Nagata, K. and Yamazoe, Y.: Assessment of human pregnane X receptor-involvement in pesticide-mediated activation of CYP3A4 gene. *Drug Metab. Dispos.*, 35: 728–733 (2007).

12) Kanegae, Y., Makimura, M. and Saito, I.: A Simple and efficient method for purification of infectious recombinant adenovirous. *Jpn. J. Med. Sci. Biol.*, 47: 157–166 (1994).

13) Barnes, P. J.: Corticosteroid effects on cell signaling. *Eur. Respir. J.*, 27: 413–426 (2006).

14) Ponec, M., Kempenaar, J., Shroot, B. and Caron, J. C.: Glucocorticoids: binding affinity and lipophilicity. *J. Pharm. Sci.*, 75: 973–975 (1986).

15) Schimmer, B. P. and Parker, K. L.: Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In Brunton, L. L., Lazo, J. S. and Parker, K. L. (eds.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 11th ed., New York, McGraw-Hill, 2006, pp. 1587–1612.

16) Hashimoto, H., Toide, K., Kitamura, R., Fujita, M., Tagawa, S., Itoh, S. and Kamataki, T.: Gene structure of CYP3A4, an adult-specific form of cytochrome P450 in human livers, and its transcriptional control. *Eur. J. Biochem.*, 218: 585–595 (1993).

17) Jounaïdi, Y., Guzelian, P. S., Maurel, P. and Vilarem, M. J.: Sequence of the 5′-flanking region of CYP3A5: comparative analysis with CYP3A4 and CYP3A7. *Biochem. Biophys. Res. Commun.*, 205: 1741–1747 (1994).

18) Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P. and Berkenstam, A.: Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc. Natl. Acad. Sci. USA*, 95: 12208–12213 (1998).

19) Blumberg, B., Sabbagh, W., Jr., Juguilon, H., Bolado, J., Jr., van Meter, C. M., Ong, E. S. and Evans, R. M.: SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.*, 12: 3195–3205 (1998).

20) Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moor, J. T. and Kliewer, S. A.: The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.*, 102: 1016–1023 (1998).

21) Maruyama, M., Matsunaga, T., Harada, E. and Ohmori, S.: Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biol. Pharm. Bull.*, 30: 2091–2097 (2007).

22) Takezawa, T., Matsunaga, T., Aikawa, K., Nakamura, K. and Ohmori, S.: Lower expression of HNF4α and PGC1α might impair rifampicin-mediated CYP3A4 induction under conditions where PXR overexpressed in human fetal liver cells. *Drug Metab. Pharmacokinet.*, 27: 430–438 (2012).

23) Pang, X. Y., Cheng, J., Kim, J. H., Matsubara, T., Krausz, K. W. and Gonzalez, F. J.: Expression and regulatjion of human fetal-specific CYP3A7 in mice. *Endocrinology*, 153: 1453–1463 (2012).

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

Drug Metab. Pharmacokinet. 27 (6): 598–604 (2012). Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

Regular Article

Evaluation of Human Embryonic Stem Cell-derived Hepatocyte-like Cells for Detection of CYP1A Inducers

Hiroyuki TSUCHIYA[1], Tamihide MATSUNAGA[2], Kaori AIKAWA[1], Noboru KAMADA[3], Katsunori NAKAMURA[1], Hinako ICHIKAWA[4], Katsunori SASAKI[4] and Shigeru OHMORI[1,*]

[1]*Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan*
[2]*Department of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan*
[3]*Basic Discovery Research, R&D Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan*
[4]*Department of Histology and Embryology, Medical School of Shinshu University, Matsumoto, Japan*

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: There is a great deal of interest in differentiation of human embryonic stem cells (hESCs) into hepatocyte-like cells for application in pharmaceutical screening. Cytochrome P450 (CYP) 1A is involved in the metabolic activation of procarcinogenic compounds as well as in detoxification of drugs. We differentiated hESCs into hepatocyte-like cells (hESC-derived hepatocyte-like cells) and examined whether CYP1A was induced in these cells by typical inducers of CYP1A. hESC-derived hepatocyte-like cells expressed albumin, α-fetoprotein, CYP3A4, CYP3A7, CYP1A1, CYP1A2, and UDP-glucuronyl transferase (UGT) 1A1 mRNA. The levels of CYP1A1, CYP1A2, and UGT1A1 mRNA expression were increased by omeprazole and 3-methylcholanthrene. Furthermore, the enzyme activity of CYP1A was also increased by these compounds. In conclusion, hESC-derived hepatocyte-like cells are available for the detection of CYP1A inducers.

Keywords: hESCs; differentiation; hESC-derived hepatocyte-like cells; CYP1A; induction

Introduction

Human hepatocytes and liver tissues are used for nonclinical tests in pharmaceutical screening. However, some problems remain, including difficulty in obtaining cells due to limitations of tissue availability as well as individual differences between such specimens. Furthermore, the expression levels of many proteins decrease rapidly in culture.[1] An alternative is to use hepatoma cell lines; however, these cells normally contain low levels of metabolic enzymes, and usually poorly reflect the phenotype of human hepatocytes *in vivo*.[2]

Human embryonic stem cells (hESCs) are able to grow indefinitely and have the ability to differentiate into most body cell types.[3] Hepatocyte-like cells, which are differentiated from hESCs, would be useful for regenerative medicine and drug discovery.[4] Therefore, there is a great deal of interest in methods of differentiating hESCs into hepatocyte-like cells for application to pharmaceutical screening.

In humans, enzymes belonging to the cytochrome P450 1 family (CYP1A) play important roles in the detoxification of therapeutic agents in the liver. On the other hand, CYP1A is involved in the metabolic activation of procarcinogenic compounds, such as polycyclic aromatic hydrocarbons and aromatic amines found in cigarette smoke and cooked foods.[5,6] For example, CYP1A1 are known to metabolize benzo[*a*]pyrene and 3-methylcholanthrene (3-MC) to their ultimate carcinogenic forms.[5,7] Therefore, it is desirable to remove drug candidates that are capable of inducing expression of the CYP1A subfamily in pharmaceutical screening.

To increase the efficiency of drug discovery, it is important to establish a new assay system that can detect enzyme induction by drug candidates without using human liver samples. In the present study, we differentiated hESCs into hepatocyte-like cells (hESC-derived hepatocyte-like cells) expressing CYP1A1 and CYP1A2. Moreover, we demonstrated induction of mRNA levels of CYP1A1 and CYP1A2,

Received February 2, 2012; Accepted April 25, 2012
J-STAGE Advance Published Date: May 29, 2012, doi:10.2133/dmpk.DMPK-12-RG-017
**To whom correspondence should be addressed:* Shigeru OHMORI, Ph.D., *Department of Pharmacy, Shinshu University Hospital, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Tel. +81-263-37-3021, Fax. +81-263-37-3021, E-mail: somori@shinshu-u.ac.jp*
This work was partly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Nos. 20926006, 23390036).

and metabolic activity by representative CYP1A inducers. In conclusion, hESC-derived hepatocyte-like cells may become a reliable substitute source of normal human hepatocytes, which are useful for clinical research and drug discovery as a tool for enzyme induction studies.

Materials and Methods

Materials: Dexamethasone (DEX), dimethyl sulfoxide (DMSO), omeprazole (OME), 3-MC, phenacetin, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride (Y-27632), oncostatin M (OSM), human fetal liver total RNA, and human normal adult liver total RNA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Human fetal liver total RNA is from 1 donor, who was male and 38 weeks old. Human adult liver total RNA is from 1 donor, who was male and 64 years old. Activin A and hepatocyte growth factor (HGF) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Wnt3a was purchased from R&D Systems (Minneapolis, MN). Murine embryonic fibroblasts (MEF) were obtained from Oriental Yeast (Tokyo, Japan). KnockOut Serum Replacement (KSR) and SuperScript III First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR) were from Invitrogen Life Technologies (Carlsbad, CA). TaKaRa SYBR Premix Ex Taq was obtained from Takara Bio Inc. (Otsu, Japan). Modified Lanford medium was obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan). Collagen Type I (collagen I)-coated microplates were obtained from Asahi Glass (Chiba, Japan). All other reagents used were of the highest quality available.

Cell culture and differentiation: This study was approved by the Shinshu University Institutional Review Board. KhES-3 cells were provided from Institute for Frontier Medical Science, Kyoto University, Japan, and cultured according to the method reported by Suemori *et al.*[8] The hESCs were used between passages 30 and 40, and maintained in 3% CO_2 at 37°C. When hESCs reached a confluence level of approximately 70%, differentiation was initiated by replacing the medium with medium A (RPMI1640 medium containing 2 mM GlutaMax, 2% B-27, 0.5% FBS, 100 ng/mL activin A, and 50 ng/mL Wnt3a). After 72 h, the medium was changed to medium B (RPMI1640 medium containing 2 mM GlutaMax, 2% KSR, 100 ng/mL activin A and 50 ng/mL Wnt3a) and cultured for 48 h. The cells were then passaged on collagen-coated 24-well plates and cultured in medium C (KnockOut-DMEM containing 20% KSR, 1 mM GlutaMax, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 1% DMSO) for 7 days, although Y-27632 was added at 10 µM during first 24 h of culture. Subsequently, the cells were cultured in medium D (Lanford modified medium containing 10 ng/mL HGF, 20 ng/mL OSM, and 100 nM DEX) for 6 days. Finally, the cells were cultured in Lanford modified medium alone for 4 days (**Fig. 1**).

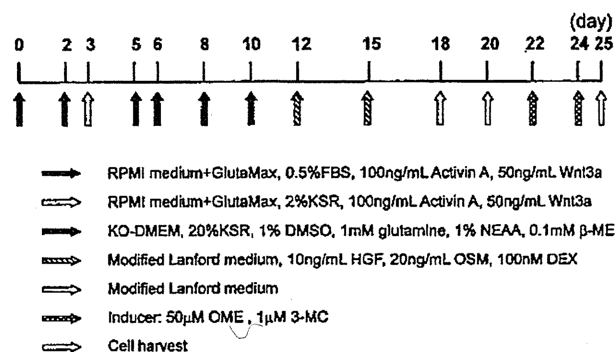


Fig. 1. Protocol for differentiation of hESCs into hepatocyte-like cells and treatment with inducers

RNA extraction and reverse transcription reaction: hESC-derived hepatocyte-like cells were treated with 50 µM OME or 1 µM 3-MC for 72 h. Total RNA was isolated from the cells using ISOGEN (Nippon Gene Co., Ltd.) according to the manufacturer's protocol. First-strand cDNA was generated from 500 ng of total RNA. Reverse transcription reaction was performed using a SuperScript III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions.

Real-time PCR: For detection of expression levels, mRNAs were analyzed by SYBR Green real-time quantitative RT-PCR. Real-time PCR analysis was performed on an ABI Prism 7300 Real-time PCR System using SYBER® Premix ExTaq (Takara Bio Inc.). PCR was performed in mixtures consisting of 10 µL of SYBER Green PCR Master Mix, 0.4 µL of 10 mM forward and reverse primers, 0.4 µL of dye, 7.8 µL of water, and 1 µL template cDNA in a total volume of 20 µL. The relative expression of each gene was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used were as follows: GAPDH: forward primer, 5′-GAG TCA ACG GAT TTG GTC GT-3′, reverse primer, 5′-GAC AAG CTT CCC GTT CTC AG-3′; albumin (ALB): forward primer, 5′-GAG CTT TTT GAG CAG CTT GG-3′, reverse primer, 5′-GGT TCA GGA CCA CGG ATA GA-3′; α-fetoprotein (AFP): forward primer, 5′-AGC TTG GTG GTG GAT GAA AC-3′, reverse primer, 5′-TCT GCA ATG ACA GCC TCA AG-3′; CYP3A4: forward primer, 5′-CTG TGT GTT TCC AAG AGA AGT TAC-3′, reverse primer, 5′-TGC ATC AAT TTC CTC CTG CAG-3′; CYP3A7: forward primer, 5′-AGA TTT AAT CCA TTA GAT CCA TTC G-3′, reverse primer, 5′-AGG CGA CCT TCT TTT ATC TG-3′; CYP1A1: forward primer, 5′-CCT CTT TGG AGC TGG GTT TG-3′, reverse primer, 5′-GCT GTG GGG GAT GGT GAA-3′; CYP1A2: forward primer, 5′-CTT TGA CAA GAA CAG TGT CCG-3′, reverse primer, 5′-AGT GTC CAG CTC CTT CTG GAT-3′; UGT1A1: forward primer, 5′-CAG CAG AGG GGA CAT GAA AT-3′, reverse primer, 5′-ACG CTG CAG GAA AGA ATC AT-3′.

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

Table 1. HPLC timetable

Time (min)	Solvent A (%)	Solvent B (%)
0.0	98	2
0.8	98	2
5.0	10	90
7.5	10	90
7.6	98	2
13.0	98	2

Solvents A and B: 10 mM ammonium acetate in water and 0.1% formic acid in methanol, respectively.

Measurement of enzymatic activity: hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h, and then incubated with 50 μM phenacetin for 24 h. After incubation, medium was collected and acetaminophen was measured by LC-MS/MS under the conditions described below.

Instrument: An Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump and a degasser linked to a CTC HTS PAL New Wash System Autosampler (AMR Inc., Tokyo, Japan) was used. Mass spectrometric detection was performed on an API 4000 triple quadruple instrument (Applied Biosystems/Sciex, Foster City, CA) equipped with a TurboIonSpray® electrospray ionization (ESI) interface. Data processing was performed with the Analyst 1.4.2 software package (Applied Biosystems/Sciex).

