in Table 4 are not guaranteed to undergo metabolism in the monocyte/macrophage-related cells. Thus, the direct immunostimulatory drugs, MBZ and LPS, were first investigated. As we previously reported (Mizuno *et al.*, 2011), MBZ strongly stimulated the expression of IL-8 and TNFα in THP-1, HL-60 and KG-1 cells. In the present study, direct immunostimulatory drugs, LPS (10 μg/mL) and MBZ (10 μM), were used to treat the cells for 3 and 6 h, and then the expression levels of these 4 biomarker's mRNAs were measured by real-time RT-PCR (Fig. 5). LPS and MBZ increased the expression level of NALP3 mRNA in K562 cells, and the expression levels of S100A8, S100A9, and RAGE mRNA were increased in HL-60 cells. In contrast, the expression levels of NALP3, RAGE, S100A8, and S100A9 in THP-1, KG-1 and differentiated THP-1 cells were not changed by their treatment with these immunostimulatory drugs. The expression level of IL-1β was not investigated in this experiment (Fig. 5) because we have previously reported that LPS and MBZ are unable to stimulate IL-1β expression in these cell lines (Mizuno *et al.*, 2011), however other drugs, such as aminopyrine and diclofenac, stimulate IL-1β expression in HL-60 cells (Supplementary Fig. 2D). For subsequent analyses, HL-60 and K562 cells were used to evaluate the immunostimulatory potency of hepatotoxic drugs.

3.5. An in vitro cell-based assay assessing the mRNA expression level of NALP3, RAGE, S100A8, S100A9, and IL-1β as biomarkers to identify hepatotoxic drugs

To investigate whether the risk of hepatotoxicity can be estimated by using these 5 biomarkers in HL-60 and K562 cells, we performed gene expression analysis of NALP3, RAGE, S100A8, S100A9, and IL-1β mRNA after exposure to 30 different drugs as listed in Table 4. The 30 representative drugs each have a different level of risk for hepatotoxicity (WDN, withdrawn; BBW, black box warning; WNG, warning; SAFE, no warning; the Physicians' Desk Reference (1995, 2000, 2004, and 2008). These drugs were used to treat HL-60 and K562 cells for 6 h in the presence or absence of HLMs. The sample of "the

absence of HLMs" was incubated with the heat-inactivated (treated at 56°C for 30 min) HLMs as mentioned in Materials and Methods. Changes of the relative expression level for each of the 5 biomarkers are shown in Supplemental Figures 1 and 2. The mRNA expression level was normalized to that of GAPDH mRNA in Supplemental Figures 1 and 2. NALP3 mRNA expression levels were not different between SAFE drugs- and hepatotoxic (WDN, BBW, and WNG) drugs-treated cells. Treatment of the cells with HLMs did not affect NALP3 mRNA expression levels (Fig. 6A). IL-1β mRNA expression levels were also not different between SAFE control drugs- and hepatotoxic drugs-treated cells without HLMs treatment, whereas under HLMs treatment, IL-1β mRNA levels were significantly higher in hepatotoxic drugs-treated cells than in SAFE control drugs-treated cells. Increase of IL-1β levels by the HLMs treatment was observed only in hepatotoxic drugs-treated cells, which might indicate that P450-mediated bioactivation of hepatotoxic drugs play a role in hepatotoxicity. However, the relative mRNA expression levels in the most hepatotoxic drugs-treated cells overlapped with those in the SAFE control drugs-treated cells despite of HLMs treatment (Fig. 6C). Taking together, these levels were unable to distinguish between the hepatotoxic and the SAFE control drugs clearly. Studies by Usui *et al.* (2009) and Nakayama *et al.* (2009) demonstrated that the maximum daily dose of a drug could be one of the factors that can determine hepatotoxicity. To investigate whether the maximum daily dose could be used to distinguish between the hepatotoxic drugs and the SAFE drugs, the relative mRNA expression levels were multiplied by the maximum daily dose (Figs. 6B and 6D). The daily doses of the drugs from the Physician's Desk Reference (1995, 2000, 2004, and 2008) are shown in Table 4. The NALP3 and IL-1β mRNA expression levels multiplied by the maximum daily dose tended to be significantly higher for hepatotoxic drugs than for the SAFE drugs; however, the distinction was still insufficient (Figs. 6B and 6D). Furthermore, we conducted the analyses of S100A8, S100A9, and RAGE in the same *in vitro* cell-based assay as in Fig. 6, and no significant difference was observed between the hepatotoxic and the SAFE control drugs

