitors are known to exhibit clinical hyperbilirubinemia via inhibition of these enzymes and/or transporters, Figure 3 (Chang, et al. 2013).

Figure 3: **Inhibition of UGT1A1, OATP1B1, OATP1B3, and BSEP by drugs and the consequence of inhibition**
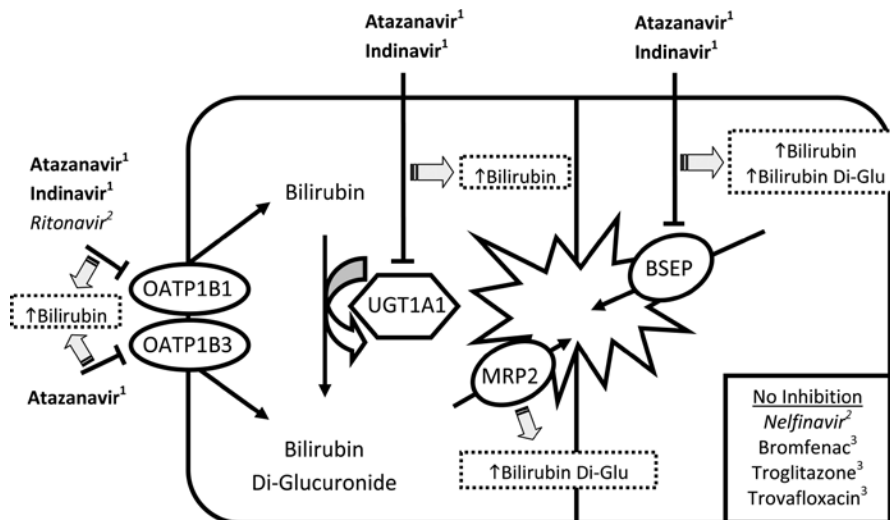
2.3. UGT1A1 polymorphism leading to low bilirubin glucuronidation activity

UGT1A1 polymorphisms are involved in altered bilirubin glucuronidation activity resulting in hyperbilirubinemia in varying degrees. Representative UGT1A1 polymorphisms and their biological features are shown in Table 1 (Takano and Sugiyama 2017). UGT1A1 polymorphisms have various variants. Among these variants, UGT1A1*28 with the insertion of a TA in the TATA box and UGT1A1*6, which has Gly71Arg, are common variants. UGT1A1 polymorphisms are related not only in hyperbilirubinemia but also in the onset and severity of toxicity of a drug that is metabolized by UGT1A1 such as irinotecan (Takano and Sugiyama 2017).

Table 1: **UGT1A1 polymorphisms and their biological features**

Denomination	Variants	Allele frequency (ethnicity)	Expression level	Enzymatic activity	Clinical consequence
UGT1A1*1	(TA)₆TA	Common allele	100%	100%	None
TATA box polymorphisms					
UGT1A1*28	c.–39_–40 ins TA: (TA)₇TA	29–45% (Caucasians); 42–51% (Africans); 16% (Asians)	Reduced	Reduced	Gilbert's syndrome, Crigler–Najjar syndrome
Polymorphisms in the promoter region					
UGT1A1*60	c.–3279 T>G	23–39% (Caucasian); 15% (African Americans); 17% (Asians)	Reduced	Unchanged	Gilbert's syndrome, Crigler–Najjar syndrome
Polymorphisms in exon 1					
UGT1A1*6	c.211 G>A p.Gly71Arg	15–20% (Asians)	Unchanged	Reduced	Gilbert's syndrome, Crigler–Najjar syndrome
UGT1A1*27	c.686 C>A p.Pro229Gln	5–28% (Asians)	Unchanged	Reduced	Gilbert's syndrome, Crigler–Najjar syndrome

2.4. 3D-culture System and hepatocytes models

In recent years, there has been a rapid development of hepatic 3D models for drug pharmacokinetics and toxicity evaluation, including spheroid systems, and various types of hepatic 3D models with different culture methods, cell types, and materials have been exhibited. Moreover, there are obvious differences in their characterization and their utility for downstream

applications (Figure 4, Lauschke et al. 2019). The ability to predict the metabolism and toxicity of pharmaceutical compounds could be improved using these models when compared to conventional 2D models.

Figure 4:   **The diversity and versatility of hepatic 3D culture models**
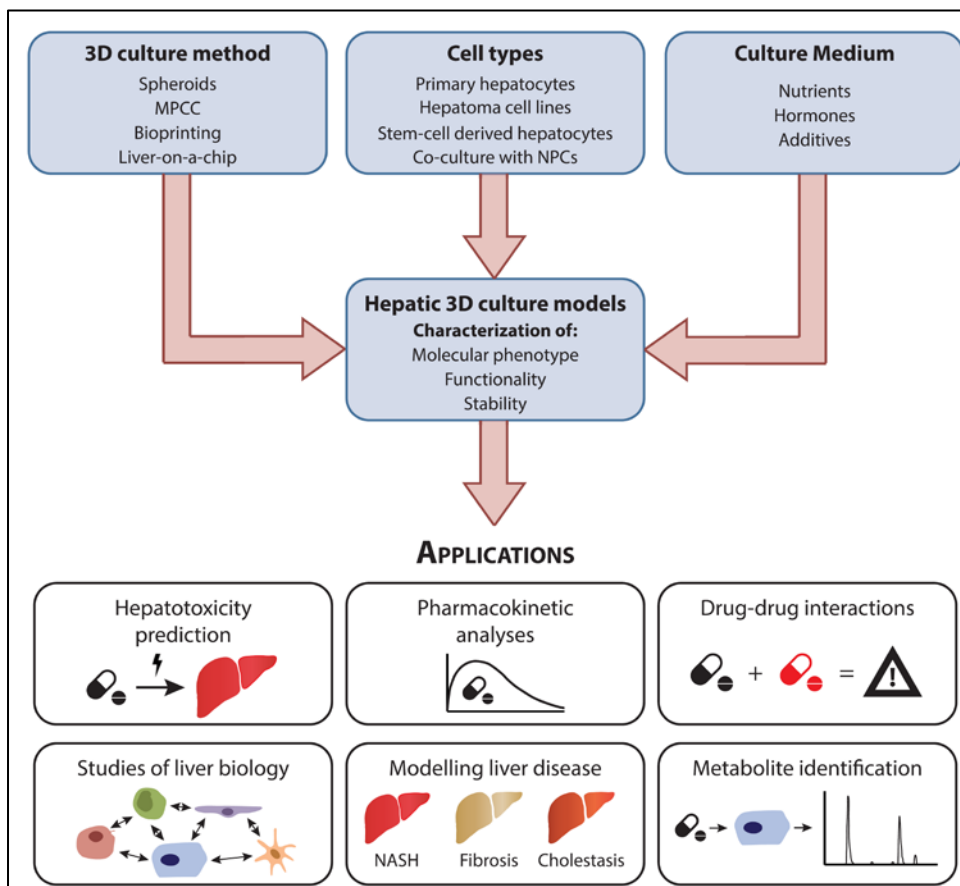
3. COMPARATIVE ANALYSIS OF BILIRUBIN GLUCURONIDATION ACTIVITY IN HUMAN AND DOGS USING HEPATOMA CELL LINES

3.1. Introduction

It is well known that 3D-culture system can increase expression of metabolizing enzymes including UGT1A1 and improve metabolic activities of primary hepatocytes and hepatoma cell lines. In this study, we attempted to show the utility of 3D culture system in evaluation for bilirubin glucuronidation activity using HepG2 (Section 3.2), to apply the evaluation system to examine species differences in bilirubin glucuronidation between human and dogs, and to discuss the possible mechanism of species difference in bilirubin glucuronidation activity (Section 3.3).

