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Regular Article

Evaluation of Human Hepatocytes Cultured by Three-dimensional Spheroid Systems for Drug Metabolism

Takako OHKURA[1,2,*], Kunihiro OHTA[1,3], Takuya NAGAO[1,4], Kumiko KUSUMOTO[1,4], Akiko KOEDA[1,5], Tadayoshi UEDA[1,6], Tomoko JOMURA[1,7], Takeshi IKEYA[1,7], Emiko OZEKI[1,7], Kazuki WADA[1,8], Kazushi NAITOH[1,9], Yukiko INOUE[1,10], Naoki TAKAHASHI[1,10], Hisakazu IWAI[1,11], Hiroshi ARAKAWA[12] and Takuo OGIHARA[1,12]

[1]*Spheroid Working Group in Safety Evaluation Forum*
[2]*Maruishi Pharmaceutical Co., Ltd., Osaka, Japan*
[3]*Pre-Clinical Research Department, Chugai Pharmaceutical Co., Ltd., Gotemba, Japan*
[4]*Bioscience Research Center, Pharmaceutical Business Division, Sumika Chemical Analysis Service, Ltd., Osaka, Japan*
[5]*Ina Research Inc., Ltd., Ina, Japan*
[6]*Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan*
[7]*Toyo Gosei Co., Ltd., Ichikawa, Japan*
[8]*Janssen Pharmaceutical K.K., Tokyo, Japan*
[9]*Charles River Laboratories Japan Inc., Ltd., Yokohama, Japan*
[10]*Sanwa Kagaku Kenkyusho Co., Ltd., Inabe, Japan*
[11]*Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan*
[12]*Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan*

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Summary: We investigated the utility of three-dimensional (3D) spheroid cultures of human hepatocytes in discovering drug metabolites. Metabolites of acetaminophen, diclofenac, lamotrigine, midazolam, propranolol and salbutamol were analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) to measure enzyme activities in this system cultured for 2 and 7 days. Sequential metabolic reactions by Phase I and then Phase II enzymes were found in diclofenac [CYP2C9 and UDP-glucuronyltransferases (UGTs)], midazolam (CYP3A4 and UGTs) and propranolol (CYP1A2/2D6 and UGTs). Moreover, lamotrigine and salbutamol were metabolized to lamotrigine-*N*-glucuronide and salbutamol 4-*O*-sulfate, respectively. These metabolites, which are human specific, could be observed in clinical studies, but not in conventional hepatic culture systems as in previous reports. Acetaminophen was metabolized to glucuronide and sulfate conjugates, and *N*-acetyl-*p*-benzo-quinoneimine (NAPQI) and its metabolites were not observed. In addition, mRNA of drug-metabolism enzymes [CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, UGT1A1, UGT2B7, sulfotransferase 1A1 (SULT1A1) and glutathione S-transferase pi 1 (GSTP1)], which were measured by qRT-PCR, were expressed in the human hepatocyte spheroids. In conclusion, these results suggest that human hepatocyte spheroids are useful in discovering drug metabolites.

Keywords: human hepatocyte; spheroid; metabolic enzymes; sequential metabolism; biotransformation

Introduction

Biotransformation of drugs is one of the most important factors affecting pharmacokinetic properties, and hence the overall therapeutic and toxicity profiles. Drug metabolisms are often assessed by human hepatic microsomes, which have various drug enzymes. However, assays using human hepatocytes are recommended for predicting metabolites in humans, because microsomes lack cytosol enzymes and several cofactors for drug metabolism. The typical use of human hepatocytes in metabolite assays involves

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To whom correspondence should be addressed: Takako OHKURA, Ph.D., Maruishi Pharmaceutical Co., Ltd., 2-4-2 Imazu-Naka, Tsurumi-ku, Osaka 538-0042, Japan. Tel. +81-6-6962-0318, Fax. +81-6-6962-6422, E-mail: takako_ohkura@maruishi-pharm.co.jp

incubation of new compounds with suspensions of fresh or cryopreserved cells, or conventional two-dimensional (2D) cells. However, studies using the fresh or cryopreserved cells are limited by the incubation period, as metabolic capacity declines within a few hours. In many cases, the metabolite profiles of compounds in such studies do not reflect those *in vivo*. Although 2D cultures of primary human hepatocytes are frequently used for biotransformation of drugs, it is difficult to maintain the hepatic physiological function in long-term cultures. For example, changes in the expression levels of various drug-metabolism enzymes have been observed.[1,2] Moreover, culturing hepatocytes in conventional 2D plates would cause morphological alterations and loss of cell polarity and specific hepatic functions.[3,4] Therefore, there is a need for long-term stability of functioning hepatocytes in drug metabolism and pharmacokinetic (DMPK) studies.

Three-dimensional (3D) cultures of human hepatocytes are considered to be a promising DMPK model *in vitro*.[5] The purpose of this study is to prove whether assays using human hepatocyte spheroids reflect human metabolic profiles. Therefore, we identically and qualitatively compared the metabolites from the tested drugs in this assay with reported metabolites in clinical studies. Moreover, we performed comprehensive mRNA expression of metabolism enzymes in 3D cultures of primary hepatocytes.