(data not shown). We found that an integrated score of the relative expression levels of S100A8, S100A9, RAGE, and IL-1β mRNA in HL-60 cells and NALP3 mRNA in K562 cells in the presence and absence of HLMs cells is likely to distinguish between the hepatotoxic WDN and BBW drugs from the SAFE control drugs (Fig. 7). This simple added score of the relative expression levels was termed the "total sum score of gene expression level". No added or external factors were processed. In the descending order of total sum score of gene expression level shown in Fig. 7, the top 10 drugs included all of the WDN and BBW drugs in Table 4 except valproic acid. These data indicate that the present cell-based assay system using the "total sum score of gene expression level" can be used to evaluate the high risk of hepatotoxicity resulting from the parent drug and/or its metabolite concurrently, without considering the maximum daily dose.

4. Discussion

DILI is an important issue for drug development and clinical drug therapy; however, prediction of DILI in preclinical studies is difficult because the underlying mechanisms are not fully understood. In this study, using data from comprehensive analyses of the gene expression levels of drug-induced hepatotoxicity *in vivo* in mice and considering metabolic activation reactions and immune- and inflammatory-related factors in humans, we aimed to establish an *in vitro* cell-based assay system with which to identify drugs with hepatotoxic potential.

To establish a novel *in vitro* cell-based screening system to identify drugs with hepatotoxic potential, we first thought that comprehensive analyses of the gene expression in the DILI mouse models are essential. In addition, it is also important to separately consider the changes in gene expression due to pharmacological effects and toxicological effects. Therefore, we selected 4 sets of hepatotoxic and non-hepatotoxic drugs for *in vivo* hepatic gene expression analyses. The hepatotoxic drugs HAL, DIC, FLU, and DIX are known to rarely cause severe hepatotoxicity in humans. Recently, the mouse DILI models of these drugs were well established by our group (Kobayashi *et al.*, 2009; Higuchi *et al.*, 2011; Yano *et al.*, 2012). The chosen non-hepatotoxic drugs, ISO, IBU, BIC, and AMP, possess pharmacological properties and chemical structures similar to HAL, DIC, FLU, and DIX, respectively, and are known to be much less hepatotoxic. These drugs showed no hepatotoxic effect with the same dosing conditions as the hepatotoxic drugs (Figs. 1A and 1B), suggesting that the pharmacological effects may not be involved in DILI under the present experimental conditions. There is no negative control drug for APAP that has similar pharmacological effects and chemical structure; therefore, APAP was used as a positive control drug.

We also reported that immune- and inflammatory-related factors are involved in the pathogenesis of DILI in HAL-, DIC-, FLU-, and DIX-treated mice (Kobayashi *et al.,* 2009; Higuchi *et al.*, 2011; Yano *et al.*, 2012). Taking the information from these DILI models and

several related reports into consideration, as shown in Table 3, 29 immune- and inflammatory-related factors, such as Th cell-related factors, cytokines, chemokines, DAMPs and their related receptors, CD8-positive cell-related factors, and TIM family members, were selected and comprehensively analyzed (Fig. 2A and 2B). In some cases, as shown in Fig. 2A, the non-hepatotoxic drugs induced the expression of inflammatory factors. For example, IBU elevated the mRNA expression of hepatic IL-1β, TNFα, IL6, and MIP-2, and AMP elevated IL-6 and MIP-2 expression even though no significant increases in plasma ALT and AST were observed. Therefore, it is necessary to subtract the gene expression changes due to the pharmacological effect. Based on the *in vivo* DILI model studies, we focused on the 5 factors, S100A8, S100A9, RAGE, IL-1β, and NALP3, for the following reasons: (1) marked increases of the mRNA expression were observed after the administration of hepatotoxic-positive drugs compared with the non-hepatotoxic drugs and (2) increased mRNA expression was observed in 3 out of the 4 hepatotoxic drugs. Based on our previous studies and other reports of DILI mouse models (Higuchi *et al.*, 2011; Yano *et al.*, 2012; Yao and Brownlee, 2010), HMGB1 was suggested to be a predictive biomarker of DILI (Fig. 4A). However, HMGB1 is actively secreted from activated immune cells and is also passively released from necrotic cells (Wang *et al.*, 2004). Thus, the release of HMGB1 measured by ELISA does not correlate with the increased expression of hepatic HMGB1 mRNA. The present cell-based screening system is designed to be an easy assay system for measuring mRNA expression changes; thus, HMGB1 was not used in the present screening system.