3.2. The utility of 3D culture system in bilirubin glucuronidation evaluation in human hepatoma cell line HepG2

3.2.1. Materials and methods

3.2.1.1. Materials

Bilirubin, acetonitrile, telmisartan, ammonium acetate, and dimethyl sulfoxide (DMSO) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The remaining chemicals and culture media were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human hepatoma cell line HepG2 was obtained from Riken BioResource Research Center (Tsukuba, Japan, RCB1886), which has authenticated that the presence of mycoplasma in the cell line was

below the detection level. Cell lines used in this study had been authenticated by STR cell identification at JCRB Cell Bank, National Institute of Biomedical Innovation on Feb. 21, 2020.

3.2.1.2. Cell culture

HepG2 cells were seeded at a density of 2.0×10^5 cells/well in 12-well 3D culture systems (Cell-able CP-12, Toyo Gosei Co. Ltd. Tokyo, Japan) and at a density of 1.0×10^5 cells/well in 12-well conventional 2D cell culture plates (VTC-P12, AS ONE Corporation, Osaka, Japan). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO2. The medium was replaced with fresh medium every 2 or 3 days.

3.2.1.3. Bilirubin glucronidation assay in cultured hepatoma cells

2D- and 3D-cultured hepatoma cells were used for bilirubin glucuronidation assessment after 3 days, and 10 days of seeding, respectively. For bilirubin glucuronidation assessment, the working solution was prepared by dissolving bilirubin (10 µM) in DMSO (20 mM) followed by the addition of DMEM containing 4 mg/mL bovine serum albumin. Subsequently, it was dispensed into the wells and incubated in a humidified incubator at 37°C with 5% CO_2. At 1 h, 10 h, 24 h, 48 h, and 72 h after incubation, the culture medium was collected and mixed with equal amount of ice-cold 100% acetonitrile to terminate the reaction. Finally, the samples were centrifuged at 12,000 rpm for 15 min at 4°C and their supernatants were stored at -80°C until analysis. Six and four replicate trials were carried out under dark conditions in 2D plates and 3D culture systems respectively.

3.2.1.4.   Measurement of bilirubin and conjugated bilirubin

Ten microliters of the supernatant from the culture medium and 10 µL of acetonitrile were mixed (the mixture). The internal standard (IS) stock solution was prepared by dissolving telmisartan in DMSO to obtain a final concentration of 100 µg/mL. The working IS solution (100 ng/mL) was prepared by diluting the stock solution with acetonitrile. Then 30 µL of 10 mM ammonium acetate/acetonitrile (50:50, v/v) and 5 µL of IS working solution was added to the mixture and centrifuged in the microcentrifuge at 20,400×g (5°C, 2 min) and the aliquots (10 µL) of supernatant were injected to the LC/MS system. The quantification of bilirubin, bilirubin monoglucuronide, and bilirubin diglucuronide was carried out using HPLC (SHIMADZU Prominence UFLC) coupled with electrospray ionization mass spectrometer (AB SCIEX Triple Quad 4500). HPLC separation was performed on Accucore C18 column (2.6 µm, 2.1 × 50 mm, Thermo Scientific). The mass spectrometer was operated in the positive ion mode. MS data were collected as single ion monitoring as [M+H]+ ions at 585 m/z for bilirubin, 761 m/z for bilirubin monoglucuronide, 937 m/z for bilirubin diglucuronide, and 515 m/z for telmisartan. The LC/MS system was controlled by Analyst 1.6.3 software, and the data were processed using Multiquant version 3.0 software.

3.2.1.5.   Statistical analysis

The relative amounts of bilirubin mono-glucuronide in 2D and 3D cultured HepG2 cells were evaluated using a t-test. $P < 0.05$ was considered statistically significant.

3.2.2. Results

3.2.2.1. Cell culture in 2D- and 3D-culture system

We used the Cell-able system, a microfabricated cell array system for 3D cell culture. The spheroid development in HepG2 cells is shown in Figure 5. The results revealed that spheroid development was easily accomplished in HepG2 cells without special technique and equipment.

Figure 5: **Spheroid development of HepG2 cells in 3D culture. Images of HepG2 cultured in 3D plate Cell-able on Day 10 at low (A, ×100) and high magnification (B, ×200), and an image of HepG2 cultured in 2D plate (C, ×200). Scale bar; 100 μm.**
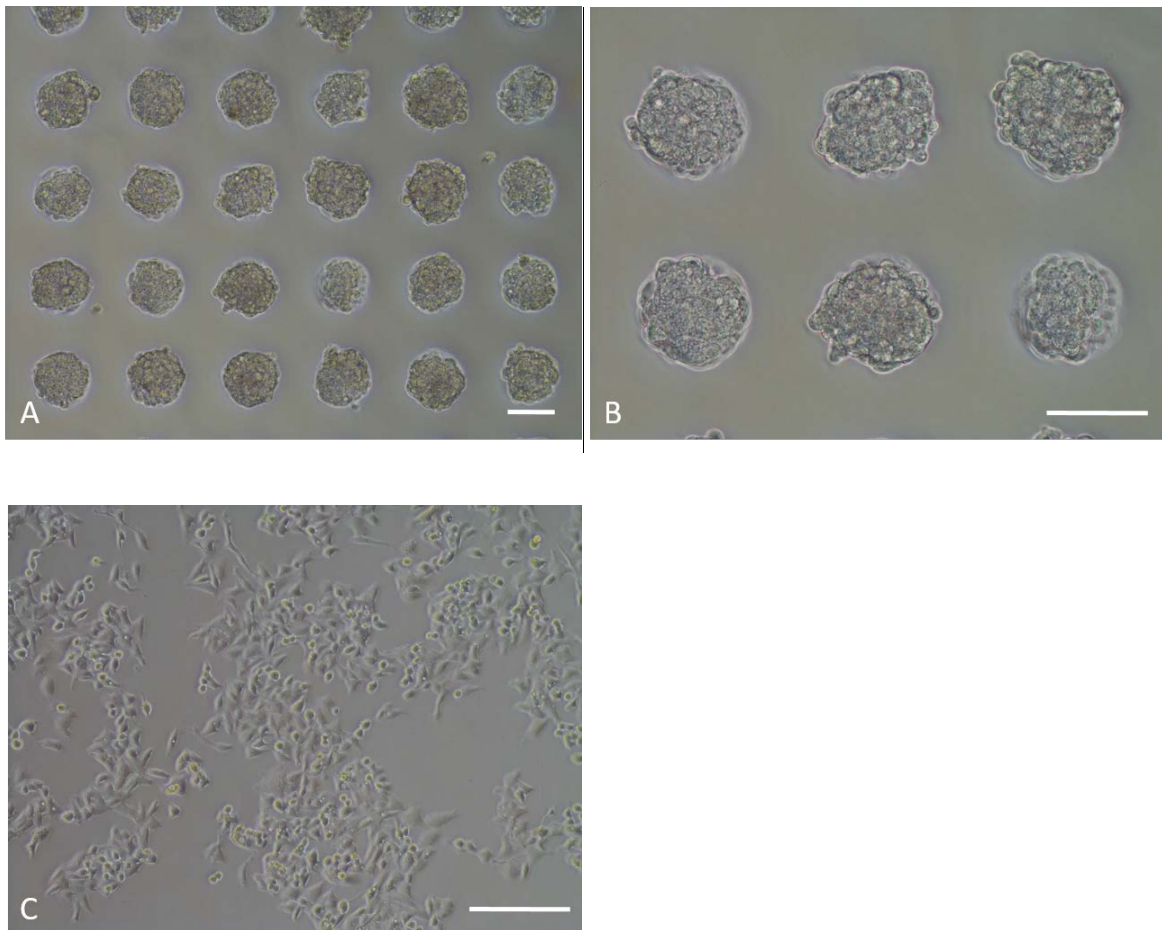
3.2.2.2. Measurement of bilirubin and conjugated bilirubin by LC-MS