Chromatographic conditions: Chromatographic separation was performed on a reversed-phase CAPCELL PAK C18 MG III column (50 × 4.6 mm i.d., 5 μm; Shiseido Co., Inc., Tokyo, Japan). The column temperature was kept constant at 40°C. The mobile phase consisted of a mixture of 10 mM ammonium acetate in water (A) with 0.1% formic acid in methanol (B) and was delivered at a flow rate of 0.6 mL/min. A stepwise gradient was used as shown in **Table 1.**

Mass spectrometric conditions: The mass spectrometer was operated using the ESI source in positive ion detection. To optimize all of the MS parameters, standard solutions (100 ng/mL) and an internal standard were infused into the mass spectrometer at a flow rate of 250 μL/min. The ion spray voltage (IS) was set at 4,500 V. The TurboIonSpray probe temperature was maintained at 600°C. The instrument parameters *viz.*, nebulizer gas, curtain gas, auxiliary gas, and collision gas, were set at 60, 15, 80, and 5, respectively. Compound parameters *viz.*, declustering potential, collision energy, entrance potential, and collision exit potential, were 40, 20, 10, and 15, respectively, for acetaminophen and [$^2H_4$]acetaminophen. Zero air was used as source gas, while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated in ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 152 precursor ion [M + H] to the m/z 110 product ion for acetaminophen (retention time: 4.7 min) and m/z 156 precursor ion [M + H] to the m/z 114 product ion for [$^2H_4$]acetaminophen (4.7 min). Quadrupoles Q1 and Q3 were set to unit resolution. Data acquisition and quantification were performed using Analyst software version 1.4.2 (Applied Biosystems, MDSSciex, Toronto, Canada).

Calibration standards: Calibration standards to cover the assay range of 1–5,000 nM of acetaminophen were prepared by adding 10 μL of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 μM working standards to 0.1 μL aliquots of control reaction mixture.

Statistical analysis: Data are expressed as means ± standard deviation (SD). Statistical significance was assessed using an unpaired t-test. Differences were regarded as statistically significant at $p < 0.05$.

Results

Basal mRNA levels of various genes in hESC-derived hepatocyte-like cells: hESCs were differentiated into hepatocyte-like cells according to the method described in **Figure 1**. The levels of mRNAs of ALB, AFP, CYP3A4, CYP3A7, CYP1A1, CYP1A2, and UGT1A1 were measured by real-time PCR and compared with those of human adult or fetal liver. The mRNAs of ALB, AFP, CYP3A4, and CYP3A7 were expressed in hESC-derived hepatocyte-like cells, whereas the levels of expression of these genes were lower than those of human adult or fetal liver except for AFP (**Figs. 2A–2D**). In addition, the mRNAs of CYP1A1, CYP1A2, and UGT1A1 were expressed in these cells. The basal mRNA levels of these genes were lower than those of human adult liver, but higher than those of human fetal liver when compared to the reference standard of human adult and fetal liver total RNA (**Figs. 2E–2G**).

Influence of typical CYP1A inducers on CYP1A1, CYP1A2, and UGT1A1 mRNA levels in hESC-derived hepatocyte-like cells: We studied whether the mRNAs of CYP1A1, CYP1A2, and UGT1A1 in hESC-derived hepatocyte-like cells were inducible by typical inducers, such as OME and 3-MC, using real-time PCR. The level of CYP1A1 mRNA expression was markedly elevated from 35- to 48-fold compared to controls by OME and 3-MC (**Fig. 3A**). The level of CYP1A2 mRNA expression was also significantly increased from 3.2- to 3.5-fold compared to controls by OME and 3-MC (**Fig. 3B**).

The level of UGT1A1 mRNA expression was also significantly increased from 6.5- to 8.0-fold compared to controls by OME and 3-MC (**Fig. 3C**).

Influence of typical CYP1A inducers on phenacetin *O*-deethylase activity in hESC-derived hepatocyte-like cells: To evaluate the effect of OME or 3-MC on CYP1A activity in hESC-derived hepatocyte-like cells, we measured the concentration of acetaminophen in medium

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

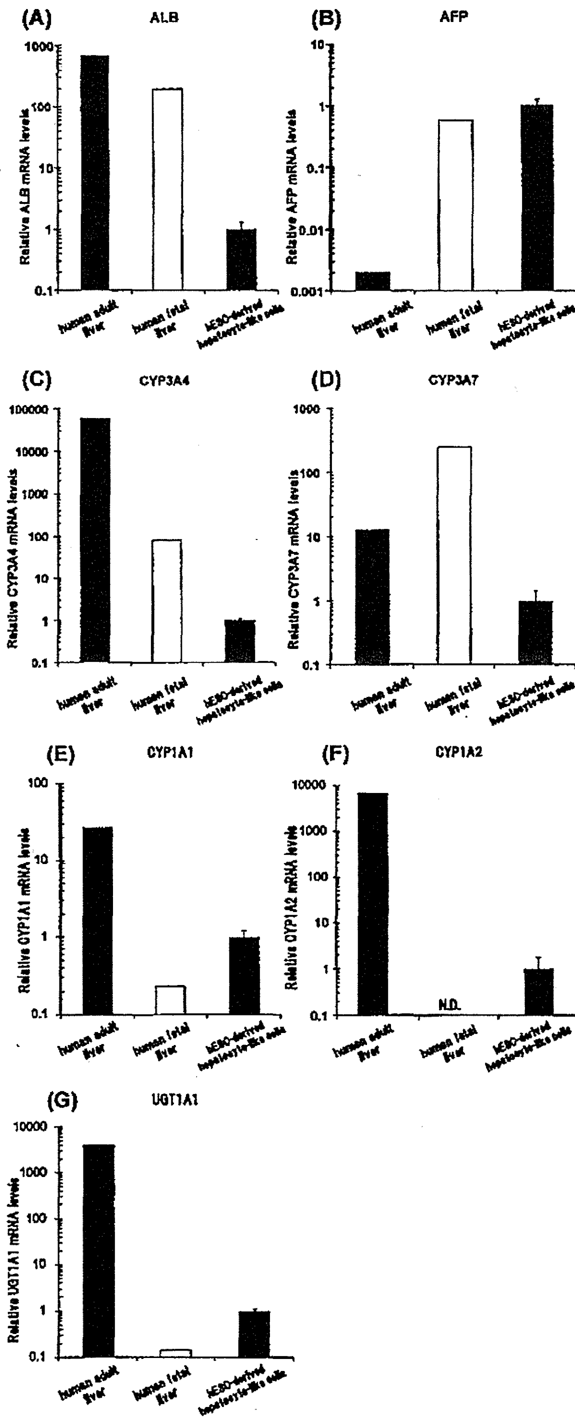


Fig. 2. Albumin (ALB), α-fetoprotein (AFP), CYP3A4, CYP3A7, CYP1A1, CYP1A2 and UGT1A1 mRNA levels in hESC-derived hepatocyte-like cells
hESCs were differentiated into hepatocyte-like cells according to the method described in Figure 1. Then, total mRNA was extracted and subjected to real-time PCR. The mRNA levels were normalized relative to that of GAPDH mRNA, and the values are shown as ratios to the average for hESC-derived hepatocyte-like cells treated with 0.1% DMSO. Results are presented as means ± SD from 6 independent experiments. N.D. means not detected.

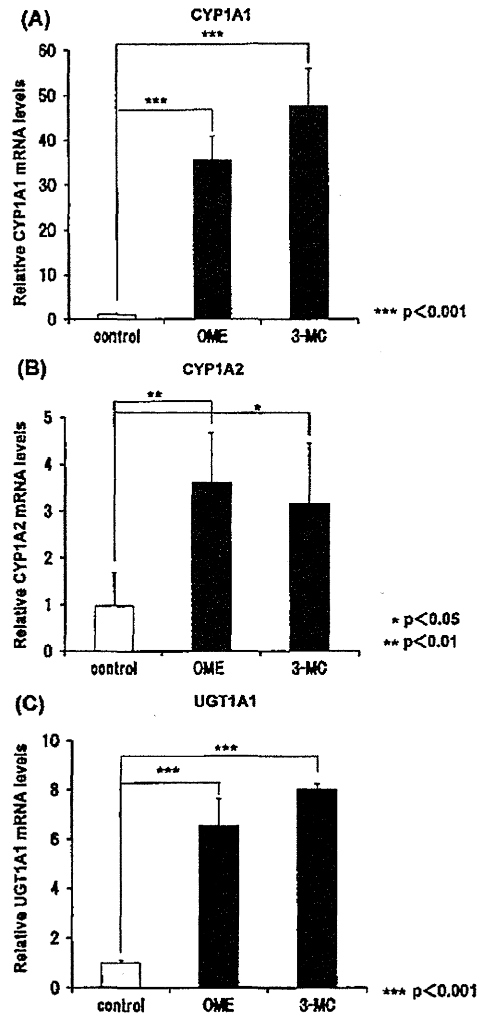


Fig. 3. The change of CYP1A1, CYP1A2 and UGT1A1 mRNA levels treated with typical CYP1A inducers in hESC-derived hepatocyte-like cells
hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h. Then, total mRNA was extracted and subjected to real-time PCR. The mRNA levels were normalized relative to that of GAPDH mRNA, and the values are shown as ratios to the average for hESC-derived hepatocyte-like cells treated with 0.1% DMSO. Results are presented as means ± SD from 6 independent experiments. Statistical analyses were performed using unpaired *t*-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

after incubation with phenacetin, which is a typical substrate for CYP1A. The phenacetin *O*-deethylase activity was elevated by 24-fold compared to controls by treatment with 3-MC, but showed only slight induction by treatment with OME (**Fig. 4**).

Discussion

In the present study, we attempted to differentiate hESCs into hepatocyte-like cells and evaluate whether CYP1A enzyme induction could be observed in these cells. A three-

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

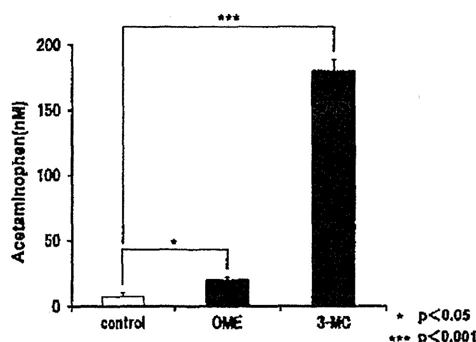


Fig. 4. The change of phenacetin O-deethylase activity treated with typical CYP1A inducers in hESC-derived hepatocyte-like cells

hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h and then incubated with 50 μM phenacetin for 24 h. After incubation, medium was collected and acetaminophen was measured with LC-MS/MS. Results are presented as means ± SD from 6 independent experiments. Statistical analyses were performed using unpaired *t*-test; *p < 0.05 and ***p < 0.001.

step process is required for induction of hESC differentiation into hepatocytes—the first step involves the differentiation of undifferentiated hESCs into endoderm, the second step involves the differentiation of endoderm into hepatoblast-like cells, and the third step involves maturation into hepatocytes. Previous studies suggested that it is important to add activin A when inducing differentiation of hESCs into endoderm.[9–11] Hay *et al.* reported that Wnt3a can also differentiate hESCs into hepatic endoderm, and its concomitant use with activin A has synergistic effects on efficient differentiation of hESCs into hepatic endoderm.[12] In the present study, we used activin A and Wnt3a concomitantly for initiation of differentiation. In addition, Y-27632, a selective inhibitor of p160-Rho-associated coiled kinase, was used to permit survival of hESCs during passage,[13,14] the use of Y-27632 decreased the death of cells during passage in the present study (data not shown).

The mRNAs of AFP and ALB, used as markers of hepatocytes, were detected in hESC-derived hepatocyte-like cells together with CYP3A4, CYP3A7, CYP1A1, CYP1A2, and UGT1A1 mRNAs (**Figs. 2A–2G**). The levels of ALB and CYP3A4 mRNAs were markedly lower than those in the human adult and fetal liver when compared to the reference standard of human adult and fetal liver total RNAs (**Figs. 2A and 2C**). On the other hand, the expression level of AFP mRNA was higher than those in the fetal and adult liver when compared to the reference standard of human adult and fetal liver total RNA (**Fig. 2B**). AFP is found to express predominantly in human fetal hepatocytes.[15] Although these results represent comparison to only one standard sample for both human adult and fetal liver, hESC-derived hepatocyte-like cells were shown to be differentiated into more fetal-like cells rather than into adult hepatocyte-like cells. It has been reported that CYP, UGT, and other liver specific genes are expressed in hepatocyte-like cells,[16] but in this report, the mRNA levels of CYP3A4 and CYP1A2 were lower than these in the present study (**Figs. 2C and 2F**).

In some studies, fibroblast growth factor, bone morphogenetic protein or sodium butyrate were used to differentiate hESCs into hepatocyte-like cells more efficiently.[11,17–19] However, when we used fibroblast growth factor, bone morphogenetic protein, and sodium butyrate to differentiate hESCs into hepatocyte-like cells in preliminary experiments, the degree of differentiation was inefficient in comparison with the conditions used in the present study (data not shown). It is necessary to develop methods to induce maturation of hepatocyte-like cells, *e.g.*, it may be useful to examine use of three-dimensional culture methods reported previously.[20–22] Besides, Takayama *et al.* recently reported that transduction of hepatocyte nuclear factor 4 into hESCs generated functional hepatocytes.[23] Therefore, it might be beneficial to utilize the transduction of a gene which differentiates hESCs efficiently into hepatocytes.