Antoine *et al.* (2009) reported that HMGB1 protein, an inflammatory indicator, in serum was linked to the mechanisms and pathological changes induced by APAP in mouse. Changes of the expression levels of S100A8 and S100A9 mRNA and HMGB1 protein were correlated in carbamazepine-induced liver injury in mouse (Higuchi *et al.*, 2012b). Reactive metabolite was suggested to generate the reactive oxygen species (ROS) in rat (Zou *et al.*, 2010), mouse (Higuchi *et al.*, 2012b) and human (Lu and Uetrecht, 2008). In human, it was

reported that ROS increased the expression of the TLR and the RAGE, as well as their ligands, such as S100 proteins and HMGB1 (Yao and Brownlee, 2010). The activation of TLR or RAGE results in the induction of inflammatory cytokines and chemokines in human (Lotze et al., 2007). Cytokines and chemokines, followed by inflammation or the infiltration of lymphocytes to hepatocytes, are involved in immune-mediated hepatotoxicity, and they are predominantly secreted from immune cells such as T lymphocytes and macrophages (Kita et al., 2001 ; Oo and Adams, 2010), suggesting that they might be involved in pathogenesis of DILI in human. Plasma ALT level and HMGB1 level was correlated in APAP-induced liver injury patients (Craig *et al.*, 2011). In the present study, the increased mRNA expression of S100A8 and S100A9 and the increased plasma protein level of HMGB1 were observed in the livers of mice administered hepatotoxic drugs (Figs. 2 and 4A), and the inhibition of TLR4 using eritoran and the neutralization of RAGE by anti-RAGE antibody attenuated drug-induced liver injury (Figs. 4B and 4C), suggesting that RAGE have an important role in the pathogenesis of DILI. Taking these results and information into consideration, the changes of the expression level of S100A8, S100A9 and RAGE would be appropriate biomarkers for prediction of DILI in human.

As reviewed by Latz (2010) and Scaffidi *et al.* (2002), the NALP3 inflammasome is activated by DAMPs that are released from injured cells. The NALP3 inflammasome generates mature IL-1β via proteolytic pathways. Recently, oxidative stress has been shown to play a principal role in the activation of the NALP3 inflammasome in cells or hepatocytes of mouse and human (Martinon *et al.*, 2009, Bryant and Fitzgerald, 2009; Zhou *et al.*, 2010). The NALP3 inflammasome plays a crucial role in the proinflammatory cytokine activation following APAP-induced liver injury in mouse (Maher, 2009; Imaeda *et al.*, 2009). In addition, we previously reported that IL-1β is involved in the early phase of DIC-induced liver injury (Yano *et al.*, 2012). Taking together, the NALP3-IL-1β signaling pathway is strongly suggested involving in DILI, however, there is no direct evidence about the relationship with

DILI severity in human. In this study, the mRNA expression of IL-1βwas also increased in hepatotoxic drug-treated mouse livers (Fig. 2); therefore, the NALP3-IL-1β signaling pathway also appears to be involved in DILI. Thus, those two factors were also thought to be useful for cell-based screening assay.

Next, we intended to apply the 5 biomarkers to an *in vitro* assay system to determine the risk of DILI of various drugs. Direct immunostimulatory drugs, MBZ and LPS, were used to screen the pro-inflammatory response of human monocytic leukemia cells (Fig. 5), according to our previous study (Mizuno *et al.*, 2011). Then, K562 and HL-60 cells were selected to detect the changes in the pro-inflammatory responses. In addition, CYP-mediated bioactivation is known to be involved in the onset of hepatotoxicity for many drugs (Hess and Rieder, 1997). Considering the metabolic activation of drugs, we performed the assay in the presence of HLM and NADPH. Thus, the anticipated mechanism of the present *in vitro* system is as follows: (1) hepatotoxic drugs are metabolized to an active metabolite by HLM; (2) the active metabolite stimulates the human monocytic leukemia cells; and (3) immune- and inflammatory-related responses are detected via changes in mRNA expression levels.

Concerning the concentration of drugs used in the cell-based assay, up to 30 times the clinically efficacious concentration or 100 μM was recommended by O'Brien *et al.* (2006). In the early stages of drug development, the clinically efficacious concentration is unavailable; thus, the use of 100 μM was adapted for the present study. However, according to many reports, the concentration of APAP was 1,000 μM.