The quantification of bilirubin, bilirubin monoglucuronide, and bilirubin diglucuronide was performed using HPLC coupled with electrospray ionization mass spectrometer (MS). MS data were collected as single ion monitoring as $[M+H]^+$ ions at 585 m/z for bilirubin, 761 m/z for bilirubin monoglucuronide, and 937 m/z for bilirubin diglucuronide. The representative chromatograms of 2D-cultured and 3D-cultured HepG2 cells for bilirubin, bilirubin mono-glucuronide, and di-glucuronide are shown in Figure 6.

Figure 6: **Representative LC-MS chromatograms of 2D-cultured (A) and 3D-cultured (B) HepG2 cells for bilirubin, bilirubin mono-glucuronide and di-glucuronide.**
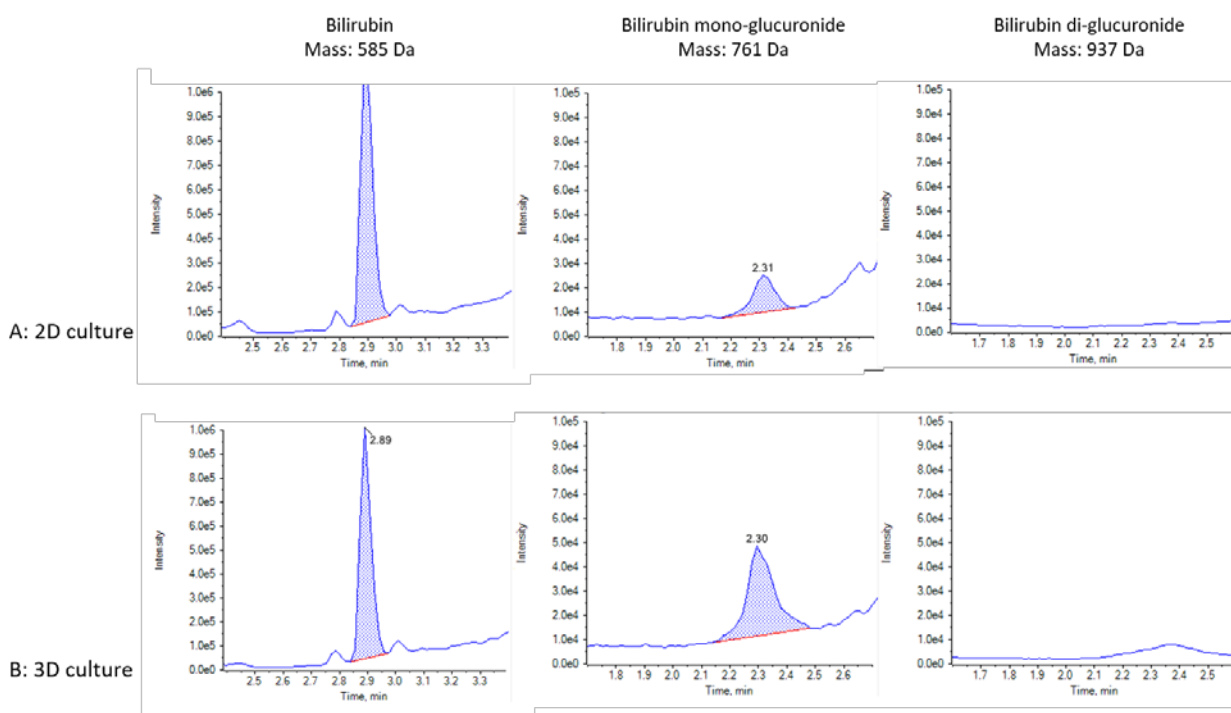
3.2.2.3. Comparative analysis of bilirubin glucuronidation activity in 2D- and 3D-cultured HepG2 cells

The glucuronidation activity of bilirubin, the bilirubin mono- and di- glucuronides were assessed from the HepG2 cells cultured in 2D plates and 3D culture systems. Bilirubin working solution (10 µM) was dispensed into the wells and incubated in a humidified incubator at 37°C with 5% CO_2. At 1 h, 10 h, 24 h, 48 h, and 72 h after incubation, each culture medium was collected and the reaction was terminated. We found that bilirubin mono-glucuronide was detected at 1 h after initiation of the incubation and bilirubin di-glucuronide was not detected in both 2D- and 3D-cultured HepG2 cells, even at 72 h after initiation of incubation under the conditions of this experiment (Figure 7 A, B). The relative amounts of bilirubin mono-glucuronide formation in HepG2 cells cultured in 2D plates and 3D culture systems were examined (Figure 7 C). The relative amounts of bilirubin mono-glucuronide increased gradually up to 72 h and were significantly higher ($P < 0.05$) in the HepG2 cells cultured by 3D culture systems than by 2D plates after 10 h of incubation.

Figure 7: Time course of bilirubin glucuronide generation (A and B) and bilirubin mono-glucuronide formation in HepG2 cells cultured in 2D plates and 3D

culture systems (C). BMG; bilirubin mono-glucuronide, BDG; bilirubin di-glucuronide. *P < 0.05. Data are expressed as mean ± SEM.