Chemical-induced expression of CYP1A is mainly regulated by the aryl hydrocarbon receptor (AhR), one of several ligand-activated transcription factors.[24–26] OME and 3-MC are typical CYP1A inducers. Although 3-MC is a strong ligand of AhR,[24] OME has been shown to activate AhR without direct binding.[27–30] The induction of UGT1A1 is also regulated by AhR as well as CYP1A1.[31] Treatment of hESC-derived hepatocyte-like cells with OME and 3-MC induced not only CYP1A1 and CYP1A2, but also UGT1A1 (**Figs. 3A–3C**), indicating that these cells possess active AhR. Induction of CYP1A1 and CYP1A2 are caused by binding of AhR to the xenobiotic-responsive element of the CYP1A1 and CYP1A2 genes.[32] Therefore, it is possible that these hESC-derived hepatocyte-like cells are available for evaluation of AhR activator. By the way, we tried to study the mRNA of CYP3A4, but the mRNA of CYP3A4 was not elevated by the treatment of rifampicin (data not shown). The character of the hESC-derived hepatocyte-like cells, *i.e.* the high mRNA levels of AFP, and the low levels of ALB and CYP3A4, suggest that these cells are fetal hepatocyte-like cells. This character is supported by the fact that the mRNA of CYP3A4 was not induced by rifampicin as shown previously.[33,34]

It is important that hESC-derived hepatocyte-like cells have inducible metabolic enzyme activity. The *O*-deethylation of phenacetin is mainly catalyzed by CYP1A2. Similar to the mRNA expression, the level of phenacetin *O*-deethylase activity was also significantly induced by treatment with OME and 3-MC (**Fig. 4**). However, the induction of phenacetin *O*-deethylase activity by OME was considerably weaker than that by 3-MC, although both showed essentially equivalent induction of not only CYP1A1 mRNA level, but also CYP1A2 mRNA level. Although the reason for this discrepancy between mRNA level and enzymatic activity is not yet clear, it may be due to the effects on posttranscrip-

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

tional regulation, *e.g.*, differences in RNA stability, translation, and protein stability.

Several reports described hESC-derived hepatocytes as having CYP1A2 activity,[12,17,19,23,35] but a few reports have previously shown inducible activity of CYP1A2 in hESC-derived hepatocytes.[23,35] In the present study, basal phenacetin *O*-deethylase activity was low, but as for elevation of phenacetin *O*-deethylase activity treated with CYP1A inducer, the hESC-derived hepatocyte-like cells may be highly sensitive to AhR activator.

In conclusion, although additional investigations are required for the development of practical applications, hESC-derived hepatocyte-like cells appear to be suitable for evaluation of CYP1A inducers.

Acknowledgments: We thank Dr. Norio Nakatsuji (Kyoto University, Kyoto, Japan) for kindly providing human embryonic stem cells (khES3).

References

1) Rodríguez-Antona, C., Donato, M. T., Boobis, A., Edwards, R. J., Watts, P. S., Castell, J. V. and Gómez-Lechón, M. J.: Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica*, 32: 505–520 (2002).

2) Jensen, J., Hyllner, J. and Björquist, P.: Human embryonic stem cell technologies and drug discovery. *J. Cell. Physiol.*, 219: 513–519 (2009).

3) Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M.: Embryonic stem cell lines derived from human blastocysts. *Science*, 282: 1145–1147 (1998).

4) Murry, C. E. and Keller, G.: Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell*, 132: 661–680 (2008).

5) Shimada, T. and Fujii-Kuriyama, Y.: Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci.*, 95: 1–6 (2004).

6) Sugimura, T., Wakabayashi, K., Nakagama, H. and Nagao, M.: Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci.*, 95: 290–299 (2004).

7) Wood, A. W., Chang, R. L., Levin, W., Thomas, P. E., Ryan, D., Stoming, T. A., Thakker, D. R., Jerina, D. M. and Conney, A. H.: Metabolic activation of 3-methylcholanthrene and its metabolites to products mutagenic to bacterial and mammalian cells. *Cancer Res.*, 38: 3398–3404 (1978).

8) Suemori, H., Yasuchika, K., Hasegawa, K., Fujioka, T., Tsuneyoshi, N. and Nakatsuji, N.: Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem. Biophys. Res. Commun.*, 345: 926–932 (2006).

9) Kubo, A., Shinozaki, K., Shannon, J. M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H. J. and Keller, G.: Development of definitive endoderm from embryonic stem cells in culture. *Development*, 131: 1651–1662 (2004).

10) D'Amour, K. A., Agulnick, A. D., Eliazer, S., Kelly, O. G., Kroon, E. and Baetge, E. E.: Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat. Biotechnol.*, 23: 1534–1541 (2005).

11) Cai, J., Zhao, Y., Liu, Y., Ye, F., Song, Z., Qin, H., Meng, S., Chen, Y., Zhou, R., Song, X., Guo, Y., Ding, M. and Deng, H.: Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology*, 45: 1229–1239 (2007).

12) Hay, D. C., Fletcher, J., Payne, C., Terrace, J. D., Gallagher, R. C., Snoeys, J., Black, J. R., Wojtacha, D., Samuel, K., Hannoun, Z., Pryde, A., Filippi, C., Currie, I. S., Forbes, S. J., Ross, J. A., Newsome, P. N. and Iredale, J. P.: Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. *Proc. Natl. Acad. Sci. USA*, 105: 12301–12306 (2008).

13) Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J. B., Nishikawa, S., Nishikawa, S., Muguruma, K. and Sasai, Y.: A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat. Biotechnol.*, 25: 681–686 (2007).

14) Takehara, T., Teramura, T., Onodera, Y., Kakegawa, R., Fukunaga, N., Takenoshita, M., Sagawa, N., Fukuda, K. and Hosoi, Y.: Rho-associated kinase inhibitor Y-27632 promotes survival of cynomolgus monkey embryonic stem cells. *Mol. Hum. Reprod.*, 14: 627–634 (2008).

15) Jones, E. A., Clement-Jones, M., James, O. F. and Wilson, D. I.: Differences between human and mouse alpha-fetoprotein expression during early development. *J. Anat.*, 198: 555–559 (2001).

16) Ek, M., Söderdahl, T., Küppers-Munther, B., Edsbagge, J., Andersson, T. B., Björquist, P., Cotgreave, I., Jernström, B., Ingelman-Sundberg, M. and Johansson, I.: Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells. *Biochem. Pharmacol.*, 74: 496–503 (2007).

17) Hay, D. C., Zhao, D., Fletcher, J., Hewitt, Z. A., McLean, D., Urruticoechea-Uriguen, A., Black, J. R., Elcombe, C., Ross, J. A., Wolf, R. and Cui, W.: Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem Cells*, 26: 894–902 (2008).

18) Agarwal, S., Holton, K. L. and Lanza, R.: Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells*, 26: 1117–1127 (2008).

19) Duan, Y., Ma, X., Zou, W., Wang, C., Bahbahan, I. S., Ahuja, T. P., Tolstikov, V. and Zern, M. A.: Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells. *Stem Cells*, 28: 674–686 (2010).

20) Baharvand, H., Hashemi, S. M., Kazemi Ashtiani, S. and Farrokhi, A.: Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. *Int. J. Dev. Biol.*, 50: 645–652 (2006).

21) Elkayam, T., Amitay-Shaprut, S., Dvir-Ginzberg, M., Harel, T. and Cohen, S.: Enhancing the drug metabolism activities of C3A—a human hepatocyte cell line—by tissue engineering within alginate scaffolds. *Tissue Eng.*, 12: 1357–1368 (2006).

22) Sasaki, K., Ichikawa, H., Takei, S., No, H. S., Tomotsune, D., Kano, Y., Yokoyama, T., Sirasawa, S., Mogi, A., Yoshie, S., Sasaki, S., Yamada, S., Matsumoto, K., Mizuguchi, M., Yue, F. and Tanaka, Y.: Hepatocyte differentiation from human ES cells using the simple embryoid body formation method and the staged-additional cocktail. *ScientificWorldJournal*, 9: 884–890 (2009).

23) Takayama, K., Inamura, M., Kawabata, K., Katayama, K., Higuchi, M., Tashiro, K., Nonaka, A., Sakurai, F., Hayakawa, T., Kusuda Furue, M. and Mizuguchi, H.: Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4α transduction. *Mol. Ther.*, 20: 127–137 (2012).

24) Whitlock, J. P., Jr.: Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.*, 39: 103–125 (1999).

25) Denison, M. S. and Nagy, S. R.: Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.*, 43: 309–334 (2003).

26) Nebert, D. W., Dalton, T. P., Okey, A. B. and Gonzalez, F. J.:

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J. Biol. Chem.*, 279: 23847–23850 (2004).

27) Daujat, M., Peryt, B., Lesca, P., Fourtanier, G., Domergue, J. and Maurel, P.: Omeprazole, an inducer of human CYP1A1 and 1A2, is not a ligand for the Ah receptor. *Biochem. Biophys. Res. Commun.*, 188: 820–825 (1992).

28) Quattrochi, L. C. and Tukey, R. H.: Nuclear uptake of the Ah (dioxin) receptor in response to omeprazole: transcriptional activation of the human CYP1A1 gene. *Mol. Pharmacol.*, 43: 504–508 (1993).

29) Lesca, P., Peryt, B., Larrieu, G., Alvinerie, M., Galtier, P., Daujat, M., Maurel, P. and Hoogenboom, L.: Evidence for the ligand-independent activation of the AH receptor. *Biochem. Biophys. Res. Commun.*, 209: 474–482 (1995).

30) Daujat, M., Charrasse, S., Fabre, I., Lesca, P., Jounaïdi, Y., Larroque, C., Poellinger, L. and Maurel, P.: Induction of CYP1A1 gene by benzimidazole derivatives during Caco-2 cell differentiation. Evidence for an aryl-hydrocarbon receptor-mediated mechanism. *Eur. J. Biochem.*, 237: 642–652 (1996).

31) Kalthoff, S., Ehmer, U., Freiberg, N., Manns, M. P. and Strassburg, C. P.: Coffee induces expression of glucuronosyltransferases by the aryl hydrocarbon receptor and Nrf2 in liver and stomach. *Gastroenterology*, 139: 1699–1710 (2010).

32) Ueda, R., Iketaki, H., Nagata, K., Kimura, S., Gonzalez, F. J., Kusano, K., Yoshimura, T. and Yamazoe, Y.: A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. *Mol. Pharmacol.*, 69: 1924–1930 (2006).

33) Matsunaga, T., Maruyama, M., Harada, E., Katsuyama, Y., Sugihara, N., Ise, H., Negishi, N., Ikeda, U. and Ohmori, S.: Expression and induction of CYP3As in human fetal hepatocytes. *Biochem. Biophys. Res. Commun.*, 318: 428–434 (2004).

34) Takezawa, T., Matsunaga, T., Aikawa, K., Nakamura, K. and Ohmori, S.: Lower expression of HNF4α and PGC1α might impair rifampicin-mediated CYP3A4 induction under conditions where PXR overexpressed in human fetal liver cells. *Drug Metab. Pharmacokinet.*, 27: 430–438 (2012).

35) Duan, Y., Catana, A., Meng, Y., Yamamoto, N., He, S., Gupta, S., Gambhir, S. S. and Zern, M. A.: Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells in vitro and in vivo. *Stem Cells*, 25: 3058–3068 (2007).

Copyright © 2012 by the Japanese Society for the Study of Xenobiotics (JSSX)

東北薬科大学研究誌，59，69－74（2012）
Journal of Tohoku Pharmaceutical University，59，69－74（2012）

鉄存在下 HepG2 細胞における TNF-α/actinomycin D 処理による肝障害モデルの構築

髙橋　昌悟，三浦　彩佳，佐々木　瞳，坂口　修平，永田　清*

TNF-α/actinomycin D-mediated HepG2 cells in the presence of iron as a model of hepatocyte injury

Shogo TAKAHASHI, Ayaka MIURA, Hitomi SASAKI, Shuhei SAKAGUCHI, and Kiyoshi NAGATA*

(Received November 20, 2012)

We examined the contribution of iron to the cytotoxicity of tumor necrosis factor (TNF)-α combined with actinomycin D (ActD) as a model of hepatocyte injury in HepG2 cells. In general, hepatocytes are resistant to TNF-α. However, a transcriptional inhibitor such as ActD can sensitize then to TNF-α. In the present study, we show that low levels of ActD (0.5 nM) sensitized HepG2 cells to the cytotoxic effects of TNF-α (20 ng/mL) for 48 h. Iron plays a critical role in catalyzing the formation of potent oxidants. To assess the toxicological significance of this TNF-α/ActD interaction, ferric-nitrilotriacetate (Fe-NTA, 2 μM) was added to the cells. Treatment with Fe-NTA significantly increased the sensitivity to the TNF-α/ActD-mediated cell death. TNF-α/ActD-mediated cell death in the presence of a lower concentration of iron did not result in DNA fragmentation. We suggest that iron increased the sensitivity to the cytotoxicity of TNF-α/ActD in HepG2 cells. It is likely that TNF-α/ActD/Fe-NTA-mediated cell death contributes to the non-apoptotic death of cells via oxidative stress caused by iron. Our experimental model may be useful for studying hepatic drug metabolism using TNF-α as a model of hepatocyte injury, especially in HepG2 cells.