We selected 17 positive (WDN, BBW, and WNG) and 13 negative (SAFE) representative compounds with regard to DILI (Table 4). As shown in Figs. 6A and 6C, there was a large overlap in the distribution of the mRNA expression levels between hepatotoxic and non-hepatotoxic drugs. Based on retrospective findings, it was reported that the daily dose is one of the most important factors for DILI, and very low-dose drugs (< 10 mg/day) seem to be devoid of any DILI induction potential (Uetrecht, 2000). In addition, studies by

Usui *et al.* (2009) and Nakayama *et al.* (2009) demonstrated that the maximum daily dose of a drug could be one of the useful factors for determining hepatotoxicity. However, in this study, discrimination between hepatotoxic and non-hepatotoxic drugs was still insufficient even when considering the maximum daily dose (Fig. 6B and 6D). We found that the use of "total sum score of gene expression level", an integrated score of the relative expression levels of S100A8, S100A9, RAGE, NALP3 and IL-1β mRNA in HL-60 or K562 cells, could help identify drugs with a high risk of hepatotoxicity. The top 10 drugs included hepatotoxic drugs with a high risk of DILI (WDN and BBW) (Fig. 7). This assay system may be one of the useful methods for assessing the metabolic activation reaction and may be beneficial for predicting the risks of liver injury by drugs in preclinical development, without considering the maximum daily dose.

In this study, we focused on metabolic activation by CYPs by addition of HLM and NADPH; however, hydrolase and/or UDP-glucuronosyltransferase enzymes should be considered because these enzymes have also been suggested to be involved in metabolic activation (Spahn-Langguth and Benet, 1992; Kobayashi *et al.*, 2012). For example, acyl-glucuronides formed from carboxylic acid-containing metabolites can bind covalently to proteins and other macromolecules because of their electrophilicity, which suggests that they are linked to drug toxicity. In addition, valproic acid, which has a high risk for hepatotoxicity, exceptionally showed the lowest total sum score of gene expression level. This might imply the mechanism of DILI caused by valproic acid could not be explained by the inflammatory- or immune-related factors. Indeed, a metabolite of valproic acid is responsible for the mitochondrial damage, impairment of fatty acid mitochondrial β-oxidation, and lipid accumulation (Baillie, 1988), which may be associated with hepatotoxicity. A predictive method that takes mitochondrial toxicity into consideration might improve predictive accuracy. On the other hand, some SAFE drugs, pravastatin, levofloxacin, and ibuprofen, showed high scores. Taking together, the predictive potential for clinical DILI of this study is

not conclusive and it should be improved in the near future.

In conclusion, using the *in vivo* mouse DILI models with 4 sets of hepatotoxic and non-hepatotoxic drugs, we found that the hepatic mRNA levels of S100A8, S100A9, RAGE, NALP3, and IL-1β could be biomarkers of DILI. These 5 biomarkers were investigated for their utility in a cell-based screening system that considers metabolic activation reactions and immune- and inflammatory-related factors. Then, we proposed the use of the total sum score of gene expression level, that is, an integrated score of the relative mRNA expression levels of biomarkers for predicting the risk of DILI in preclinical drug development.

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Conflict of interest

Authors have no conflicts to declare.

References

Antoine, D.J., Williams, D.P., Kipar, A., Jenkins, R.E., Regan, S.L., Sathish, J.G., Kitteringham, N.R., Park, B.K., 2009. High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis and apoptosis in vivo. Toxicol Sci 112, 521-531.

Antoine, D.J., Williams, D.P., Kipar, A., Laverty, H., Park, B.K., 2010. Diet restriction inhibits apoptosis and HMGB1 oxidation and promotes inflammatory cell recruitment during acetaminophen hepatotoxicity. Mol Med 16, 479-490.

Baillie, T.A., 1988. Metabolic activation of valproic acid and drug-mediated hepatotoxicity. Role of the terminal olefin, 2-n-propyl-4-pentenoic acid. Chem Res Toxicol 1, 195-199.

Bryant, C., Fitzgerald, K.A., 2009. Molecular mechanisms involved in inflammasome activation. Trends Cell Biol 19, 455-464.

Cheng, L., You, Q., Yin, H., Holt, M.P., Ju, C., 2010. Involvement of natural killer T cells in halothane-induced liver injury in mice. Biochem Pharmacol 80, 255-261.

Craig, D.G., Lee, P., Pryde, E.A., Masterton, G.S., Hayes, P.C., Simpson, K.J., 2011. Circulating apoptotic and necrotic cell death markers in patients with acute liver injury. Liver Int 31, 1127-1136.

Deng, X., Liguori, M.J., Sparkenbaugh, E.M., Waring, J.F., Blomme, E.A., Ganey, P.E., Roth, R.A., 2008. Gene expression profiles in livers from diclofenac-treated rats reveal intestinal bacteria-dependent and -independent pathways associated with liver injury. J Pharmacol Exp Ther 327, 634-644.