A

B

C
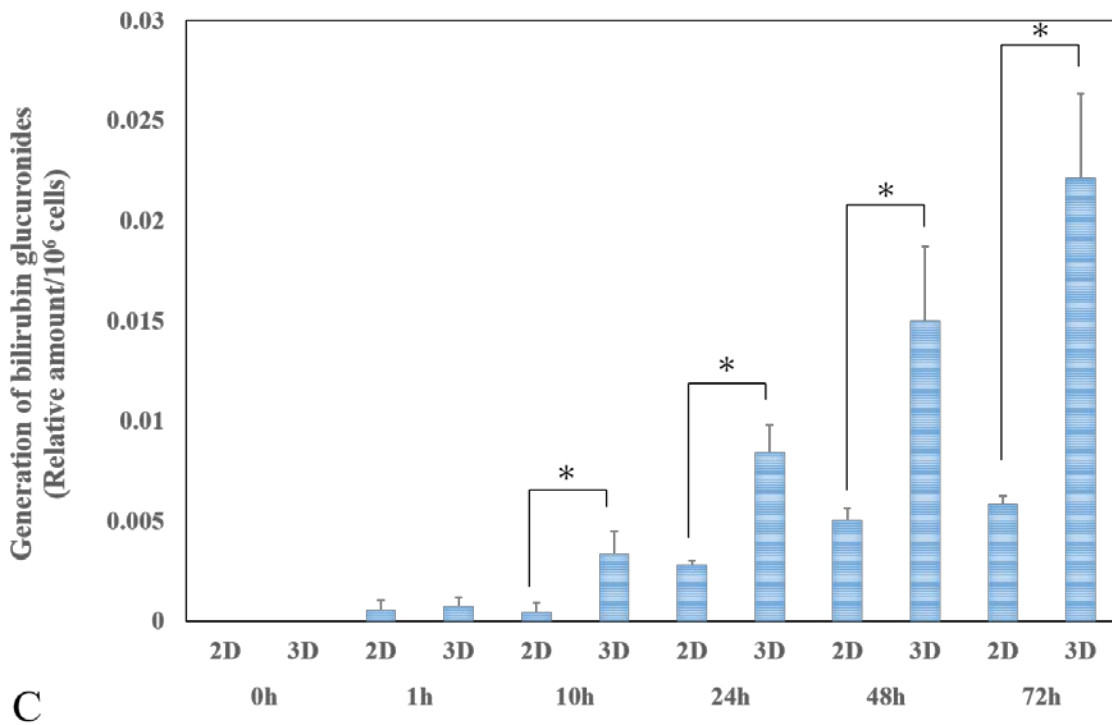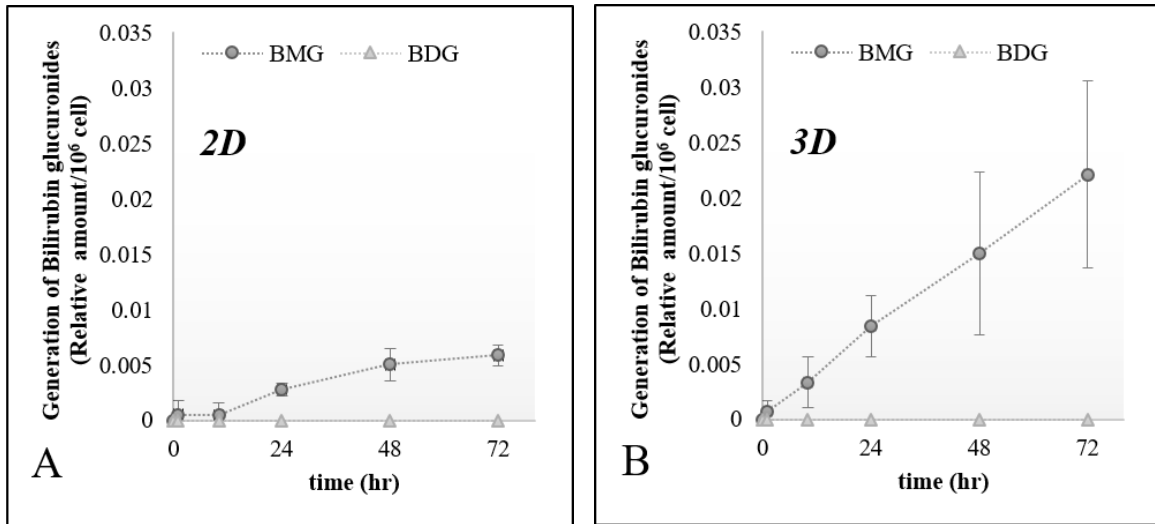
3.2.3.   Discussion

We analyzed and compared the bilirubin glucuronidation activity of HepG2 cells cultured in conventional 2D plates and 3D culture systems to demonstrate the efficiency of 3D-cultured hepatoma for evaluation of bilirubin metabolism properties.

In this experiment, we detected bilirubin mono-glucuronides in both 2D- and 3D-cultured HepG2 cells from 1 h after initiation of incubation and relative amounts of bilirubin mono-glucuronides increased gradually up to 72 h especially in 3D-cultured cells and were significantly higher in the HepG2 cells cultured by 3D culture systems than by 2D plates after 10 h of incubation.

At present, to our knowledge, little information is available concerning the evaluation of bilirubin glucuronidation activity in hepatocytes. In previous reports, the incubation time of primary hepatocytes cultured with bilirubin was only 60 min (Jemnitz, et al. 2002; Lengyel, et al. 2005). Although we also detected bilirubin glucuronides after 1 h-incubation, the amounts of glucuronides were not sufficient to evaluate the utility of 3D-culture system. Therefore, in this experiment, we set longer incubation time up to 72 h to obviously show the difference between 2D plates and 3D-culture system. Generally, hepatoma cell lines demonstrate lower metabolic enzyme activities than primary hepatocytes, including UGT1A1 that catalyzes bilirubin glucuronidation reaction (Guo, et al. 2011; Westerink and Schoonen 2007; Wilkening, et al. 2003). Thus, longer incubation time might be required for the generation of bilirubin glucuronides in our culture system.

The hepatoma cells cultured by the 3D culture system exhibited a higher metabolic activity of bilirubin glucuronidation than those by 2D culture plates, indicating the efficacy of 3D culture system in bilirubin glucuronidation activity evaluation using hepatoma cell lines. In earlier study,

higher expression of metabolizing enzymes, including UGT1A1 and transporters and higher metabolic activities for exogenous compounds in 3D-cultured hepatoma cell lines have also been reported (Kobayashi, et al. 2012; Ramaiahgari, et al. 2014). In this experiment, we showed that the hepatoma cells HepG2 cultured in the 3D culture systems exhibited a higher metabolic activity of glucuronidation to bilirubin, which is an endogenous toxic product. It is important to establish the easy-to-use *in vitro* culture system for the comprehensive evaluation of bilirubin glucuronidation since it has been reported that the impairment of bilirubin metabolism causes many clinical consequences and detailed exploration for bilirubin metabolism is required to reveal these characteristics. This evaluation system will enable us to conduct detailed study for bilirubin glucuronidation easily using hepatoma cell lines.

3.3. Comparative analysis of bilirubin glucuronidation activity in human and dog

3.3.1. Materials and methods

3.3.1.1. Materials

Bilirubin, MgCl$_2$, telmisartan, acetonitrile, methanol, and dimethyl sulfoxide (DMSO) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Uridine-diphosphate glucuronic acid (UDPGA), alamethicin, D-saccharic acid 1, 4-lactone, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human hepatoma cell line HepG2 was obtained from the Riken BioResource Research Center (Tsukuba, Japan, RCB1886), which has authenticated that the presence of mycoplasma in the cell line was below the detection level. Cell lines used in this study had been authenticated by STR cell identification at JCRB Cell Bank, National Institute of Biomedical Innovation, on Feb. 21, 2020.

Canine hepatoma cell (code No. AZACH) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Pooled human liver microsomes (HLMs) and dog liver microsomes (DLMs) were purchased from Sekisui XenoTech, LLC (Kansas City, KS, USA).

3.3.1.2. Cell culture

HepG2 cells and canine hepatoma cells were seeded at a density of 2.0×10^5 cells/well on 12-well 3D culture plates (Cell-able CP-12, Toyo Gosei Co., Ltd., Tokyo, Japan). HepG2 cells and canine hepatoma cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every 2 or 3 days. These cell cultures were performed at 37℃ with 5% CO_2.

3.3.1.3. Bilirubin glucuronidation assay in 3D-cultured hepatoma cells and liver microsomes

3D-cultured hepatoma cells were applied for bilirubin glucuronidation assays 10 days after the seeding. Bilirubin was dissolved in DMSO (20 mM) and added to DMEM containing 4 mg/mL BSA to make a working solution. The incubation was initiated by the addition of the working solution to the wells at 37℃, with 5% CO_2. The final concentration of bilirubin was 10 µM. At 1, 10, 24, 48, and 72 h after, the incubation was terminated by mixing with an equal amount of ice-cold 100% acetonitrile. Each samples were centrifuged at 12000 rpm for 15 min at 4℃. The supernatant was stored at -80℃ until analysis.