Methods

Chemicals: Acetaminophen, diclofenac, lamotrigine, midazolam, salbutamol, propranolol and dextrophan were purchased from Sigma Aldrich (St. Louis, MO). Cryopreserved human hepatocytes were purchased from Charles River (Lot No. Hu8110, Tokyo, Japan). William's Medium E, Cryopreserved Hepatocytes Recovery Medium (CHRM®) and Hepatocyte Plating Supplement Pack (CM3000) were purchased from Invitrogen (Durham, NC). A 3D culture system, Cell-able™ 96-well and 24-well plates, and RM101 medium were purchased from Transparent (Chiba, 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.

Three-dimensional culture of human hepatocytes and metabolic assays: Feeder cells (3T3-Swiss Albino cells) were cultured in DMEM (Wako Pure Chemical Industries, Osaka, Japan) with penicillin (100 µg/mL, Nacalai Tesque, Kyoto, Japan), streptomycin (100 µg/mL, Wako Pure Chemical Industries) and 10% fetal bovine serum (Invitrogen). The 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 96-well format Cell-able™. After 4 days, cryopreserved human hepatocytes were seeded. Briefly, they were stored in liquid nitrogen until use, and immediately immersed in water pre-warmed to 37°C. After dissolution of the hepatocytes, they were decanted into CHRM® and centrifuged at $100 \times g$ for 10 min. The hepatocytes were re-suspended in William's Medium E containing CM3000. The viability of the hepatocytes was assessed by trypan blue exclusion, and suspensions with viability of over 90% were used. They were seeded at a density of 2.0×10^4 cells/well on the Cell-able™ and maintained using RM101 medium (containing 1% FBS, Transparent) for spheroid-formation periods of 2, 7, 14 or 21 days before assays. In metabolism assays, the hepatocyte spheroids were cultured with the culture medium (RM101) containing test compounds for 2 or 7 days.

LC/MS/MS analysis: The amount of unchanged compound remaining was determined using a Xevo™ TQ MS (Waters, MA) tandem quadrupole mass spectrometer coupled with an ACQUITY UPLC® system (Waters) as previously described.[6] The mass numbers of the molecular and product ions for each compound were as follows: acetaminophen (152.0 → 110.1), diclofenac (296.0 → 214.6), lamotrigine (256.0 → 109.0), midazolam (326.1 → 128.9), salbutamol (240.1 → 148.0) and propranolol (260.1 → 116.0). The metabolites were identified by comparing the retention times and molecular weights of the standard compounds. When corresponding metabolites were not commercially available, they were estimated using these calculated molecular weights and ion fragment patterns. The amounts of metabolites were determined by relative ratio to the ion peak areas of dextrorphan, an internal standard (IS).

mRNA expression assay: The feeder cells were plated at a density of 4.0×10^4 cells/well on 24-well format Cell-able™. After 4 days, cryopreserved human hepatocytes were seeded at a density of 1.0×10^5 cells/well on the Cell-able™. The total RNA was extracted from the hepatocytes cultured on the Cell-able™ after 2 and 7 days. The mRNA expression of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, UGT1A1, UGT2B7, SULT1A1 and GSTP1 was measured by quantitative RT-PCR (qRT-PCR), using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). Since hypoxanthine guaninephosphoribosyl-transferase 1 (HPRT1) mRNA exhibited stable expression in human hepatocytes,[7] the mRNA expression of target genes was normalized to HPRT1, and was calculated relative to that of the cryopreserved hepatocytes (set to 100%) using the 2-ΔΔ threshold cycles (Ct) method.[8] The gene-specific primer sequences are listed in **Table 1**.

Results

Substrate depletion in the human hepatocyte spheroids: The tested compounds (acetaminophen, diclofenac, lamotrigine, midazolam, salbutamol and propranolol) were incubated in hepatocyte spheroids for 2 (**Fig. 1A**) and 7 days (**Fig. 1B**) after spheroid-formation periods of 2, 7, 14 and 21 days. The metabolic activity of hepatocyte spheroids increased for up to 7 days during the culture periods. However, the metabolic activities for spheroid-formation periods of 14 and 21 days decreased compared with those for 2 and 7 days. Therefore, the period of spheroid formation was set at 2 days for subsequent examinations.

Biotransformation of drugs in the human hepatocyte spheroids: The biotransformation assays of drugs were measured after incubation with the hepatocyte spheroids for 2 and 7 days (**Table 2**).

Diclofenac, a substrate of CYP2C9, was converted to hydroxyl diclofenac, diclofenac acyl glucuronide and 4′-hydroxy diclofenac acylglucuronide at 2 days. In addition, 4′,5-dihydroxy diclofenac was observed at 7 days (**Table 2**; see **Supplemental Fig. 1A**). The production of *N*,5-dihydroxy diclofenac, known as a hepatotoxic metabolite of diclofenac,[9] was not observed.

Midazolam, a substrate of CYP3A4, was converted to 1′-hydroxy midazolam, midazolam *N*-glucuronide, 1′-hydroxymidazolam *O*-glucuronide, 1′-hydroxy midazolam *N*-glucuronide, 4-hydroxy midazolam and 4-hydroxy midazolam *N*-glucuronide at 2 days (**Table 2**; see **Supplemental Fig. 1B**). At 7 days of incubation, 1′-hydroxymidazolam and 4-hydroxymidazolam were not observed, suggesting that these metabolites were converted to

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