Deng, X., Stachlewitz, R.F., Liguori, M.J., Blomme, E.A., Waring, J.F., Luyendyk, J.P., Maddox, J.F., Ganey, P.E., Roth, R.A., 2006. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J Pharmacol Exp Ther 319, 1191-1199.

Dugan, C.M., Fullerton, A.M., Roth, R.A., Ganey, P.E., 2011. Natural killer cells mediate

severe liver injury in a murine model of halothane hepatitis. Toxicol Sci 120, 507-518.

Dugan, C.M., MacDonald, A.E., Roth, R.A., Ganey, P.E., 2010. A mouse model of severe halothane hepatitis based on human risk factors. J Pharmacol Exp Ther 333, 364-372.

Duplay, D., editor, 2004. Physicians' Desk Reference. Thomson Healthcare Inc., Montvale, NJ.

Evans, D.C., Watt, A.P., Nicoll-Griffith, D.A., Baillie, T.A., 2004. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. Chem Res Toxicol 17, 3-16.

Gole, W., editor, 1995. Physicians' Desk Reference. Medical Economics Co., Montvale, NJ.

Hess, D.A., Rieder, M.J., 1997. The role of reactive drug metabolites in immune-mediated adverse drug reactions. Ann Pharmacother 31, 1378-1387.

Higuchi, S., Kobayashi, M., Yano, A., Tsuneyama, K., Fukami, T., Nakajima, M., Yokoi, T., 2012a. Involvement of Th2 cytokines in the mouse model of flutamide-induced acute liver injury. J Appl Toxicol 32, 815-822.

Higuchi, S., Kobayashi, M., Yoshikawa, Y., Tsuneyama, K., Fukami, T., Nakajima, M., Yokoi, T., 2011. IL-4 mediates dicloxacillin-induced liver injury in mice. Toxicol Lett 200, 139-145.

Higuchi, S., Yano, A., Takai, S., Tsuneyama, K., Fukami, T., Nakajima, M., Yokoi, T., 2012b. Metabolic activation and inflammation reactions involved in carbamazepine-induced liver injury. Toxicol Sci 130, 4-16.

Imaeda, A.B., Watanabe, A., Sohail, M.A., Mahmood, S., Mohamadnejad, M., Sutterwala, F.S., Flavell, R.A., Mehal, W.Z., 2009. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. J Clin Invest 119, 305-314.

Ishida, Y., Kondo, T., Tsuneyama, K., Lu, P., Takayasu, T., Mukaida, N., 2004. The pathogenic roles of tumor necrosis factor receptor p55 in acetaminophen-induced liver injury in mice.

J Leukoc Biol 75, 59-67.

Kita, H., Mackay, I.R., Van De Water, J., Gershwin, M.E., 2001. The lymphoid liver: considerations on pathways to autoimmune injury. Gastroenterology 120, 1485-1501.

Kobayashi, E., Kobayashi, M., Tsuneyama, K., Fukami, T., Nakajima, M., Yokoi, T., 2009. Halothane-induced liver injury is mediated by interleukin-17 in mice. Toxicol Sci 111, 302-310.

Kobayashi, Y., Fukami, T., Higuchi, R., Nakajima, M., Yokoi, T., 2012. Metabolic activation by human arylacetamide deacetylase, CYP2E1, and CYP1A2 causes phenacetin-induced methemoglobinemia. Biochem Pharmacol 84, 1196-1206.

Latz, E., 2010. The inflammasomes: mechanisms of activation and function. Curr Opin Immunol 22, 28-33.

Lee, W.M., 2003. Drug-induced hepatotoxicity. N Engl J Med 349, 474-485.

Lotze, M.T., Zeh, H.J., Rubartelli, A., Sparvero, L.J., Amoscato, A.A., Washburn, N.R., Devera, M.E., Liang, X., Tör, M., Billiar, T., 2007. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol Rev 220, 60-81.

Lu, W., Uetrecht, J.P., 2008. Peroxidase-mediated bioactivation of hydroxylated metabolites of carbamazepine and phenytoin. Drug Metab Dispos 36, 1624-1636.

Maher, J.J., 2009. DAMPs ramp up drug toxicity. J Clin Invest 119, 246-249.

Martinon, F., Mayor, A., Tschopp, J., 2009. The inflammasomes: guardians of the body. Annu Rev Immunol 27, 229-265.

Mizuno, K., Toyoda, Y., Fukami, T., Nakajima, M., Yokoi, T., 2011. Stimulation of pro-inflammatory responses by mebendazole in human monocytic THP-1 cells through an ERK signaling pathway. Arch Toxicol 85, 199-207.