HLMs and DLMs were incubated with bilirubin dissolved in DMSO at 37℃. The incubation mixture included HLMs or DLMs (0.5 mg protein/ml), bilirubin (25 µM), alamethicin (25

µg/ml), D-saccharic acid 1, 4-lactone (5 mM), MgCl$_2$ (5 mM), and potassium phosphate buffer (100 mM, pH 7.4). Each mixture was incubated for 5 min at 37℃ in a shaking water bath before initiation of the incubation. The reaction was initiated by the addition of UDPGA (2 mM). The incubation was performed at 37℃ for 60 min and terminated by adding the double volume of acetonitrile/methanol (3:1) containing 0.1% phosphoric acid solution. The samples were centrifuged at 12000 rpm for 15 min at 4℃, and each supernatant was stored at -80℃ until analysis. All experiments were performed in duplicate under dark conditions.

3.3.1.4. Measurement of bilirubin and conjugated bilirubin

Ten microliters of the supernatant from the culture medium and 10 µL of acetonitrile were mixed (the mixture). The internal standard (IS) stock solution was prepared by dissolving telmisartan in DMSO to obtain a final concentration of 100 µg/mL. The working IS solution (100 ng/mL) was prepared by diluting the stock solution with acetonitrile. Then 30 µL of 10 mM ammonium acetate/acetonitrile (50:50, v/v) and five µL of IS working solution was added to the mixture and centrifuged in the microcentrifuge at 20,400×g (5°C, 2 min). The aliquots (10 µL) of supernatant were injected into the LC/MS system. The quantification of bilirubin, bilirubin monoglucuronide, and bilirubin diglucuronide was carried out using HPLC (SHIMADZU Prominence UFLC) coupled with electrospray ionization mass spectrometer (AB SCIEX Triple Quad 4500). HPLC separation was performed on the Accucore C18 column (2.6 µm, 2.1 × 50 mm, Thermo Scientific). The mass spectrometer was operated in the positive ion mode. MS data were collected as single ion monitoring as [M+H]+ ions at 585 m/z for bilirubin, 761 m/z for bilirubin monoglucuronide, and 937 m/z for bilirubin diglucuronide. The LC/MS system was

controlled by Analyst 1.6.3 software, and the data were processed using Multiquant version 3.0 software.

3.3.2. Results

3.3.2.1. Cell culture of canine hepatoma cells in the 3D-culture system

We used the Cell-able system, a microfabricated cell array system for 3D cell culture of canine hepatoma cells. The spheroid development in canine hepatoma cells is shown in Figure 8. The results revealed that spheroid development was easily accomplished also in canine hepatoma cells. However, adhesion of cells to the interstitial area between spheroids and suspended cells were observed.

Figure 8: **Spheroid development of canine hepatoma cells in 3D culture. Images of canine hepatoma cells cultured in 3D plate Cell-able on Day 10 at low (A, ×100) and high magnification (B, ×200) and an image of canine hepatoma cells cultured in 2D plate (C, ×200). Scale bar; 100 μm.**
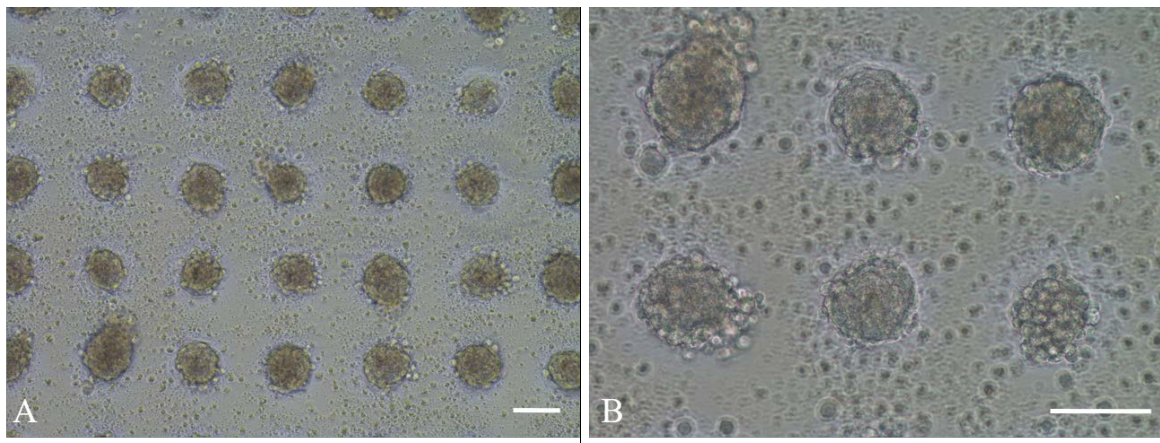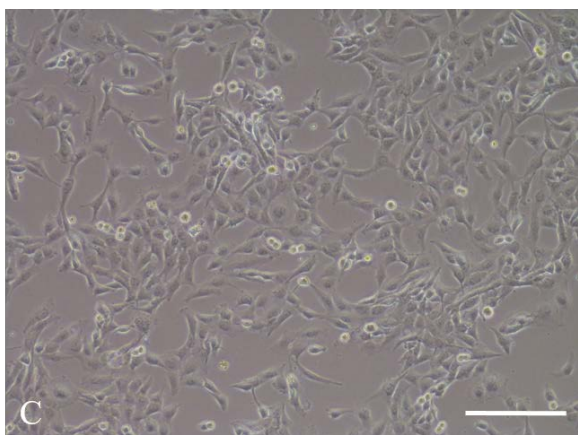
3.3.2.2. Bilirubin glucuronidation activity in HLMs and DLMs

The glucuronidation activity were assessed in HLMs and DLMs by quantification of bilirubin, the bilirubin mono- and di- glucuronides. At 5, 15, 30, 45, and 60 min after incubation, each sample was collected, and the reaction was terminated. Consequently, bilirubin mono- and di-glucuronides were detected in both HLMs and DLMs (Figure 9). The relative amounts of bilirubin mono- and di-glucuronides generated after 60 min incubation in DLMs were lower than those of HLMs.

Figure 9: Time course of bilirubin glucuronides generation (A and B). BMG; bilirubin mono-glucuronide, BDG; bilirubin di-glucuronide.
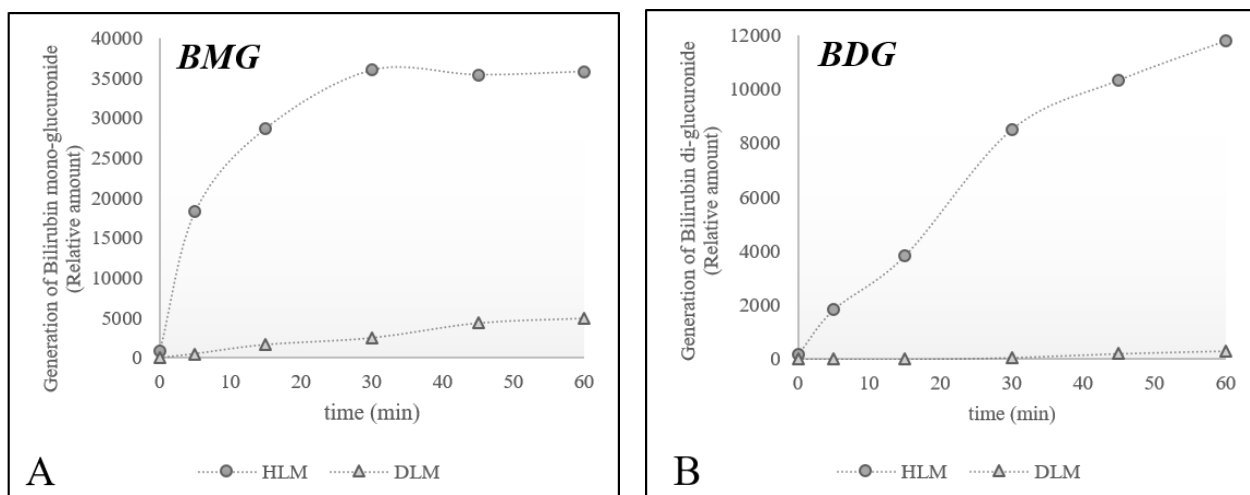
3.3.2.3. Bilirubin glucuronidation activity in HepG2 and canine hepatoma cells

Bilirubin glucuronidation activity in HepG2 and canine hepatoma cells were examined using a 3D culture system according to Section 3.2. 3D-cultured HepG2 and canine hepatoma cells were applied for bilirubin glucuronidation assays 10 days after the seeding. Bilirubin glucuronides were assessed at 1, 10, 24, 48, and 72 h after incubation.