Key words —— Tumor necrosis factor, cytotoxicity, actinomycin D, oxidative stress, iron

緒　　　言

創薬において，医薬品候補化合物の開発中止における重大な要因は，ヒトに対する効能不足および毒性発現とされている．[1] すなわち，非臨床試験で有効とされていた新薬候補化合物が，臨床試験において有効性が認められない，または強い副作用により開発中止を余儀なくされている．[2-4] その中でも，薬物暴露により肝臓に生じる毒性（薬物性肝障害）は，非常に高頻度で発現する副作用である．[5] 実際，薬物性肝障害は開発中止，警告あるいは販売中止に至る主要な薬剤関連有害事象となっている．[6] 薬物性肝障害は非臨床試験および市販後試験のいずれの段階においても発現し得るため，肝障害ポテンシャルおよび肝障害機序を可能な限り早期かつ正確に評価し，リスク・ベネフィット分析等を通じて，医薬品開発に関するgo/no-goの決定は，新薬の生産性に大きく貢献すると考えられる．近年の実験動物から得られた知見として，穏やかな炎症状態が毒性への閾値を低下させ，副作用への個々の感受性を上昇させるとの報告があり，[7] 肝障害に対する炎症反応の寄与が注目されている．また，Wei らにより，非ステロイド性抗炎症薬（NSAIDs）のスリンダクが，中用量のLipopolysaccharide（LPS）供処理により肝障害が誘発されるとの報告がなされた．[8,9] 加えて，そのスリンダク誘発性肝障害は，tumor necrosis factor (TNF)-α により増強されることが示唆されている．TNF-α は本来，生体防御機構に関わる炎症性サイトカインであり，肝において炎症性変化の中心的役割を演じている．一方で，近年の TNF-α による肝細胞死誘導のメカニズムの研究により，この TNF-α は肝細胞の TNF-α 受容体に結合し reactive oxygen species（ROS）産生とともに c-jun N-terminal kinase（JNK）経路を活性化し，炎症を起こした肝細胞の細胞死を誘導し，炎症を収束させると考えられている．[10] これは，活性化された JNK が，caspase-8 に対する阻害作用をもつ cellular FLICE-inhibitory protein（c-Flip）を抑制し引き起こされると考えられている．[10] しかしなが

ら，*In vitro* では多くの細胞が TNF-α の細胞死誘導に対して耐性であり，特に肝細胞や肝がん細胞においては顕著である．近年の研究から，それには，nuclear factor-kappa B（NF-κB）による JNK 経路の抑制が寄与しており，[11] また，NF-κB の発現量をノックダウンした細胞では，少量の TNF-α でも細胞死が起こることが知られている．[12] 従って，薬物性肝障害を起こしやすいモデル細胞を樹立するには，NF-κB 抑制による JNK 経路の感受性を高めることが必要である．一方，転写阻害剤である actinomycin D（ActD），D-galactosamine および α-amanitin の存在下で，TNF-α による細胞死が誘導されることが明らかとなっている．[13] また，酸化ストレスの誘導が細胞死に重要であることが報告されているが，[14] 近年 TNF-α 刺激による細胞死においても，活性酸素による細胞シグナル制御（ROS シグナル）が関与することが明らかになってきている．[15] そこで本稿では *in vitro* における薬物性肝障害評価系の構築を目的として転写阻害剤 ActD および酸化ストレス誘発剤鉄ニトリロ三酢酸（Fe(Ⅲ)-nitrilotriacetic acid，Fe-NTA）を肝モデル細胞として汎用される HepG2 細胞へ添加し，TNF-α 誘導性肝細胞障害モデルの構築を試みた．

実験方法および実験材料

1．細胞培養

HepG2 細胞は，東北大学加齢医学研究所医用細胞資源センターより供与されたものを用いた．この細胞を 10% fetal calf serum（FCS, WAKO 社製）および Antibiotic-Antimycotic［100 U/mL penicillin G sodium, 100 μg/mL amphotericin B（Invitrogen 社製)］，0.45% glucose，2 mM-glutamine 含有 Dulbecco's modified Eagle's medium，（DMEM，WAKO 社製）中で，5% CO_2-95% air を気相とし，37℃でインキュベーションを行った．

2．Fe-NTA 調製

Awai らの方法に従った．[16] 硝酸鉄（Ⅲ）九水和物（WAKO 社製）は 1 M HCl 溶液を用いて 50 mM に調整した．また，NTA（Nacalai tesque 社製）は 1M NaOH 溶液（Nacalai tesque 社製）を用いて 150 mM に調整した．50 mM 硝酸鉄（Nacalai tesque 社製）および 150 mM NTA を 1:3 で混合し，$NaHCO_3$ を用いて pH 7.4 に合わせた後，0.45 μm membrane filter でろ過滅菌し，Fe-NTA とし使用した．

3．MTT assay

細胞毒性試験は MTT 試薬（[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]，Nacalai tesque 社製）を用いて，同社のプロトコールに従って行った．細胞を 96 well-plate（Becton, Dickinson 社製）に 2×10^4 cells/well の密度で播種し，CO_2 インキュベーター内で 24 時間前培養した後，ActD を処置し，30 分後 TNF-α，さらに 1 時間後 Fe-NTA を細胞に処理した．48 時間培養後，MTT 溶液を各 well に 10 μL ずつ添加し，定色反応を 4 時間行った．可溶化液（0.04 M 塩酸を含むイソプロピルアルコール）を 100 μL 添加し，沈殿したホルマザンをピペッテイングにより可溶化し，その後マイクロプレートリーダー（TOSOH 社製）を用いて 570 nm の吸光度測定を行った．

4．アガロースゲル電気泳動法による DNA 断片化の検出

100 mm 培養ディッシュで培養した細胞を回収し，滅菌済み Dulbecco's phosphate-buffered saline（DPBS, WAKO 社製）200 μL に浮遊させ，1.5 mL チューブに移した．遠心分離（250×g, 10 分間）後上清を取り除き，細胞ペレットに細胞溶解バッファー［0.1 M Tris-HCl, 0.1 M-2-({2-[bis(carboxymethyl) amino]ethyl}(carboxymethyl) amino) acetic acid（EDTA），5% Triton］100 μL を加え，細胞を溶解させ，DNA 断片を抽出した．4℃，10 分間放置後，15,000 rpm，5 分間遠心分離し，上清を新たな 1.5 mL チューブに取り，TE バッファー 300 μL，フェノール/クロロホルム（Nacalai tesque 社製）400 μL 加え再度，遠心分離（15,000 rpm，5 分間）した．上清を新たな 1.5 mL チューブに取り，Ribonuclease A（RNase A, Invitrogen 社製）溶液を 1 μL 加え，37℃，1 時間温置した後，proteinase K（Nacalai tesque 社製）溶液を 8 μL 加え，20℃で一晩放置した．遠心分離後（15,000 rpm，15 分間），上清を除去し，70% エタノール 1 mL 加え，再度遠心分離した．上清を除去後 TE バッファー 20 μL 添加し，1.2% アガロースゲル電気泳動を行った．

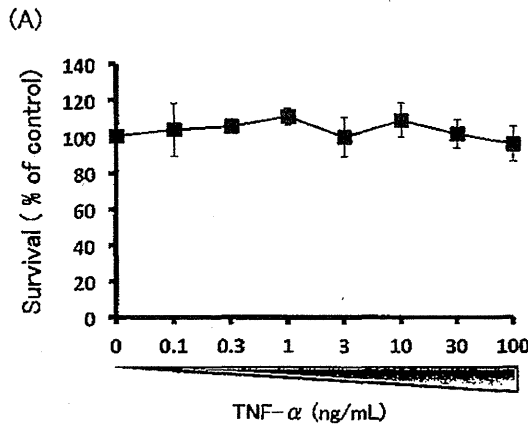
5．統計学的解析

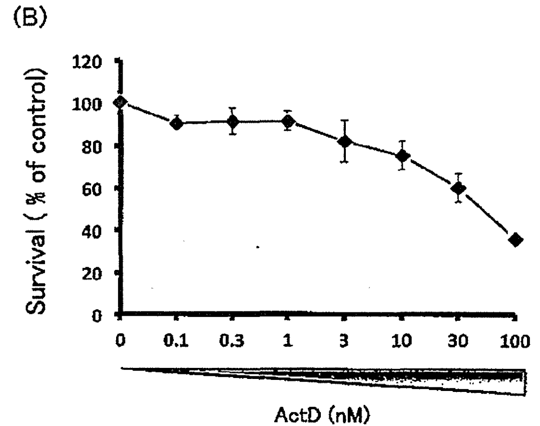
得られた実験値は平均値±標準偏差（mean±S.D.）で示し，比較検定には Student の *t* 検定を行った．統計学的有意差は危険率 1%を基準として判定した．

結　　果

1．TNF-α (A)，ActD (B)，Fe-NTA (C) 単独処理での HepG2 細胞への細胞毒性

我々はまず，TNF-α，ActD，および Fe-NTA 単独処置による細胞毒性を検討した．HepG2 細胞において，TNF-α 単独添加（0.1～100 ng/mL）の 48 時間後では有意な細胞毒性を示さなかった（Fig. 1A）．また，HepG2 細胞は TNF-α 誘発性肝障害に対する耐性を示し，それは ActD 等により減少することが知られている．[17] そこで，ActD を 0.1～100 nM の範囲で処理し，48 時間後の細胞毒性を検討した．その結果，Fig. 1B に示すように量依存性で細胞毒性が増大し，100 nM の ActD では生存率は 36%に低下した．一方，0.1～1 nM Act D 処置群では細胞毒性は示さなかった．さらに，我々は酸化ストレスモデルを構築する目的で代表的な遷移金属である鉄を導入して検討を行った．一般に鉄イオン単独での細胞内導入率は低いことから，導入効率の良い Fe-NTA を使用し，HepG2 細胞にそれを 0.5～150 μM の範囲で曝露した．48 時間で 0.5～10 μM では有意な細胞毒性は認められなかったが，50～150 μM では有意な毒性が認められた（Fig. 1C）．





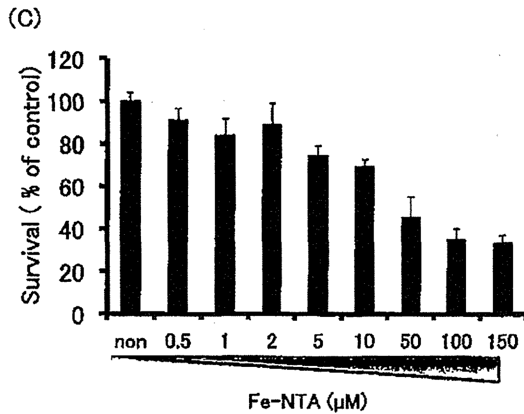


Fig. 1. Changes in cell viability of the treatments with TNF-α (A), ActD (B) and Fe-NTA (C) in HepG2 cells.

These cells were preincubated for 24 h with DMEM. After the addition of TNF-α (0.1～100 ng/mL), ActD (0.1～100 nM) and Fe-NTA (0.5～150 μM) to the culture medium, the cells were incubated for 48 h. The cell survival was measured by the MTT assay as described in materials and methods. Data represent the means ± SD (n = 4 experiments).

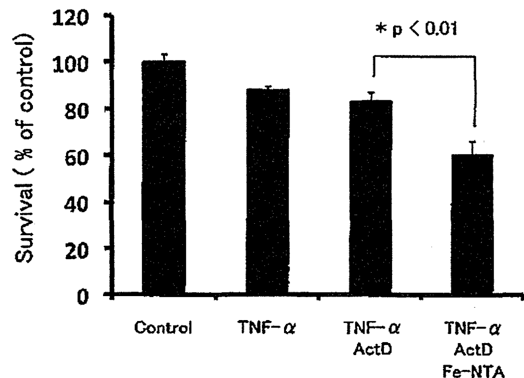


Fig. 2. Effect of iron on TNF-α/ActD-induced cytotoxicity in HepG2 cells.

These cells were incubated with TNF-α (20 ng/mL) combined with ActD (0.5 nM) for 48 h in the presence of Fe-NTA (2 μM). Cell survival was measured by the MTT assay as described in materials and methods. The surviving fraction was determined by dividing the absorbance of treated cells by that of control cells. A: Control, B: TNF-α, C: TNF-α/ActD, D: TNF-α/ActD/Fe-NTA. Data represent the mean ± SD (n = 4 experiments). *$p < 0.01$, compared to cells treated with TNF-α/ActD (Student's t-test).

2．鉄存在下での ActD-感受性 HepG2 細胞における TNF-α の細胞毒性

本研究において，ActD (0.5 nM)，Fe-NTA (2 μM) では HepG2 細胞における細胞毒性は認められなかったことから，TNF-α (20 ng/mL) /ActD (0.5 nM) /Fe-NTA (2 μM) の低添加量を選択し，48 時間後での鉄存在下 TNF-α /ActD による HepG2 細胞における細胞毒性を検討した．ActD は TNF-α 処理 30 分前に細胞に添加した．その 1 時間後に Fe-NTA を処理した (Fig. 2)．その結果，TNF-α /ActD では無処置細胞に比べ細胞生存率は 82% であるが，鉄存在下での TNF-α /ActD/Fe-NTA 群においては顕著な細胞死が観察され，細胞生存率は 60% まで低下した (Fig. 2)．

3．HepG2 細胞での TNF-α /ActD/Fe-NTA 処理による DNA の断片化

上記に示すように鉄存在下 TNF-α /ActD が有意な細胞死を認めたため，この肝細胞死の形態がアポトーシスあるいはネクローシスかについて検討した．

本研究においてアガロースゲル電気泳動法で DNA 断片化に基づく細胞死の判定を行ったところ，アポトーシス陽性対照として用いた 10 μM camptothecin 処置群においては，ラダーリングは

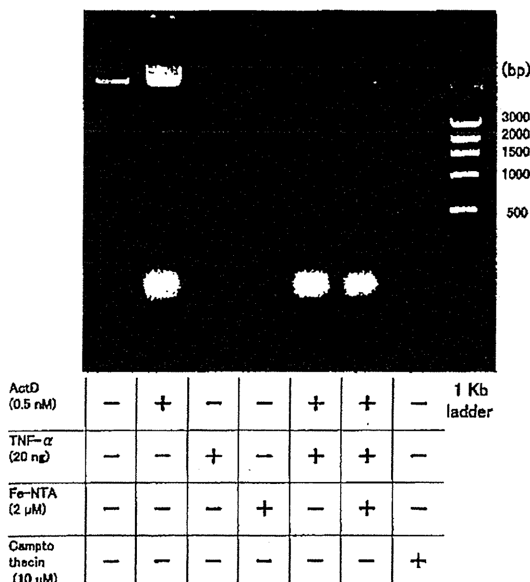


Fig. 3. Effects of TNF-α/ActD/Fe-NTA-treated HepG2 cells on DNA laddering.