Nakayama, S., Atsumi, R., Takakusa, H., Kobayashi, Y., Kurihara, A., Nagai, Y., Nakai, D., Okazaki, O., 2009. A zone classification system for risk assessment of idiosyncratic drug

toxicity using daily dose and covalent binding. Drug Metab Dispos 37, 1970-1977.

O'Brien, P.J., Irwin, W., Diaz, D., Howard-Cofield, E., Krejsa, C.M., Slaughter, M.R., Gao, B., Kaludercic, N., Angeline, A., Bernardi, P., Brain, P., Hougham, C., 2006. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch Toxicol 80, 580-604.

Oo, Y.H., Adams, D.H., 2010. The role of chemokines in the recruitment of lymphocytes to the liver. J Autoimmun 34, 45-54.

Racanelli, V., Rehermann, B., 2006. The liver as an immunological organ. Hepatology 43, S54-62.

Ramaiah, S.K., Jaeschke, H., 2007. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. Toxicol Pathol 35, 757-766.

Sanborn, K., editor, 2008. Physicians' Desk Reference. Thomson Healthcare Inc., Montvale, NJ.

Scaffidi, P., Misteli, T., Bianchi, M.E., 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418, 191-195.

Spahn-Langguth, H., Benet, L.Z., 1992. Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? Drug Metab Rev 24, 5-47.

Takakusa, H., Masumoto, H., Yukinaga, H., Makino, C., Nakayama, S., Okazaki, O., Sudo, K., 2008. Covalent binding and tissue distribution/retention assessment of drugs associated with idiosyncratic drug toxicity. Drug Metab Dispos 36, 1770-1779.

Tukov, F.F., Luyendyk, J.P., Ganey, P.E., Roth, R.A., 2007. The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. Toxicol Sci 100, 267-280.

Uetrecht, J.P., 1999. New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. Chem Res Toxicol 12, 387-395.

Uetrecht, J.P., 2000. Is it possible to more accurately predict which drug candidates will cause idiosyncratic drug reactions? Curr Drug Metab 1, 133-141.

Usui, T., Mise, M., Hashizume, T., Yabuki, M., Komuro, S., 2009. Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. Drug Metab Dispos 37, 2383-2392.

Walgren, J.L., Mitchell, M.D., Thompson, D.C., 2005. Role of metabolism in drug-induced idiosyncratic hepatotoxicity. Crit Rev Toxicol 35, 325-361.

Walsh, P., editor, 2000. Physicians' Desk Reference. Medical Economics Co., Montvale, NJ.

Wang, H., Yang, H., Tracey, K.J., 2004. Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med 255, 320-331.

Yano, A., Higuchi, S., Tsuneyama, K., Fukami, T., Nakajima, M., Yokoi, T., 2012. Involvement of immune-related factors in diclofenac-induced acute liver injury in mice. Toxicology 293, 107-114.

Yao, D., Brownlee, M., 2010. Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. Diabetes 59, 249-255.

You, Q., Cheng, L., Reilly, T.P., Wegmann, D., Ju, C., 2006. Role of neutrophils in a mouse model of halothane-induced liver injury. Hepatology 44, 1421-1431.

Zhou, R., Tardivel, A., Thorens, B., Choi, I., Tschopp, J., 2010. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat Immunol 11, 136-140.

Zou, W., Roth, R.A., Younis, H.S., Burgoon, L.D., Ganey, P.E. 2010. Oxidative stress is important in the pathogenesis of liver injury induced by sulindac and lipopolysaccharide cotreatment. Toxicology 272, 32-38.

Figure legends

Fig. 1. Time-dependent changes of plasma ALT and AST levels in mice administered hepatotoxic drugs.

Hepatotoxic drugs (APAP: 300 mg/kg, *i.p.*; HAL: 30 mmol/kg, *i.p.*; DIC: 150 mg/kg, *i.p.*; FLU: 1500 mg/kg, *p.o.*; DIX: 600 mg/kg, *i.p.*) and non-hepatotoxic control drugs (ISO: 30 mmol/kg, *i.p.*; IBU: 150 mg/kg, *i.p.*; BIC: 1500 mg/kg, *p.o.*; AMP: 1000 mg/kg, *i.p.*) were administered. Each drug was administered once to mice, and blood samples were collected for the assessment of ALT and AST levels 1, 3, 6, and 24 h after drug administration. Data are shown as the mean ± SEM of results from 5 mice. Differences compared to the control group were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Fig. 2. Time-dependent changes of the mRNA expression profiles of inflammatory- and immune-related factors in drug-induced liver injury.