In both HepG2 and canine hepatoma cells, bilirubin mono-glucuronide was detected from 1 h after the initiation and increased time-dependently to 72 h after the initiation and bilirubin di-glucuronide was not detected (Figure 10 A, B). However, remaining bilirubin in canine hepatoma cell culture medium drastically decreased especially until 10 h after incubation, whereas the amount of remaining bilirubin in the HepG2 culture medium was almost stable throughout the culture period (Figure 10 C).

Figure 10: Time course of bilirubin glucuronides generation (A and B) and remaining bilirubin (C). BMG; bilirubin mono-glucuronide, BDG; bilirubin di-glucuronide.
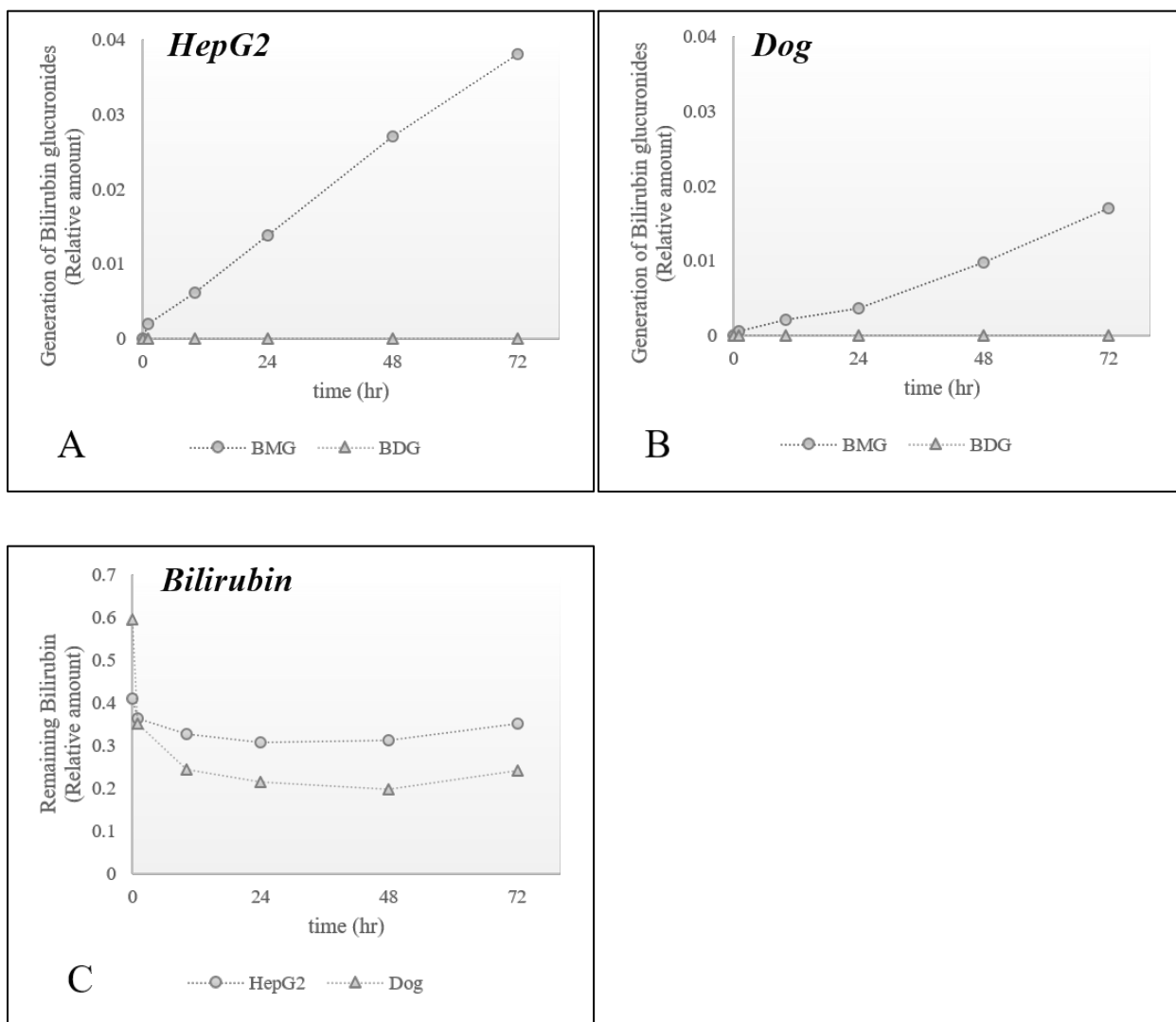
3.3.3. Discussion

We tried to examine species differences in bilirubin glucuronidation activity between human and dog using microsomes and the 3D culture evaluation system established in Section 3.2.

The microsomes experiment exhibited that bilirubin glucuronidation activity in DLMs was obviously lower than that of HLMs in consistency with previous reports (Soars, et al. 2001;

Troberg, et al. 2015). In the 3D culture experiment, we detected bilirubin mono-glucuronide in both HepG2 and canine hepatoma cells. However, remaining bilirubin in canine hepatoma cell culture medium obviously decreased especially until 10 h after incubation, whereas the amount of remaining bilirubin in the HepG2 culture medium was almost stable throughout the culture period. Theoretically, remaining bilirubin should change little in amounts as the behavior in HepG2 since the amount of bilirubin applied is much larger than generated bilirubin glucuronides in this system. Therefore, the behavior of the amount for remaining bilirubin in canine hepatoma cell culture medium was not reasonable, and the bilirubin glucuronide detected in canine hepatoma cells might not reflect the correct activity of bilirubin catabolism of the cells. The cause of the decrease in remaining bilirubin in canine hepatoma cell culture medium remains unclear. The further optimization for cell culture and bilirubin glucuronidation assay conditions is required to solve the problem because there was adhesion of cells to the interstitial area between spheroids and suspended cells that might affect the behavior of bilirubin and bilirubin glucuronides in canine hepatoma cell culture medium.