These cells were incubated with TNF-α (20 ng/mL) combined with ActD (0.5 nM) for 48 h in the presence of Fe-NTA (2 μM). DNA was prepared from supernatant of 15,000 rpm of cell homogenates and electrophoresed as described in materials and methods. DNA fragments were used as molecular markers. DNA laddering was absent in control cells.

認められたが，低濃度 TNF-α /ActD/Fe-NTA 処置群においては整数倍のラダーパターン (180-200 bp) は見られなかった．この結果より，本鉄存在下での TNF-α /ActD 誘導細胞死はネクローシス様の細胞死であることが示唆された (Fig. 3)．

考　　察

TNF-α は腫瘍部位に出血壊死を誘導する因子として見いだされたが，現在では炎症での生体防御機構に広く関わる炎症性サイトカインとして理解されている．[4] 肝細胞においても，炎症反応を惹起するサイトカインとして中心的な役割を演じ，生体の恒常性の維持に重要な役割を果たしている．また，TNF-α は，過剰に生産されると肝障害を誘発することから，TNF-α は肝障害 mediator とも考えられている．[23] 我々は，薬物性肝障害モデル細胞として，薬物代謝や肝毒性研究に汎用されるヒト肝癌由来細胞株の HepG2 細胞を使用してきた．しかしながら，この細胞は NF-κB による JNK 経路の抑制を示すことが知られているため，転写阻害剤である ActD および酸化ストレス誘発材である Fe-NTA により，TNF-α 誘発性肝障害に感受性を持たせることを試みた．ActD は，転写阻害剤として知られており，*in vivo*, *in vitro* でアポトーシスを誘導する．[18] Leist らは [13] HepG2 細胞への ActD 333 nM，24 時間処理で TNF-α の感受性増大を報告しているが，Fig. 1B に示すように著者らの検討ではこれらの ActD 濃度では細胞障害が強く不適であったため，0.5 nM 処置を行った (Fig. 1B)．また，肝障害においては，活性酸素による酸化ストレスが起こることが知られており，[24] 2 価のフリー鉄は Fenton/Haber-Weiss 反応により有毒なヒドロラジカル (·OH) を生じ酸化ストレスを生起する．[19] HepG2 細胞へ Fe-NTA を添加しない ActD/TNF-α に比べ，Fe-NTA 添加群では，明らかに TNF-α による細胞障害が認められた (Fig. 2)．これらの結果から 2 価鉄と ActD が TNF-α 誘導細胞に対する感受性因子に重要な役割を演じることが示された．

スリンダク/LPS 誘導性肝障害などの炎症反応が関与する薬物性肝障害において，TNF-α が媒介する経路は重要な役割を担っている．[9] それは，TNF-α による death receptor を介した経路の他に JNK 活性化経路，さらに NF-κB 活性化によるアポ

トーシス抑制経路が主なものである．一般に，肝細胞における TNF-α の細胞死に対する耐性は，NF-κB の肝細胞と炎症細胞における作用の違いに起因すると考えられている．すなわち，NF-κB は，肝細胞においては TNF-α 誘導性の細胞死シグナルに対して拮抗する生存シグナルを伝えるが，一方，炎症細胞においては，TNF-α の産生を誘導する二面性をもつことから説明されている．本モデルにおいては，ActD により NF-κB 経路が阻害され，細胞死が誘導された可能性が示唆される．

また，細胞死は形態学的にアポトーシスとネクローシスに区別され，TNF-α による肝障害においても，肝細胞ではアポトーシスおよびネクローシスが関与し細胞死を誘導することが報告されている．[13,20] 従って，その細胞死を識別することは，その原因を探索する上で重要な手がかりとなる．DNA 断片化（DNA fragmentation）は，アポトーシスの生化学的指標とされており，通常，アポトーシスが生じた細胞より抽出した DNA をアガロースゲル電気泳動で泳動後，180～200 bp の整数倍の“ラダー（ladder）”として検出されている．[21] 一方，ネクローシスによる DNA の断片化はアポトーシスに比べて少ないのが特徴である．ActD はアポトーシスを誘導するが，Kleeff らは ActD 100～1000 ng/mL では DNA 断片化が認められるが，10 ng/mL では認められないと述べている．[22] 本条件下 ActD は 0.5 nM を使用しているため，この ActD の濃度ではアポトーシスを誘導しないと考えられた．実際，この条件下での TNF-α/ActD/Fe-NTA 添加の細胞死誘導は，アポトーシスの特徴である DNA の断片化は見られず，非アポトーシスによることを示唆した（Fig. 3）．スリンダクなどの薬物による TNF-α を媒介した肝障害においても，その細胞死は，主にネクローシスであることが示唆されている．[8] また，そのような薬物性肝障害は，ROS の発生が重要な因子となることが報告されている．[24,25] そこで，我々の肝障害モデルでは，Fe-NTA 添加により，細胞内の H_2O_2 を反応性の強い・OH に変換させ（Fenton 反応），ROS の産生能を増加させている．これにより，薬物およびその代謝物により産生された ROS に対する感受性が増大し，薬物性肝障害を感度よく予測することができると考えられる．

これまでカスパーゼを介するアポトーシスが分子生物学的および生化学的に解明されてきており，ネクローシスは偶発的なものと考えられてきた．しかし，近年，TNF-α による細胞死が細胞の種類によってアポトーシスではなく，ネクローシス様の細胞死を誘導することが知られており，[26,27]“偶発的でない制御されたネクローシス”の存在が示唆されていた．その分子機構は全く不明であったが，最近，TNF-α によって誘導されるネクローシス様の細胞死は RIPK1 および RIPK3（RIPK; receptor-interacting protein kinase）というキナーゼに依存性であることが明らかとなり，[27] ネクローシスもアポトーシスと同様に高度に遺伝子によって制御されたプログラムネクローシス（necroptosis）の存在が認知されるようになった．TNF-α 受容体の活性化は細胞死および細胞生存という相互に排他的な 2 つのシグナル伝達経路を誘発するが，[28] 前者はさらに，カスパーゼに依存性のアポトーシスと RIPK に依存性のネクローシスという 2 つの形態の細胞死につながるシグナル伝達経路に分岐する．近年，薬物性肝障害における TNF-α など炎症性サイトカインの寄与が示唆されているが，[6] このシグナル伝達経路の均衡の崩れが肝障害に寄与する可能性が考えられる．また，酸化ストレスにおいても，その強弱によりアポトーシスあるいはネクローシスが誘導されると推測されている．[29] ヒト白血病 T 細胞株である Jurkat 細胞において，H_2O_2 は 0.7 μM 以下では細胞増殖に作用し，1～3 μM 以下ではアポトーシス，そして 3 μM 以上ではネクローシスを生じることが報告されている．[30] 薬物性肝障害においても，ネクローシスとアポトーシスの発生の差異は，酸化ストレスによっても担われると推測される．すなわち，TNF-α および酸化ストレスに対して，高感受性である TNF-α/ActD/Fe-NTA による本モデルは，薬物性肝障害モデルとして有用な情報を提供するツールになると考えられる．更なる検討により，薬物性肝障害の病態をより正確に評価できる系が確立されれば，創薬において，医薬品の開発プロセスにおける精度良い予測を可能にし，創薬シーズのヒット率の上昇に寄与できると考えられる．

REFERENCES

1）Frank R., Hargreaves R., *Nat. Rev. Drug Discov.*, 2, 566－580（2003）.

2）Ebert A. D., Svendsen C. N., *Nat. Rev. Drug Discov.*,

9, 367－372（2010）.

3）Teranishi M., Manabe S., *Nihon Yakurigaku Zasshi*, **132**, 347－350（2008）.

4）Kola I., Landis J., *Nat. Rev. Drug Discov.*, **3**, 711－715（2004）.

5）Lee W. M., *N. Engl. J. Med.*, **349**, 474－485（2003）.

6）Kaplowitz N., *Nat. Rev. Drug Discov.*, **4**, 489－499（2005）.

7）Roth R. A., Luyendyk J. P., Maddox J. F., Ganey P. E., *J. Pharmacol. Exp. Ther.*, **307**, 1－8（2003）.

8）Zou W., Devi S. S., Sparkenbaugh E., Younis H. S., Roth R. A., Ganey P. E., *Toxicol. Sci.*, **108**, 184－193（2009）.

9）Zou W., Beggs K. M., Sparkenbaugh E. M., Jones A. D., Younis H. S., Roth R. A., Ganey P. E., *J. Pharmacol. Exp. Ther.*, **331**, 114－121（2009）.

10）Liu J., Lin A., *Cell Res.*, **15**, 36－42（2005）.

11）Wullaert A., Heyninck K., Beyaert R., *Biochem. Pharmacol.*, **72**, 1090－1101（2006）.

12）Liu H., Lo C. R., Czaja M. J., *Hepatology*, **35**, 772－778（2002）.

13）Leist M., Gantner F., Bohlinger I., Germann P. G., Tiegs G., Wendel A., *J. Immunol.*, **153**, 1778－1788（1994）.

14）Novo E., Parola M., *Fibrogenesis Tissue Repair*, **5**, S4（2012）.

15）Papa S., Bubici C., Zazzeroni F., Franzoso G., *Biol. Chem.*, **390**, 965－976（2009）.

16）Awai M., Narasaki M., Yamanoi Y., Seno S., *Am. J. Pathol.*, **95**, 663－673（1979）.

17）Karin M., *Nature*, **441**, 431－436（2006）.

18）Perry R. P., Kelley D. E., *J. Cell Physiol.*, **76**, 127－139（1970）.

19）Aust S. D., Morehouse L. A., Thomas C. E., *J. Free Radic. Biol. Med.*, **1**, 3－25（1985）.

20）Fiers W., Beyaert R., Declercq W., Vandenabeele P., *Oncogene*, **18**, 7719－7730（1999）.

21）Carson D. A., Ribeiro J. M., *Lancet*, **341**, 1251－1254（1993）.

22）Kleeff J., Kornmann M., Sawhney H., Korc M., *Int. J. Cancer*, **86**, 399－407（2000）.

23）Schwabe R. F., Brenner D. A., *Am. J. Physiol. Gastrointest. Liver Physiol.*, **290**, G583－589（2006）.

24）Sun Y., Chen J., Rigas B., *Carcinogenesis*, **30**, 93－100（2009）.

25）Tafazoli S., Spehar D. D., O'Brien P. J., *Drug Metab. Rev.*, **37**, 311－325（2005）.

26）Festjens N., Vanden Berghe T., Vandenabeele P., *Biochim. Biophys. Acta*, **1757**, 1371－1387（2006）.

27）Zhang D. W., Shao J., Lin J., Zhang N., Lu B. J., Lin S. C., Dong M. Q., Han J., *Science*, **325**, 332－336（2009）.

28）Nagaki M., Moriwaki H., *Hepatol. Res.*, **38**, S19－28（2008）.

29）Morgan M. J., Kim Y. S., Liu Z. G., *Cell Res.*, **18**, 343－349（2008）.

30）Antunes F., Cadenas E., *Free Radic. Biol. Med.*, **30**, 1008－1018（2001）.

Drug Metab. Pharmacokinet. 28 (1): 38–43 (2013). Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX)

Review

Clinical Evidence of Pharmacokinetic Changes in Thalidomide Therapy

Katsunori NAKAMURA[1], Naoki MATSUZAWA[2], Shigeru OHMORI[2], Yuichi ANDO[3], Hiroshi YAMAZAKI[4] and Tamihide MATSUNAGA[1,*]

[1]*Department of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan*
[2]*Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan*
[3]*Department of Clinical Oncology and Chemotherapy, Nagoya University Hospital, Nagoya, Japan*
[4]*Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Japan*

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: The teratogenic effects of thalidomide have been studied for more than 50 years. However, there have been few studies of the pharmacokinetic changes occurring during thalidomide therapy. Thalidomide was originally developed as a sedative. However, thalidomide induces multiple birth defects when used in pregnant women. Thalidomide is now used in the treatment of multiple myeloma (MM) and erythema nodosum leprosum (ENL) in Japan. Rational use of thalidomide is problematic due to a lack of basic research regarding its mechanism of action and serum concentration/effect relationships. There are a number of hypotheses for pharmacokinetic changes in thalidomide therapy. Genetic factors including single nucleotide polymorphisms (SNPs) that change cytochrome P450 (CYP) activity and epigenetic regulation that modifies CYP expression levels may contribute to the changes in pharmacokinetics and adverse drug reactions (ADRs) of thalidomide. Environmental factors include the pharmacological context of drug-drug interactions and the physiological context of liver diseases. Liver and kidney diseases do not play important roles in pharmacokinetic changes or ADRs in thalidomide therapy. To date, most research has focused on teratogenic activity, while the impact of polymorphisms in genes encoding drug metabolic enzymes and drug-drug interactions could mediate ADRs. Here, we discuss clinical evidence of pharmacokinetic changes in thalidomide therapy.