Each drug was administered to the mice. At 1, 3, 6, and 24 h after administration, the hepatic mRNA expression level of 29 different types of inflammatory- and immune-related factors listed in Table 3 were measured by real-time RT-PCR. (A) Results of S100A8, S100A9, RAGE, TLR4, IL-1β, TNFα IL-6, NALP3, MIP-2 and TIM1 are shown. The expression level of hepatic mRNA was normalized to that of Gapdh mRNA. The change in the expression level is indicated by the fold-change compared to the vehicle-administered control group. Data are shown as the mean from 5 independent experiments. (B) Graphical expression of the data for S100A8, S100A9, RAGE, NALP3, and IL-1β. Data are presented as the log2 value ± SEM of the results from 5 mice.

Fig. 3. Time-dependent changes of the plasma ALT level and the hepatic mRNA expression of S100A8 and S100A9 in HAL-administered mice.

The plasma ALT level and hepatic S100A8 or S100A9 mRNA levels were measured 1, 3, 6, and 24 h after the HAL administration (30 mmol/kg, *i.p.*). The expression level of hepatic mRNA was normalized to that of Gapdh mRNA. Data are shown as the mean ± SEM of results from 5 mice. Differences of ALT levels compared to the control group were considered significant at $*p < 0.05$ and $***p < 0.001$. Differences of mRNA levels compared to the control group were considered significant at $\#p < 0.05$ and $\#\#p < 0.01$.

Fig. 4. Time-dependent changes of plasma HMGB1 levels and the effect of the inhibition of TLR4 and RAGE on plasma ALT levels during drug-induced liver injury.

(A) The plasma HMGB1 level was measured by ELISA 1, 3, 6, and 24 h after the administration of hepatotoxic (HAL, DIC, FLU, and DIX) and non-hepatotoxic control (ISO, IBU, BIC and AMP) drugs. (B) Eritoran (50 μg/mouse, *i.v.*), a TLR4 antagonist, was administered to the mice simultaneously with HAL (30 mmol/kg, *i.p.*), DIC (150 mg/kg, *i.p.*), or DIX (600 mg/kg, *i.p.*). The plasma ALT level was measured 6 h after DIC or DIX administration, or 24 h after HAL administration. (C) Anti-mouse RAGE antibody (0.1 mg/mouse, *i.p.*) or IgG2a (control) was simultaneously administered with DIX (600 mg/kg, *i.p.*). The plasma ALT level was measured 6 h after DIX administration. Data are shown as the mean ± SEM of results from 5 mice. Differences compared to the control mice (A), the drug alone treatment group (B) or the IgG2a-administered mice (C) were considered significant at $*P < 0.05$ and $**P < 0.01$.

Fig. 5. Effects of LPS and MBZ on the mRNA expression levels of NALP3, RAGE, S100A8, and S100A9 in human monocytic leukemia cells.

(A) THP-1, (B) K562, (C) KG-1, (D) HL-60, and (E) differentiated THP-1 cells were treated with 10 μg/mL LPS or 10 μM MBZ for 3 and 6 hours. The mRNA expression level was measured by real-time RT-PCR analysis. The mRNA expression level was normalized to

that of GAPDH mRNA. Data are shown as the mean from 2 independent experiments. ND; not detectable.

Fig. 6. Effect of the incubation of hepatotoxic and control SAFE drugs with HLMs on the expression levels of NALP3 and IL-1β mRNA and those expression levels multiplied by the maximum daily dose of drugs.

The comparison of the expression levels of NALP3 mRNA in K562 (A) and IL-1β mRNA in HL-60 (C) cells exposed to the hepatotoxic and control SAFE drugs incubated with HLMs are shown. The relative expression levels of NALP3 or IL-1β mRNA multiplied with the maximum daily dose of the hepatotoxic and control SAFE drugs are shown in Figs. 6B and 6D, respectively. Differences between two groups were considered significant at $*p < 0.05$ and $**p < 0.01$

Fig. 7. Total sum score of gene expression level of 30 drugs with different levels of risk for hepatotoxicity (WDN, withdrawn; BBW, black box warning; WNG, warning; SAFE, no warning, Table 4).

The "total sum score of gene expression level" was defined as an integrated score of the relative expression levels of NALP3 mRNA in K562 cells, and RAGE, S100A8, S100A9 and IL-1β mRNA in HL-60 cells incubated with and without HLMs. The expression level of hepatic mRNA was normalized to that of GAPDH mRNA. Drugs are ranked in descending order of total sum score of gene expression level. Red indicates WDN drugs, orange indicates BBW drugs, purple indicates WNG drugs, and black indicates SAFE drugs, as shown in Table 4.

Table 1. Primer sequences used for real-time RT-PCR analyses in the *in vivo* study.