It is important to understand the similarities and differences of metabolism between human and animals in order to extrapolate the results of non-clinical toxicity evaluations to human in pharmaceutical compound development. Furthermore, when we could reveal the mechanism of species difference in detail, for instance, at the level of gene or protein, the accuracy of extrapolation of animal data to human would be improved. Regarding bilirubin glucuronidation activity, species difference between human and dog that dog has lower activity, has been reported previously and reconfirmed in this experiment using microsomes. Several kinds of genetic polymorphism of UGT1A1 that catalyzes bilirubin glucuronidation have been reported in human, and they show defective bilirubin conjugation in varying degrees, leading to a decrease

in bilirubin clearance (Erlinger, et al. 2014). For example, UGT1A1*6, a single nucleotide substitution causing a missense mutation (Gly71Arg), is common polymorphism and causes a decreased bilirubin glucuronidation activity. Interestingly, the 71th amino acid of UGT1A1 in dog is also different from other species, including human (Figure 11). In addition, it has been reported that mutations of UGT1A1, including Gly71Arg, alters the number of orientations of bilirubin and the bilirubin conjugation capacity of UGT1A1 is mainly regulated by the orientation of substrate (Takaoka, et al. 2010). Therefore, we hypothesize that the difference in the 71th amino acid of UGT1A1 leads to lower bilirubin glucuronidation activity in dogs as patients with UGT1A1*6 (Gly71Arg). We hope that there will be future studies to verify the hypothesis. For example, we can consider an experiment that assesses bilirubin glucuronidation activity before and after the conversion of the 71th amino acid of UGT1A1 in canine hepatoma cells to human form (alanine to glycine) and the conversion of the 71th amino acid of UGT1A1 in HepG2 to canine form (glycine to alanine) using genome editing. We do not doubt that the 3D culture bilirubin glucuronidation evaluation system using hepatoma cell lines will be of help for these further experiments because hepatoma cell lines, including HepG2, are generally thought to be easier to obtain, handle, and operate in genome editing than primary hepatocytes.

Figure 11: Excerpt of sequences and alignment of UGT1A1 proteins for human (UGT1A1*6), dog, human (normal), monkey, mouse and rat, information

```
Human_UGT1A1*6   M A V E S Q G G R P L V L G - - L L L C V L G P V V S H A G K I L L I P V D G S H W L S M L G A I Q Q L Q Q R G H E I V   58
         Dog     M A A E A P G P R P L V L G - - L L L C A L S A P V S Q G G K L L L I P V D G S H W L S M L G V V K Q L H Q R G H E V V   58
  Human_normal   M A V E S Q G G R P L V L G - - L L L C V L G P V V S H A G K I L L I P V D G S H W L S M L G A I Q Q L Q Q R G H E I V   58
       Monkey    M A V E S Q G R H P L V L G - - L L L C V L G P V L C H A G K M L L I P V D G S H W L S M L G T I Q Q L Q Q R G H E I V   58
        Mouse    M T V V C W S S R L L L L L P Y L L L C V F G P S A S H A G R L L V F P M D G S H W L S M L G V I Q Q L Q Q K G H E V V   58
         Rat     M S V V C R S S C S L L L L P C L L L C V L G P S A S H A G K L L V I P I D G S H W L S M L G V I Q Q L Q Q K G H E V V   58
                 * : .   .   .         * : *           * * * * . : . .       . : . * : : * : : * : * * * * * * * * * . : : * * : * : * * * : *

Human_UGT1A1*6   V L A P D A S L Y I R D R A F Y T L K T Y P V P F Q R E D V K E S F V S L G H N V F E N D S F L Q R V I K T Y K K I K K   118
         Dog     V I A S E A S V Y I K G A A F Y T L K R Y P V P F R R E D V E A T F T S L G R G V F E N V P L L R R V I K T Y K K V K E   118
        Human    V L A P D A S L Y I R D G A F Y T L K T Y P V P F Q R E D V K E S F V S L G H N V F E N D S F L Q R V I K T Y K K I K K   118
       Monkey    V L A P D A S L Y I R E G A F Y T L K T Y P V P F Q R E D V K E S F V S L G H N V F E N D S F L R R V I K T Y K K I K K   118
        Mouse    V I A P E A S I H I K E G S F Y T L R K F P V P F Q K E N V T A T L V E L G R T A F N Q D S F L L R V V K I Y M K V K R   120
         Rat     V I A P E A S I H I K E G S F Y T M R K Y P V P F Q N E N V T A A F V E L G R S V F D Q D P F L L R V V K T Y N K V K R   120
                 * : * . : * * : : * :   : * * * : :   : * * * * : . * : *       : : . . * * :   . * : :   . : *   * * : *   *   * : * .
                                  ↑
                                  71
```

from (Li and Wu 2007)

4.   SUMMARY

In the first experiment, we conducted experiments to analyze and compare the bilirubin glucuronidation activity of HepG2 cells cultured in the usual 2D plates and 3D culture systems to exhibit the efficiency of the 3D-cultured hepatoma cell line for evaluation of bilirubin metabolism features. In this experiment, we found that bilirubin mono-glucuronide was detected at 1 h after initiation of the incubation in both 2D- and 3D-cultured HepG2 cells and the relative amounts of bilirubin mono-glucuronides elevated in a time-dependent manner up to 72 h especially in 3D-cultured HepG2 cells. The relative amounts of conjugated bilirubin in the HepG2 cells cultured by 3D culture systems significantly increased as compared with those generated by 2D plates after 10 h of incubation. Although bilirubin glucuronides were detected after 1 h-incubation, we judged that the amounts of glucuronides were too low to compare the bilirubin glucuronidation activity and to evaluate the utility of the 3D-culture systems. Therefore, in this experiment, we set longer incubation time up to 72 h to demonstrate the difference between 2D and 3D-culture conditions. Consequently, the HepG2 cells cultured by the 3D culture systems exhibited a higher metabolic activity of bilirubin glucuronidation than those by 2D culture plates, and we confirmed the efficacy of the 3D culture systems for the evaluation of bilirubin glucuronidation activity using hepatoma cell lines. It is crucial to establish the easy-to-use in vitro culture system for the comprehensive evaluation of bilirubin glucuronidation because it is well known that the impairment of bilirubin metabolism is related to many clinical outcome and detailed exploration for bilirubin metabolism can help to reveal these characteristics. With this evaluation system of 3D culture, we can conduct a detailed study for bilirubin glucuronidation easily using hepatoma cell lines.

In the second experiment, we tried to compare the bilirubin glucuronidation activity between humans and dogs by the microsome experiments and the experiment using the 3D culture systems established in the first experiment. In the microsomes experiment, bilirubin glucuronidation activity in DLMs was lower than that of HLMs in consistency with previous reports. In the 3D culture experiment, although bilirubin mono-glucuronide was generated in both HepG2 and canine hepatoma cells, remaining bilirubin in canine hepatoma cell culture medium obviously decreased especially until 10 h after incubation. In contrast, the amount of remaining bilirubin in the HepG2 culture medium was almost stable throughout the culture period. Theoretically, remaining bilirubin should change little in amounts because the amount of applied bilirubin is much larger than generated bilirubin glucuronides in this system. Therefore, the behavior for the amount of residual bilirubin in canine hepatoma cell culture medium was not reasonable, and the conjugated bilirubin detected in the culture medium of canine hepatoma cells might not reflect the correct activity of bilirubin catabolism of the cells. The cell culture and bilirubin glucuronidation assay conditions need to be optimized further because there were adhesion of cells in the interstitial area between spheroids and also suspended cells that could affect the behavior of bilirubin and bilirubin glucuronides in canine hepatoma cell culture medium.