Keywords: thalidomide; CYP2C19; CYP3A5; polymorphism; inter-individual variability

Introduction

Thalidomide was once used as a nonbarbiturate sedative-hypnotic with only low toxicity, but was withdrawn from the market because of its teratogenic side effects in the early 1960s.[1] Cereblon, a protein encoded by a candidate gene for mild mental retardation, was shown to be a primary target of thalidomide teratogenicity due to the inhibition of ubiquitin ligase activity.[2] Thalidomide has attracted renewed interest in recent years for use on a restricted basis in certain countries as a novel antineoplastic agent with immunomodulatory and antiangiogenic activities.[3–6] This drug is now commonly used in the treatment of multiple myeloma (MM).[7–9] Thalidomide has also been shown to have some activity against renal cell carcinoma, Kaposi's sarcoma, agnogenic myeloid metaplasia, Waldenström's macroglobulinemia, and myelodysplastic syndrome.[10,11] However, limited efficacy data are available in these conditions compared to myeloma, and its use in these settings is still investigational. Studies of its use in several other malignant and nonmalignant disorders are currently ongoing.[12] The incidence of toxicity is correlated with the dose of the drug. Patients receiving 200 mg or less seem to tolerate the treatment well with minimal side effects. Conversely, almost all patients taking thalidomide at doses of more than 400 mg/day experience drug-related toxicity.[12] Thalidomide is eliminated mainly through nonenzymatic hydrolysis and urinary excretion in humans, although animal studies have suggested that it may also be metabolized by the hepatic CYP enzyme system.[13–16] (*S*)-Thalidomide was reported to induce apoptosis.[17] However, (*S*)-thalidomide was reported to undergo rapid conversion into the racemate *in vitro*.[18,19] The transition of (*R*)- and (*S*)-thalidomide could influence the therapeutic effects. The increasing use of thalidomide raises the possibility of metabolic interactions with other prescription medications.[20] Clinically important interactions between thalidomide and coadministered therapeutic agents may

Received August 17, 2012; Accepted October 26, 2012
J-STAGE Advance Published Date: November 20, 2012, doi:10.2133/dmpk.DMPK-12-RV-089
**To whom correspondence should be addressed:* Tamihide MATSUNAGA, Ph.D., *Department of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan. Tel: +81-52-836-3751, Fax: +81-52-836-3751, E-mail: tmatsu@phar.nagoya-cu.ac.jp*

affect the activation or detoxification of thalidomide and other drugs, giving rise to attenuation or amplification of biological effects and/or toxicities. While metabolic studies have been performed in animals,[13] to date there have been only a few human studies. This review summarizes the clinical evidence of pharmacokinetic changes in thalidomide therapy.

Metabolism

Metabolism of thalidomide in humans: Thalidomide is an optical isomer, and (*S*)-thalidomide was reported to induce apoptosis.[17] However, rapid conversion occurs from (*S*)-thalidomide into the racemate *in vitro*.[18,19] It has been suggested that the transition of (*R*)- and (*S*)-thalidomide in the blood influences the therapeutic effects. Although thalidomide is mainly eliminated through nonenzymatic hydrolysis and urinary excretion in humans, animal studies have suggested that it may also be metabolized by the hepatic CYPs.[13–15] Thalidomide was oxidized to 5-hydroxythalidomide and 5′-hydroxythalidomide by NADPH-fortified liver microsomes from humans as observed in monkeys, rats, mice, rabbits, and dogs (**Fig. 1**).[16] (*R*)-Thalidomide was hydroxylated more efficiently than (*S*)-thalidomide. Thalidomide has been reported to be metabolized by human recombinant CYP2C19, 3A4, and 3A5 *in vitro*.[21] CYP3A is known to be the main enzyme involved in the metabolism of many currently available medications. It is commonly accepted that CYP3A5 substrate specificity is similar to that of CYP3A4, although some differences in catalytic properties of thalidomide metabolism have been found.[21] Human CYP2C19, 3A4, and 3A5 mediate thalidomide 5-hydroxylation and further oxidation leading to a glutathione conjugate, which may be of relevance in the pharmacological actions of thalidomide (**Fig. 1**).[22] These metabolic pathways were confirmed by humanized TK-NOG mice, prepared by the introduction of thymidine kinase followed by induction with ganciclovir, and transplantation of human liver cells.[21] Experiments in these humanized mice could be expected to reveal the pharmacokinetic differences between humans and other animals. As thalidomide metabolites were not stable, degradation was avoided by rapid chilling and acidification of the samples. This property makes it more difficult to perform kinetic analysis of thalidomide. After incubation of thalidomide with the S9 fraction from human liver, formation of the 5-hydroxy and 5′-hydroxy metabolites was observed. The 5′-hydroxy metabolite was found in plasma samples from eight healthy male volunteers who had received thalidomide orally, but the concentrations were low.[23] Thalidomide does not undergo significant metabolism by human CYP and clinically important interactions between thalidomide and drugs that are also metabolized by this enzyme system are unlikely (**Table 1**). The major route of thalidomide breakdown in humans and animals is through spontaneous hydrolysis with subsequent elimination in the urine.[24] As both enzymatic metabolism and renal excretion play minor roles in the elimination of thalidomide, the risk of drug interactions seems to be low.[1]

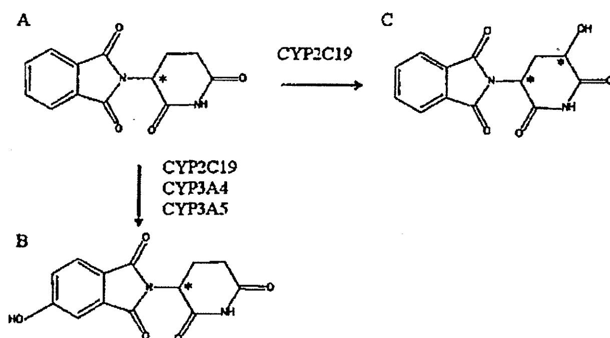


Fig. 1. Structures of thalidomide and two hydroxylated metabolites
These two hydroxylated metabolites are further oxidized leading to a glutathione conjugate. A, Thalidomide; B, 5-Hydroxy thalidomide; C, 5′-Hydroxy thalidomide.

CYPs and Transporters

Genetic polymorphisms and thalidomide pharmacokinetic changes: CYP is one of the most important groups of enzymes

Table 1. Summary of thalidomide-related pharmacokinetic changes from drug-drug interactions

Substrate and inhibitor	Effect	Mechanism of pharmacokinetic change
Drugs affecting the pharmacokinetics of thalidomide		
Cyclophosphamide	inhibition	Cyclophosphamide increased the thalidomide t(1/2) and AUC in plasma and tumor tissue in mice[54]
CPT-11	no change	CPT-11 did not significantly alter the pharmacokinetics of thalidomide[55]
	no change	Concurrent administration of CPT-11/thalidomide did not influence pharmacokinetics[56]
Thalidomide affecting the pharmacokinetics of other drugs		
Cyclosporin A	enhancement	Cyclosporin metabolism was enhanced by thalidomide in CYP3A5 and in liver microsomes expressing CYP3A5 in the presence of thalidomide[21]
	enhancement	Cyclosporin A clearance was enhanced in CYP3A5 and liver microsomes expressing CYP3A5 by thalidomide[34]
CPT-11 and SN-38	no change	Thalidomide inhibited CPT-11 metabolism, unlikely to be clinically significant[57,58]
	inhibition	Thalidomide increased the AUC of CPT-11[38,56]
	inhibition	Thalidomide decreased the AUC and t(1/2) of SN-38[38,56]
	inhibition	Thalidomide decreased metabolism of CPT-11 into SN-38[58]
Midazolam	inhibition	Midazolam 4-hydroxylation activities were suppressed by thalidomide[34]
	enhancement	1′-Hydroxylation and total midazolam oxidation were enhanced in the presence of thalidomide[34]
	enhancement	Midazolam hydroxylation was enhanced by thalidomide in CYP3A5 and in liver microsomes expressing CYP3A5 in the presence of thalidomide[21]
(*S*)-Mephenytoin	inhibition	Thalidomide inhibited (*S*)-mephenytoin 4′-hydroxylation activities of recombinant CYP2C19 and human liver microsomes[34]
Testosterone	enhancement	Testosterone 6β-hydroxylation were enhanced in the presence of thalidomide[34]
DMXAA	inhibition	Thalidomide reduced clearance of DMXAA[59]
	no change	(*S*)-Thalidomide did not alter plasma DMXAA AUC in rats[60]

Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX)

in thalidomide metabolism. Many CYPs are polymorphic and catalytic alterations of allelic variant proteins can affect the metabolic activities of many drugs. The *CYP2C19*, *3A4*, and *3A5* genes, the gene products of which catalyze thalidomide hydroxylation, are particularly polymorphic. *In vitro* studies using cDNA expression systems are useful tools for evaluating functional alterations of the allelic variants of CYP, particularly for low-frequency alleles.[25–28] There was some influence of genetic polymorphism in *CYP2C19* on the blood concentration of thalidomide in MM, amyloid light chain amyloidosis, and polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes (POEMS) syndrome related to MM in Japanese patients.[29] It has been reported that decreased formation of thalidomide metabolites would be expected with defective alleles of *CYP2C19* compared to wild-type in clinical treatment with thalidomide plus dexamethasone.[30] Association studies of genetic variation and treatment effect may serve as predictive markers for the effects of treatment and can also uncover biological pathways behind drug effects. The SNPs have been studied in relation to high-dose treatment, thalidomide- and bortezomib-based therapy, maintenance treatment with interferon-α, and therapy-related adverse effects caused by treatment. In thalidomide- and bortezomib-based therapy, candidate genes include tumor necrosis factor-α (TNF-α) and genes involved in the nuclear factor kappa B (NFκB) pathway, respectively. In maintenance treatment with interferon-α, a polymorphism in gene NFκB1 is a candidate for prediction of efficacy. Adverse drug reactions (ADRs) include infection, osteonecrosis of the jaw, venous thrombotic events (VTE), and peripheral neuropathy (PN). A SNP in the *CYP2C8* gene was strongly associated with osteonecrosis of the jaw.[31] Several SNPs in genes encoding proteins involved in DNA repair, apoptosis, and inflammation as well as genes involved in nervous system function are associated with VTE induced by thalidomide and PN induced by bortezomib. Further studies of SNPs in clinical trials are needed.[31]

Relation of transporters to the pharmacokinetic changes of thalidomide: Since studies in patients have indicated that the oral absorption of thalidomide is considerably variable at high doses, the contributions of transporters to the pharmacokinetics/pharmacodynamics of thalidomide were examined by Zhou *et al.*[32] and Zimmermann *et al.*[33] using human colon cancer cell lines, which have been widely used to investigate drug permeability. Thalidomide did not induce P-glycoprotein (P-gp) expression in LS180 cells. The uptake of rhodamine 123 in CCRF cells overexpressing P-gp was not influenced by coincubation with thalidomide. Transport through Caco-2 monolayers was linear and the permeability was similar in both directions. There were no differences between the thalidomide enantiomers. From this study, thalidomide was concluded to be neither a substrate nor an inhibitor or inducer of P-gp, and P-gp-related drug-drug interactions with thalidomide are unlikely.[33] Zhou *et al.* reported that the uptake of thalidomide by Caco-2 cells was very limited (up to 2.1%).[32] The transport of thalidomide appeared to be linear up to 1 h. The permeability coefficients (Papp) of thalidomide at 2.5–300 μM from apical (AP) to basolateral (BL) and from BL to AP were 2×10^{-5}–6×10^{-5} cm/s, with a marked decrease in Papp values from AP to BL at increased thalidomide concentration. The transport of thalidomide was dependent on sodium, temperature, and pH, as replacement of extracellular sodium chloride or reducing temperature and apical pH resulted in significant decreases in the Papp values. Additional data indicated that transport of thalidomide is energy-dependent, as it was significantly ($p < 0.05$) inhibited by the ATP inhibitors sodium azide and 2,4-dinitrophenol. In addition, DL-glutamic acid, cytidine, dipyridamole, papaverine, quinidine, and cyclophosphamide significantly ($p < 0.05$) inhibited the transport of thalidomide, while the P-gp inhibitor verapamil and other nucleosides and nucleotides, such as thymidine and guanine, had no effect. These results suggested that thalidomide may be rapidly transported by a saturable energy-dependent transporter in Caco-2 monolayers.[32]

Drug-drug interaction of thalidomide: The increasing use of thalidomide raises the possibility of metabolic interactions with other prescription medications. Clinically important interactions between thalidomide and coadministered therapeutics may affect the activation or detoxification of thalidomide and other drugs giving rise to attenuation or amplification of biological effects and/or toxicities. While metabolic studies have been performed in animals,[13] to date there have been only a few human studies (**Table 1**). The possible drug interactions that could be mediated by thalidomide were investigated in human liver microsomes. (*S*)-Mephenytoin 4′-hydroxylation activity was inhibited by thalidomide in recombinant CYP2C19 and human liver microsomes with apparent IC_{50} of approximately 270 μM for CYP2C19.[34] Interestingly, midazolam 4-hydroxylation activity was suppressed by the presence of thalidomide, but 1′-hydroxylation activities, total midazolam oxidation activity, and testosterone 6β-hydroxylation activities were enhanced in the presence of thalidomide. Recombinant CYP3A5 altered kinetics at clinical concentrations of thalidomide. CYP3A4 was affected only at higher thalidomide concentrations. Enhancement of midazolam hydroxylation by thalidomide was seen in liver microsomes from *CYP3A5*1* subjects. Cyclosporin A clearance was similarly enhanced by thalidomide in recombinant CYP3A5 and liver microsomes expressing CYP3A5. Close interaction between thalidomide and the heme of CYP3A5 was observed in docking studies. As total midazolam metabolism or cyclosporin A clearance may be increased by thalidomide in a dose-dependent manner, unexpected drug interactions could occur *via* heterotropic cooperativity of CYP3A5.[34] Both (*S*)-thalidomide and diclofenac increased the plasma DMXAA AUC in mice. In the case of diclofenac, this may be due to direct competitive inhibition of DMXAA metabolism, but this mechanism is not always applied to (*S*)-thalidomide. The *in vivo* predictive model is inappropriate for the (*S*)-thalidomide-DMXAA interactions when based on direct inhibition of metabolism in mice and humans.[35] Thalidomide tends not to affect the pharmacokinetics of orally administered hormonal contraceptives.[36] In contrast, conversion of CPT-11 into the active metabolite SN-38 was significantly inhibited by thalidomide. The possibility of an interaction of thalidomide with CPT-11 metabolism may explain the previously described improvement in tolerability of CPT-11 therapies.[37]