Target		Sequence
CXCL1	F (5'-3')	GATTCACCTCAAGAACATCCAGAG
	R (5'-3')	GAAGCCAGCGTTCACCAGAC
FasL	F (5'-3')	AGAAGGAACTGGCAGAACTC
	R (5'-3')	GCGGTTCCATATGTGTCTTC
GATA-3	F (5'-3')	GGAGGACTTCCCCAAGAGCA
	R (5'-3')	CATGCTGGAAGGGTGGTGA
Granzyme B	F (5'-3')	TCGAGAGGACTTTGTGCTG
	R (5'-3')	CCTCTTGGCCTTACTCTTC
HMGB1	F (5'-3')	GGAGATCCTAAAAAGCCGAG
	R (5'-3')	ATAACGAGCCTTGTCAGCCT
HSP70	F (5'-3')	GGAGTTCAAGAGGAAGCACA
	R (5'-3')	TGGATGTGTAGAAGTCGATG
IFN-γ	F (5'-3')	TCAAGTGGCATAGATGTGGAAGAA
	R (5'-3')	TGGCTCTGCAGGATTTTCATG
IL-1β	F (5'-3')	GTTGACGGACCCCAAAAGAT
	R (5'-3')	CACACACCAGCAGGTTATCA
IL-6	F (5'-3')	CCATAGCTACCTGGAGTACA
	R (5'-3')	GGAAATTGGGGTAGGAAGGA
IL-12p35	F (5'-3')	TGCTGAAGACCACAGATGAC
	R (5'-3')	GAAGTCTCTCTAGTAGCCAG
MCP-1	F (5'-3')	TGTCATGCTTCTGGGCCTG
	R (5'-3')	CCTCTCTCTTGAGCTTGGTG
MIP-2	F (5'-3')	AAGTTTGCCTTGACCCTGAAG
	R (5'-3')	ATCAGGTACGATCCAGGCTTC
NALP3	F (5'-3')	AGCCTTCCAGGATCCTCTTC
	R (5'-3')	CTTGGGCAGCAGTTTCTTTC
Perforin	F (5'-3')	ACAGTAGAGTGTCGCATG
	R (5'-3')	ACAGCCGTGATAAAGTGC
RAGE	F (5'-3')	GAAACTTCTGATTCCCGATGG
	R (5'-3')	GCTCAACCAACAGCTGAATG
ROR-γt	F (5'-3')	ACCTCCACTGCCAGCTGTGTGCTGTC
	R (5'-3')	TCATTTCTGCACTTCTGCATGTAGACTGTCCC
STAT1	F (5'-3')	GTTTCAGCTCTGCTCCATAC
	R (5'-3')	CTGCTGAAGCTCGAACCAC
STAT3	F (5'-3')	TGCAGAGCAGGTATCTTGAG
	R (5'-3')	TGCTGCTTCTCTGTCACTAC

Table 2. Primer sequences used for real-time RT–PCR analyses for the *in vitro* study.

Target		Sequence
Human NALP3	F (5'-3')	CCA CGC TAA TGA TCG ACT TC
	R (5'-3')	GTA AAC CCA TCC ACT CCT CT
Human RAGE	F (5'-3')	GGC TGG AAT GGA AAC TGA AC
	R (5'-3')	TGG TCT CCT TTC CAT TCC TG
Human S100A8	F (5'-3')	CTG GAG AAA GCC TTG AAC TC
	R (5'-3')	GAA TGA GGA ACT CCT GGA AG
Human S100A9	F (5'-3')	ACA GAG TGC AAG ACG ATG AC
	R (5'-3')	AGG TCC TCC ATG ATG TGT TC
Human IL-1β	F (5'-3')	GCTGATGGCCCTAAACAGATG
	R (5'-3')	TTCTCCTGGAAGGTCTGTGG
Human GAPDH	F (5'-3')	CCA TGA GGT CCA CCA CCC TGT T
	R (5'-3')	TGG GTG GCA GTG ATG GCA TGG A

F: Forward primer, R: Reverse primer.

Table 3. Inflammatory- and immune-related factors.

Type	Factors
Th cells-related factor	RORγt, T-bet, GATA3, STAT1, STAT3, STAT6
Cytokine	TNFα, IFN-γ, IL-1β, IL-6, IL-12
Chemokine	MIP-2, MCP-1, CXCL1, VCAM1
DAMP	S100A8, S100A9, HSP70, HMGB1
Receptor	TLR4, NALP3, RAGE
CD8 positive cells-related factor	Granzyme B, Perforin, FasL
TIM family	TIM1, TIM2, TIM3, TIM4