It is important to understand the similarities and differences in metabolism properties between humans and animals to extrapolate the results of non-clinical toxicity studies to humans in pharmaceutical compound development. In addition, if we could reveal the mechanism of species difference at the level of gene or protein, an extrapolation of animal data to humans would be more accurate. Regarding bilirubin glucuronidation activity, the dog has been reported previously to have lower activity than human, and we have reconfirmed that in the microsome

experiment of this study. Some genetic polymorphisms of UGT1A1 (enzyme for bilirubin glucuronidation) have been reported in humans. For example, UGT1A1*6, a single nucleotide substitution causing a missense mutation (Gly71Arg), is a common polymorphism that causes a lower bilirubin glucuronidation activity. The 71th amino acid of UGT1A1 in the dog is also different from human, and the difference in the 71th amino acid of UGT1A1 might be involved in lower bilirubin glucuronidation activity in dogs. We believe that the 3D culture systems for bilirubin glucuronidation evaluation using hepatoma cell lines will be helpful for the further experiments that address the detail for species difference at the level of gene or protein because hepatoma cell lines, including HepG2, are generally easier to obtain and handle than primary hepatocytes.

5.   ACKNOWLEDGEMENT

6.   REFERENCES

Aono S, Adachi Y, Uyama E, Yamada Y, Keino H, Nanno T, Koiwai O, Sato H (1995) Analysis
of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. Lancet
(London, England) 345:958-959

Bell CC, Hendriks DF, Moro SM, Ellis E, Walsh J, Renblom A, Fredriksson Puigvert L, Dankers
AC, Jacobs F, Snoeys J, Sison-Young RL, Jenkins RE, Nordling A, Mkrtchian S, Park
BK, Kitteringham NR, Goldring CE, Lauschke VM, Ingelman-Sundberg M (2016)
Characterization of primary human hepatocyte spheroids as a model system for drug-
induced liver injury, liver function and disease. Scientific reports 6:25187

Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, Lindhout D, Tytgat GN,
Jansen PL, Oude Elferink RP, et al. (1995) The genetic basis of the reduced expression of
bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. The New England
journal of medicine 333:1171-1175

Brandon EF, Raap CD, Meijerman I, Beijnen JH, Schellens JH (2003) An update on in vitro test
methods in human hepatic drug biotransformation research: pros and cons. Toxicology
and applied pharmacology 189:233-246

Chang JH, Plise E, Cheong J, Ho Q, Lin M (2013) Evaluating the in vitro inhibition of UGT1A1,
OATP1B1, OATP1B3, MRP2, and BSEP in predicting drug-induced hyperbilirubinemia.
Molecular pharmaceutics 10:3067-3075

Dalvie D, Obach RS, Kang P, Prakash C, Loi CM, Hurst S, Nedderman A, Goulet L, Smith E,
Bu HZ, Smith DA (2009) Assessment of three human in vitro systems in the generation
of major human excretory and circulating metabolites. Chemical research in toxicology
22:357-368

Erlinger S, Arias IM, Dhumeaux D (2014) Inherited disorders of bilirubin transport and

conjugation: new insights into molecular mechanisms and consequences.

Gastroenterology 146:1625-1638

Guo L, Dial S, Shi L, Branham W, Liu J, Fang JL, Green B, Deng H, Kaput J, Ning B (2011)

Similarities and differences in the expression of drug-metabolizing enzymes between

human hepatic cell lines and primary human hepatocytes. Drug metabolism and

disposition: the biological fate of chemicals 39:528-538

Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L,

Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM,

Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of

metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of

hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity

studies. Drug metabolism reviews 39:159-234

Jemnitz K, Lengyel G, Vereczkey L (2002) In vitro induction of bilirubin conjugation in primary

rat hepatocyte culture. Biochemical and biophysical research communications 291:29-33

Kadakol A, Ghosh SS, Sappal BS, Sharma G, Chowdhury JR, Chowdhury NR (2000) Genetic

lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1)

causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype.

Human mutation 16:297-306

Kobayashi K, Yoshida A, Ejiri Y, Takagi S, Mimura H, Hosoda M, Matsuura T, Chiba K (2012)

Increased expression of drug-metabolizing enzymes in human hepatocarcinoma FLC-4

cells cultured on micro-space cell culture plates. Drug metabolism and pharmacokinetics

27:478-485

Lauschke VM, Shafagh RZ, Hendriks DFG, Ingelman-Sundberg M (2019) 3D Primary Hepatocyte Culture Systems for Analyses of Liver Diseases, Drug Metabolism, and Toxicity: Emerging Culture Paradigms and Applications. Biotechnology journal 14:e1800347

Lengyel G, Veres Z, Szabo P, Vereczkey L, Jemnitz K (2005) Canalicular and sinusoidal disposition of bilirubin mono- and diglucuronides in sandwich-cultured human and rat primary hepatocytes. Drug metabolism and disposition: the biological fate of chemicals 33:1355-1360

Li C, Wu Q (2007) Adaptive evolution of multiple-variable exons and structural diversity of drug-metabolizing enzymes. BMC evolutionary biology 7:69

Ma G, Lin J, Cai W, Tan B, Xiang X, Zhang Y, Zhang P (2014) Simultaneous determination of bilirubin and its glucuronides in liver microsomes and recombinant UGT1A1 enzyme incubation systems by HPLC method and its application to bilirubin glucuronidation studies. Journal of pharmaceutical and biomedical analysis 92:149-159

Ohkura T, Ohta K, Nagao T, Kusumoto K, Koeda A, Ueda T, Jomura T, Ikeya T, Ozeki E, Wada K, Naitoh K, Inoue Y, Takahashi N, Iwai H, Arakawa H, Ogihara T (2014) Evaluation of human hepatocytes cultured by three-dimensional spheroid systems for drug metabolism. Drug metabolism and pharmacokinetics 29:373-378

Ramaiahgari SC, den Braver MW, Herpers B, Terpstra V, Commandeur JN, van de Water B, Price LS (2014) A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Archives of toxicology 88:1083-1095

Soars MG, Riley RJ, Findlay KA, Coffey MJ, Burchell B (2001) Evidence for significant differences in microsomal drug glucuronidation by canine and human liver and kidney. Drug metabolism and disposition: the biological fate of chemicals 29:121-126

Sticova E, Jirsa M (2013) New insights in bilirubin metabolism and their clinical implications. World journal of gastroenterology 19:6398-6407

Takahashi Y, Hori Y, Yamamoto T, Urashima T, Ohara Y, Tanaka H (2015) 3D spheroid cultures improve the metabolic gene expression profiles of HepaRG cells. Bioscience reports 35

Takano M, Sugiyama T (2017) UGT1A1 polymorphisms in cancer: impact on irinotecan treatment. Pharmacogenomics and personalized medicine 10:61-68

Takaoka Y, Ohta M, Takeuchi A, Miura K, Matsuo M, Sakaeda T, Sugano A, Nishio H (2010) Ligand orientation governs conjugation capacity of UDPglucuronosyltransferase 1A1. J Biochem 148:25-28

Troberg J, Jarvinen E, Muniz M, Sneitz N, Mosorin J, Hagstrom M, Finel M (2015) Dog UDP-glucuronosyltransferase enzymes of subfamily 1A: cloning, expression, and activity. Drug metabolism and disposition: the biological fate of chemicals 43:107-118

Wang Q, Dai Z, Wen B, Ma S, Zhang Y (2015) Estimating the Differences of UGT1A1 Activity in Recombinant UGT1A1 Enzyme, Human Liver Microsomes and Rat Liver Microsome Incubation Systems in Vitro. Biological & pharmaceutical bulletin 38:1910-1917

Westerink WM, Schoonen WG (2007) Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. Toxicology in vitro : an international journal published in association with BIBRA 21:1592-1602

Wilkening S, Stahl F, Bader A (2003) Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug metabolism and disposition: the biological fate of chemicals 31:1035-1042