Factors contributing to adverse effects of thalidomide: Given the increasing clinical use of thalidomide, it is becoming important to study the adverse effects of this drug. In addition to teratogenicity, thalidomide has certain unique and frequent toxicities, such as constipation, sedation, fatigue, and neuropathy. Matsuzawa *et al.*[29] evaluated the genotype, age, sex, diagnosis, and concurrent CYP2C19 substrate medications as factors important for the adverse effects of thalidomide. Age and sex did not show clear effects on thalidomide dose in the Japanese population. In patients taking concurrent drugs, thalidomide dosages were not reduced

Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX)

in patients taking CYP2C19 substrates. *CYP2C19* genetic polymorphism may be one of the factors underlying the individual differences in thalidomide toxicity. The mean (*R*)-thalidomide AUC was 35.9% lower in the *CYP2C19*1/*3* and **1/*2* groups than in the *CYP2C9*2/*2* group, and the mean (*S*)-thalidomide AUC was 33.5% lower in the *CYP2C19*1/*3* and **1/*2* groups than the *CYP2C9*2/*2* group. All patients developed adverse reactions to thalidomide. Major adverse drug effects were constipation, somnolence, and peripheral neuropathy. Only one patient with the *CYP2C19*2/*2* genotype taking thalidomide developed dyspnea as a side effect, but it improved following the termination of thalidomide.[29] Peripheral neuropathy is a common side effect of thalidomide and often calls for the cessation of therapy when the symptoms are severe.[12] The thalidomide-related peripheral neuropathy associated with ABCA1 (rs363717), ICAM1 (rs1799969), PPARD (rs2076169), SERPINB2 (rs6103), and SLC12A6 (rs7164902) SNPs and an individual's risk of developing peripheral neuropathy after thalidomide treatment can be mediated by polymorphisms in genes governing repair mechanisms and inflammation in the peripheral nervous system.[38] No association was observed between the number of functional *CYP2C19* and *CYP2D6* alleles and outcome in a population of 166 MM patients treated with thalidomide.[39] There were also no associations between the numbers of functional *CYP2C19* and *CYP2D6* alleles and neurological adverse reactions to thalidomide. Further studies in larger numbers of patients may be needed to determine the roles of polymorphic *CYP* alleles in treatment outcome.[39] VTE with the subsequent risk of pulmonary embolism (PE) is a major concern in the treatment of MM patients with thalidomide. Deep venous thrombosis (DVT) and/or PE occurs in only about 1–3% of patients receiving single-agent thalidomide for myeloma.[40–46] The risk of thalidomide-induced thrombosis is highest in newly diagnosed patients when the drug is given in combination with dexamethasone, doxorubicin, or other chemotherapeutic drugs. The risk is elevated in the elderly and in patients with an underlying inherited or acquired thrombotic predisposition. The use of routine prophylactic warfarin or low molecular weight heparin or aspirin for all patients receiving thalidomide in combination with dexamethasone is currently under consideration.[12] The susceptibility to the development of VTE in response to thalidomide therapy is likely to be influenced by both genetic and environmental factors. SNPs associated with thalidomide-related VTE were enriched in genes and pathways important in drug transport/metabolism, DNA repair, and cytokine balance.[46]

Other Factors

Effects of liver or kidney dysfunction: The absorption and elimination of thalidomide are not significantly different in patients with hepatic dysfunction.[47] Thalidomide is mainly hydrolyzed and passively excreted, and its pharmacokinetics are not expected to be altered in patients with impaired liver or kidney function.[48] The inter- and intra-patient variability in liver or kidney dysfunction was low. There was no correlation between thalidomide clearance and renal function. Although clearance during dialysis is doubled, thalidomide dose need not be changed for patients with decreased kidney function. There is also no need for a supplementary dose due to hemodialysis.[49] The serum concentration of thalidomide in MM patients with renal insufficiency was investigated in Japanese patients.[50] The serum concentration of thalidomide 12 and 16 h after administration in patients with MM on hemodialysis (HD) taking 100–200 mg/day were similar with or without HD. The thalidomide concentration was not significantly increased by renal insufficiency. In this study, there is no correlation between the concentration of thalidomide and its clinical effect. In Japanese patients, the thalidomide dosage need not be modified for renal insufficiency or HD.[50]

Effects of food on the pharmacokinetics of thalidomide: Although food often delays and/or decreases drug absorption, the absorption of a few drugs is increased by food. The effects of food on the oral pharmacokinetics of thalidomide and the relative bioavailability of two oral thalidomide formulations were determined by Teo *et al.*[24] Five male and eight female healthy volunteers received a single oral dose of 200 mg thalidomide in capsule form under fasting and non-fasting conditions. A high-fat breakfast delayed the onset of absorption of thalidomide by 0.5–1.5 h.

Gender difference on the pharmacokinetics of thalidomide: Although there were no statistically significant differences in any of the pharmacokinetic parameters of thalidomide pharmacokinetics between men and women, tendencies toward gender differences in the pharmacokinetics of thalidomide were observed for some of the parameters.[24] For example, women tended to have slightly larger C_{max} and AUC values than men. These differences can be explained by the greater body weight of the male subjects. The mean half-life and mean residence time were also slightly larger for females than for males.[24]

Differences in pharmacokinetic parameters among formulations: Terminal half-life showed two- to three-fold differences among tested formulations and is clear evidence for absorption rate limitations.[51] Fujita *et al.* compared the dissolution profile and plasma thalidomide concentrations of Japanese and British capsules and Mexican tablets. The dissolution rate of the Japanese capsule was the fastest, followed by the British and Mexican formulations. The pharmacokinetic profiles of the Japanese and British capsules were similar, while the 100 mg Japanese thalidomide capsule showed a 1.6-fold higher maximum plasma concentration than the 200 mg Mexican thalidomide tablet, greatly shortened T_{max}, and an apparent half life that was only one-third that of the Mexican tablet. Thus, pharmacokinetic changes may occur in plasma thalidomide concentration when switching between different formulations.[52]

Conclusions

In conclusion, this review provided insights into the contribution of gene variations, drug-drug interactions, liver and kidney dysfunction, and thalidomide formulations to pharmacokinetic changes of thalidomide (**Table 2**). *CYP2C19* poor metabolizer (PM) patients

Table 2. Effects of various factors on thalidomide pharmacokinetics in humans

Factors	Thalidomide pharmacokinetics	References
Gender	No significant difference (slightly higher C_{max} and AUC in females)	24)
Age	No significant difference	29)
Renal function	No significant difference	48–50)
Hepatic function	No significant difference	47)
Food	High-fat meal delayed the absorption of thalidomide	24)
Transporter polymorphisms	Unreported (*in vivo* data)	
CYP polymorphisms	No significant difference (larger AUC in *CYP2C9*2/*2* genotype)	29)
Formulations	Pharmacokinetic changes occur when switching to other formulations	51, 52)

Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX)

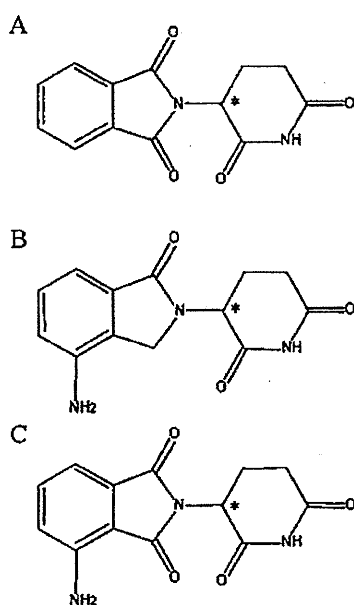


Fig. 2. Structures of IMiDs
A, Thalidomide; B, Lenalidomide; C, Pomalidomide.

tend to have high serum thalidomide concentrations and high risks of adverse drug effects, such as constipation, somnolence, and peripheral neuropathy. Further studies to clarify the mechanisms underlying the pharmacokinetic changes of thalidomide are required. Several next-generation antiepileptic drugs with improved tolerability profiles and reduced potential for drug interactions have been added to the therapeutic armamentarium. Thalidomide analogs termed immunomodulatory drugs (IMiDs, **Fig. 2**) have been developed that are more effective and have less toxicity than thalidomide. Thalidomide and its co-stimulatory IMiD analogs are currently being assessed in patients with advanced myeloma and some solid tumors, with promising effects.[9] However, despite the promising effects of thalidomide on a broad range of serious diseases, further careful studies on the pharmacological and pharmacodynamic properties of thalidomide are necessary.[12] Although thalidomide and IMiDs show similar biological activities, IMiDs are more potent than thalidomide and achieve responses at lower doses. Lenalidomide, a thalidomide derivative, has also been shown to have a different toxicity profile.[53] Pharmacokinetic and clinical interactions between lenalidomide and other drugs seemed to occur, as *in vitro* data indicated that lenalidomide is a P-gp substrate.[53]

Overall, these advances in studies of thalidomide pharmacokinetics have expanded the opportunities for individualization of drug therapy with antiepileptic drugs, to enhance effectiveness and minimize the risk of ADRs.

References

1) Eriksson, T., Björkman, S. and Höglund, P.: Clinical pharmacology of thalidomide. *Eur. J. Clin. Pharmacol.*, **57**: 365–376 (2001).
2) Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y. and Handa, H.: Identification of a primary target of thalidomide teratogenicity. *Science*, **327**: 1345–1350 (2010).
3) Juliusson, G., Celsing, F., Turesson, I., Lenhoff, S., Adriansson, M. and Malm, C.: Frequent good partial remissions from thalidomide including best response ever in patients with advanced refractory and relapsed myeloma. *Br. J. Haematol.*, **109**: 89–96 (2000).
4) Calabrese, L. and Resztak, K.: Thalidomide revisited: pharmacology and clinical applications. *Expert Opin. Investig. Drugs*, **7**: 2043–2060 (1998).
5) Seldin, D. C., Choufani, E. B., Dember, L. M., Wiesman, J. F., Berk, J. L., Falk, R. H., O'Hara, C., Fennessey, S., Finn, K. T., Wright, D. G., Skinner, M. and Sanchorawala, V.: Tolerability and efficacy of thalidomide for the treatment of patients with light chain-associated (AL) amyloidosis. *Clin. Lymphoma*, **3**: 241–246 (2003).
6) Palladini, G., Perfetti, V., Perlini, S., Obici, L., Lavatelli, F., Caccialanza, R., Invernizzi, R., Comotti, B. and Merlini, G.: The combination of thalidomide and intermediate-dose dexamethasone is an effective but toxic treatment for patients with primary amyloidosis (AL). *Blood*, **105**: 2949–2951 (2005).
7) Rajkumar, S. V.: Thalidomide in the treatment of multiple myeloma. *Expert Rev. Anticancer Ther.*, **1**: 20–28 (2001).
8) Kyle, R. A. and Rajkumar, S. V.: Therapeutic application of thalidomide in multiple myeloma. *Semin. Oncol.*, **28**: 583–587 (2001).
9) Kyle, R. A.: Current therapy of multiple myeloma. *Intern. Med.*, **41**: 175–180 (2002).
10) Cavenagh, J. D. and Oakervee, H.: UK Myeloma Forum and the BCSH Haematology/Oncology Task Forces. Thalidomide in multiple myeloma: current status and future prospects. *Br. J. Haematol.*, **120**: 18–26 (2003).
11) Bertolini, F., Mingrone, W., Alietti, A., Ferrucci, P. F., Cocorocchio, E., Peccatori, F., Cinieri, S., Mancuso, P., Corsini, C., Burlini, A., Zucca, E. and Martinelli, G.: Thalidomide in multiple myeloma, myelodysplastic syndromes and histiocytosis: analysis of clinical results and of surrogate angiogenesis markers. *Ann. Oncol.*, **12**: 987–990 (2001).
12) Ghobrial, I. M. and Rajkumar, S. V.: Management of thalidomide toxicity. *J. Support. Oncol.*, **1**: 194–205 (2003).
13) Meyring, M., Mühlbacher, J., Messer, K., Kastner-Pustet, N., Bringmann, G., Mannschreck, A. and Blaschke, G.: In vitro biotransformation of (R)- and (S)-thalidomide: application of circular dichroism spectroscopy to the stereochemical characterization of the hydroxylated metabolites. *Anal. Chem.*, **74**: 3726–3735 (2002).
14) Teo, S. K., Sabourin, P. J., O'Brien, K., Kook, K. A. and Thomas, S. D.: Metabolism of thalidomide in human microsomes, cloned human cytochrome P-450 isozymes, and Hansen's disease patients. *J. Biochem. Mol. Toxicol.*, **14**: 140–147 (2000).
15) Ando, Y., Price, D. K., Dahut, W. L., Cox, M. C., Reed, E. and Figg, W. D.: Pharmacogenetic associations of CYP2C19 genotype with in vivo metabolisms and pharmacological effects of thalidomide. *Cancer Biol. Ther.*, **1**: 669–673 (2002).
16) Ando, Y., Fuse, E. and Figg, W. D.: Thalidomide metabolism by the CYP2C subfamily. *Clin. Cancer Res.*, **8**: 1964–1973 (2002).
17) Liu, W. M., Strauss, S. J., Chaplin, T., Shahin, S., Propper, D. J., Young, B. D., Joel, S. P. and Malpas, J. S.: S-thalidomide has a greater effect on apoptosis than angiogenesis in a multiple myeloma cell line. *Hematol. J.*, **5**: 247–254 (2004).
18) Knoche, B. and Blaschke, G.: Investigations on the *in vitro* racemization of thalidomide by high-performance liquid chromatography. *J. Chromatogr. A*, **666**: 235–240 (1994).
19) Nishimura, K., Hashimoto, Y. and Iwasaki, S.: (S)-form of alpha-methyl-N(alpha)-phthalimidoglutarimide, but not its (R)-form, enhanced phorbol ester-induced tumor necrosis factor-alpha production by human leukemia cell HL-60: implication of optical resolution of thalidomidal effects. *Chem. Pharm. Bull. (Tokyo)*, **42**: 1157–1159 (1994).
20) Furuta, T., Ohashi, K., Kobayashi, K., Iida, I., Yoshida, H., Shirai, N., Takashima, M., Kosuge, K., Hanai, H., Chiba, K., Ishizaki, T. and Kaneko, E.: Effects of clarithromycin on the metabolism of omeprazole in relation to CYP2C19 genotype status in humans. *Clin. Pharmacol. Ther.*, **66**: 265–274 (1999).
21) Yamazaki, H., Nakamoto, M., Shimizu, M., Murayama, N. and Niwa, T.: Potential impact of cytochrome P450 3A5 in human liver on drug interactions with triazoles. *Br. J. Clin. Pharmacol.*, **69**: 593–597 (2010).
22) Chowdhury, G., Murayama, N., Okada, Y., Uno, Y., Shimizu, M., Shibata, N., Guengerich, F. P. and Yamazaki, H.: Human liver microsomal cytochrome P450 3A enzymes involved in thalidomide 5-hydroxylation and formation of a glutathione conjugate. *Chem. Res. Toxicol.*, **23**: 1018–1024 (2010).
23) Eriksson, T., Björkman, S., Roth, B. and Höglund, P.: Intravenous formulations of the enantiomers of thalidomide: pharmacokinetic and initial pharmacodynamic characterization in man. *J. Pharm. Pharmacol.*, **52**: 807–817 (2000).
24) Teo, S. K., Scheffler, M. R., Kook, K. A., Tracewell, W. G., Colburn, W. A., Stirling, D. I. and Thomas, S. D.: Effect of a high-fat meal on thalidomide pharmacokinetics and the relative bioavailability of oral formulations in healthy men and women. *Biopharm. Drug Dispos.*, **21**:

Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX)

33–40 (2000).
25) Hiratsuka, M.: In vitro assessment of the allelic variants of cytochrome P450. *Drug Metab. Pharmacokinet.*, **27**: 68–84 (2012).
26) Miyazaki, M., Nakamura, K., Fujita, Y., Guengerich, F. P., Horiuchi, R. and Yamamoto, K.: Defective activity of recombinant cytochromes P450 3A4.2 and 3A4.16 in oxidation of midazolam, nifedipine, and testosterone. *Drug Metab. Dispos.*, **36**: 2287–2291 (2008).
27) Parikh, A., Gillam, E. M. and Guengerich, F. P.: Drug metabolism by Escherichia coli expressing human cytochromes P450. *Nat. Biotechnol.*, **15**: 784–788 (1997).
28) Iwata, H., Fujita, K., Kushida, H., Suzuki, A., Konno, Y., Nakamura, K., Fujino, A. and Kamataki, T.: High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in Escherichia coli. *Biochem. Pharmacol.*, **55**: 1315–1325 (1998).
29) Matsuzawa, N., Nakamura, K., Matsuda, M., Ishida, F. and Ohmori, S.: Influence of cytochrome P450 2C19 gene variations on pharmacokinetic parameters of thalidomide in Japanese patients. *Biol. Pharm. Bull.*, **35**: 317–320 (2012).
30) Li, Y. H. and Hou, J.: Effect of *CYP2C19* gene polymorphism on efficacy of thalidomide-based regimens for the treatment of multiple myeloma. *Zhonghua Xue Ye Xue Za Zhi*, **28**: 651–654 (2007).
31) Vangsted, A., Klausen, T. W. and Vogel, U.: Genetic variations in multiple myeloma II: association with effect of treatment. *Eur. J. Haematol.*, **88**: 93–117 (2012).
32) Zhou, S., Li, Y., Kestell, P., Schafer, P., Chan, E. and Paxton, J. W.: Transport of thalidomide by the human intestinal caco-2 monolayers. *Eur. J. Drug Metab. Pharmacokinet.*, **30**: 49–61 (2005).
33) Zimmermann, C., Gutmann, H. and Drewe, J.: Thalidomide does not interact with P-glycoprotein. *Cancer Chemother. Pharmacol.*, **57**: 599–606 (2006).
34) Okada, Y., Murayama, N., Yanagida, C., Shimizu, M., Guengerich, F. P. and Yamazaki, H.: Drug interactions of thalidomide with midazolam and cyclosporine A: heterotropic cooperativity of human cytochrome P450 3A5. *Drug Metab. Dispos.*, **37**: 18–23 (2009).
35) Zhou, S., Kestell, P., Tingle, M. D., Ching, L. M. and Paxton, J. W.: In vitro and in vivo kinetic interactions of the antitumour agent 5,6-dimethylxanthenone-4-acetic acid with thalidomide and diclofenac. *Cancer Chemother. Pharmacol.*, **47**: 319–326 (2001).
36) Scheffler, M. R., Colburn, W., Kook, K. A. and Thomas, S. D.: Thalidomide does not alter estrogen-progesterone hormone single dose pharmacokinetics. *Clin. Pharmacol. Ther.*, **65**: 483–490 (1999).
37) Allegrini, G., Di Paolo, A., Cerri, E., Cupini, S., Amatori, F., Masi, G., Danesi, R., Marcucci, L., Bocci, G., Del Tacca, M. and Falcone, A.: Irinotecan in combination with thalidomide in patients with advanced solid tumors: a clinical study with pharmacodynamic and pharmacokinetic evaluation. *Cancer Chemother. Pharmacol.*, **58**: 585–593 (2006).
38) Johnson, D. C., Corthals, S. L., Walker, B. A., Ross, F. M., Gregory, W. M., Dickens, N. J., Lokhorst, H. M. and Goldschmidt, H.: Genetic factors underlying the risk of thalidomide-related neuropathy in patients with multiple myeloma. *J. Clin. Oncol.*, **29**: 797–804 (2011).
39) Vangsted, A. J., Soeby, K., Klausen, T. W., Abildgaard, N., Andersen, N. F., Gimsing, P., Gregersen, H., Vogel, U., Werge, T. and Rasmussen, H. B.: No influence of the polymorphisms CYP2C19 and CYP2D6 on the efficacy of cyclophosphamide, thalidomide, and bortezomib in patients with Multiple Myeloma. *BMC Cancer*, **10**: 404–411 (2010).
40) Rajkumar, S. V., Hayman, S., Gertz, M. A., Dispenzieri, A., Lacy, M. Q., Greipp, P. R., Geyer, S., Iturria, N., Fonseca, R., Lust, J. A., Kyle, R. A. and Witzig, T. E.: Combination therapy with thalidomide plus dexamethasone for newly diagnosed myeloma. *J. Clin. Oncol.*, **20**: 4319–4323 (2002).
41) Barlogie, B., Desikan, R., Eddlemon, P., Spencer, T., Zeldis, J., Munshi, N., Badros, A., Zangari, M., Anaissie, E., Epstein, J., Shaughnessy, J., Ayers, D., Spoon, D. and Tricot, G.: Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase II study of 169 patients. *Blood*, **98**: 492–494 (2001).
42) Osman, K., Comenzo, R. and Rajkumar, S. V.: Deep venous thrombosis and thalidomide therapy for multiple myeloma. *N. Engl. J. Med.*, 344: 1951–1952 (2001).
43) Zangari, M., Anaissie, E., Barlogie, B., Badros, A., Desikan, R., Gopal, A. V., Morris, C., Toor, A., Siegel, E., Fink, L. and Tricot, G.: Increased risk of deep-vein thrombosis in patients with multiple myeloma receiving thalidomide and chemotherapy. *Blood*, **98**: 1614–1615 (2001).
44) Bowcock, S. J., Rassam, S. M., Ward, S. M., Turner, J. T. and Laffan, M.: Thromboembolism in patients on thalidomide for myeloma. *Hematology*, **7**: 51–53 (2002).
45) Camba, L., Peccatori, J., Pescarollo, A., Tresoldi, M., Corradini, P. and Bregni, M.: Thalidomide and thrombosis in patients with multiple myeloma. *Haematologica*, **86**: 1108–1109 (2001).
46) Johnson, D. C., Corthals, S., Ramos, C., Hoering, A., Cocks, K., Dickens, N. J., Haessler, J., Goldschmidt, H., Child, J. A., Bell, S. E., Jackson, G., Baris, D., Rajkumar, S. V., Davies, F. E., Durie, B. G., Crowley, J., Sonneveld, P., Van Ness, B. and Morgan, G. J.: Genetic associations with thalidomide mediated venous thrombotic events in myeloma identified using targeted genotyping. *Blood*, **112**: 4924–4934 (2008).
47) Shiah, H. S., Chao, Y., Chen, L. T., Yao, T. J., Huang, J. D., Chang, J. Y., Chen, P. J., Chuang, T. R. and Chin, Y. H.: Phase I and pharmacokinetic study of oral thalidomide in patients with advanced hepatocellular carcinoma. *Cancer Chemother. Pharmacol.*, **58**: 654–664 (2006).
48) Teo, S. K., Colburn, W. A., Tracewell, W. G., Kook, K. A., Stirling, D. I., Jaworsky, M. S., Scheffler, M. A., Thomas, S. D. and Laskin, O. L.: Clinical pharmacokinetics of thalidomide. *Clin. Pharmacokinet.*, **43**: 311–327 (2004).
49) Eriksson, T., Höglund, P., Turesson, I., Waage, A., Don, B. R., Vu, J., Scheffler, M. and Kaysen, G. A.: Pharmacokinetics of thalidomide in patients with impaired renal function and while on and off dialysis. *J. Pharm. Pharmacol.*, **55**: 1701–1706 (2003).
50) Arai, A., Hirota, A., Fukuda, T., Tohda, S., Mori, Y., Terada, Y., Sasaki, S. and Miura, O.: Analysis of plasma concentration of thalidomide in Japanese patients of multiple myeloma with renal dysfunction. *Rinsho Ketsueki*, **50**: 295–299 (2009).
51) Teo, S. K., Colburn, W. A. and Thomas, S. D.: Single-dose oral pharmacokinetics of three formulations of thalidomide in healthy male volunteers. *J. Clin. Pharmacol.*, **39**: 1162–1168 (1999).
52) Fujita, Y., Yamamoto, K., Aomori, T., Murakami, H. and Horiuchi, R.: Comparison of dissolution profile and plasma concentration-time profile of the thalidomide formulations made by Japanese, Mexican and British companies. *Yakugaku Zasshi*, **128**: 1449–1457 (2008).
53) Hofmeister, C. C., Yang, X., Pichiorri, F., Chen, P., Rozewski, D. M., Johnson, A. J., Lee, S., Liu, Z., Garr, C. L., Hade, E. M., Ji, J., Schaaf, L. J., Benson, D. M., Jr., Kraut, E. H., Hicks, W. J., Chan, K. K., Chen, C. S., Farag, S. S., Grever, M. R., Byrd, J. C. and Phelps, M. A.: Phase I trial of lenalidomide and CCI-779 in patients with relapsed multiple myeloma: evidence for lenalidomide-CCI-779 interaction via P-glycoprotein. *J. Clin. Oncol.*, **29**: 3427–3434 (2011).
54) Chung, F., Wang, L. C., Kestell, P., Baguley, B. C. and Ching, L. M.: Modulation of thalidomide pharmacokinetics by cyclophosphamide or 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in mice: the role of tumour necrosis factor. *Cancer Chemother. Pharmacol.*, **53**: 377–383 (2004).
55) Yang, X. X., Hu, Z. P., Chan, S. Y., Duan, W., Ho, P. C., Boelsterli, U. A., Ng, K. Y., Chan, E., Bian, J. S., Chen, Y. Z., Huang, M. and Zhou, S. F.: Pharmacokinetic mechanisms for reduced toxicity of irinotecan by coadministered thalidomide. *Curr. Drug Metab.*, **7**: 431–455 (2006).
56) Villalona-Calero, M., Schaaf, L., Phillips, G., Otterson, G., Panico, K., Duan, W., Kleiber, B., Shah, M., Young, D., Wu, W. H. and Kuhn, J.: Thalidomide and celecoxib as potential modulators of irinotecan's activity in cancer patients. *Cancer Chemother. Pharmacol.*, **59**: 23–33 (2007).
57) Ramirez, J., Wu, K., Janisch, L., Karrison, T., House, L. K., Innocenti, F., Cohen, E. E. and Ratain, M. J.: The effect of thalidomide on the pharmacokinetics of irinotecan and metabolites in advanced solid tumor patients. *Cancer Chemother. Pharmacol.*, **68**: 1629–1632 (2011).
58) Allegrini, G., Di Paolo, A., Cerri, E., Cupini, S., Amatori, F., Masi, G., Danesi, R., Marcucci, L., Bocci, G., Del Tacca, M. and Falcone, A.: Irinotecan in combination with thalidomide in patients with advanced solid tumors: a clinical study with pharmacodynamic and pharmacokinetic evaluation. *Cancer Chemother. Pharmacol.*, **58**: 585–593 (2006).
59) Chung, F., Liu, J., Ching, L. M. and Baguley, B. C.: Consequences of increased vascular permeability induced by treatment of mice with 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and thalidomide. *Cancer Chemother. Pharmacol.*, **61**: 497–502 (2008).
60) Zhou, S., Kestell, P., Tingle, M. D., Ching, L. M. and Paxton, J. W.: A difference between the rat and mouse in the pharmacokinetic interaction of 5,6-dimethylxanthenone-4-acetic acid with thalidomide. *Cancer Chemother. Pharmacol.*, **47**: 541–544 (2001).

Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX)