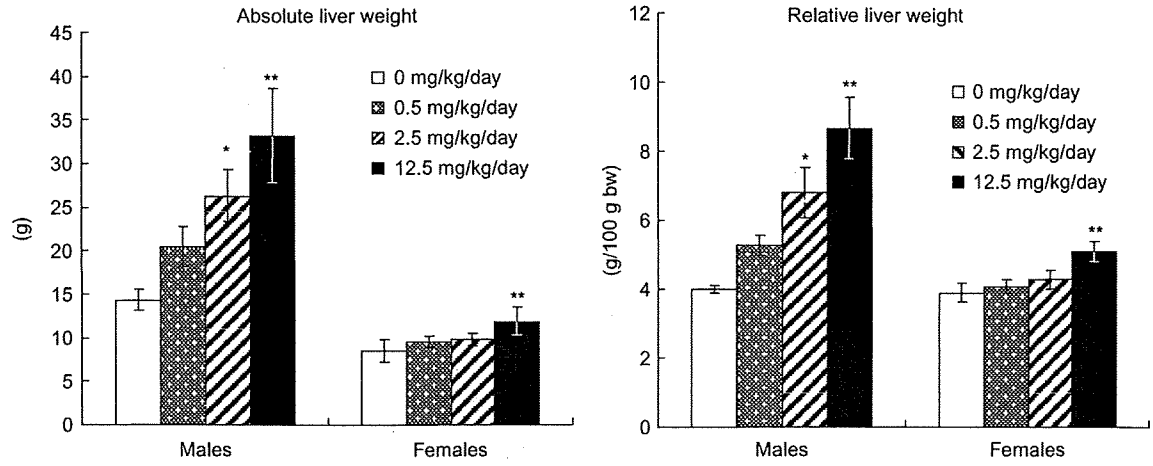

Figure 1. Absolute and relative liver weight of male and female rats given HDBB by gavage for 28 days. Data are expressed as the mean ± standard deviation (SD). *Significantly different from the control, $P < 0.05$; **Significantly different from the control, $P < 0.01$.

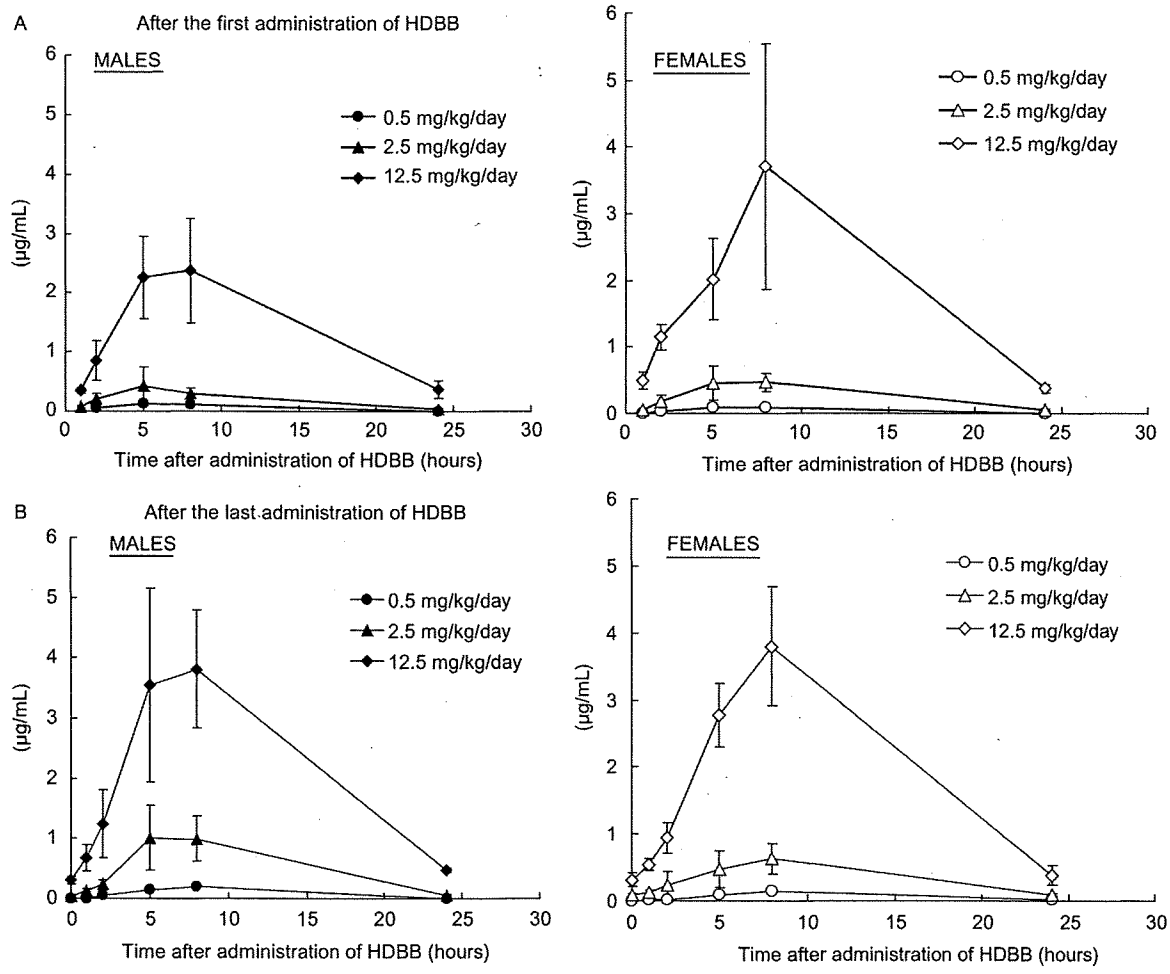

Figure 2. Plasma HDBB concentrations against time after the administration of HDBB to male and female rats.Data are expressed as the mean ± SD.

eliminated from the plasma in both sexes. No clear gender-related differences were found in the plasma profiles of HDBB at any doses. After 28-day repeated-dose administration, similar plasma HDBB profiles were observed, and there were no gender-related differences in the profiles (Figure 2B). In all dose groups, HDBB metabolites were not detected in the plasma at any sampling times in either sex.

The calculated values of C_{max}, T_{max}, and $AUC_{0\text{-}24h}$ for HDBB in plasma are given in Table 1. The data showed that $AUC_{0\text{-}24h}$ as well as C_{max} values increased in rat plasma with the higher HDBB dose. Comparison of data for males and females indicated no gender-related differences.

Table 1. Toxicokinetic parameters of HDBB.

Doses	Sexes	C_{max} (μg/mL)	T_{max} (h)	$AUC_{0\text{-}24h}$ (μg·h/mL)
After the first administration of HDBB				
0.5 mg/kg/day	Males	0.145±0.031	5.75±1.50	1.59±0.32
	Females	0.116±0.036	5.75±1.50	1.25±0.10
2.5 mg/kg/day	Males	0.484±0.276	5.75±1.50	4.99±1.45
	Females	0.573±0.165	7.25±1.50	6.65±1.61
12.5 mg/kg/day	Males	2.85±0.64	6.50±1.73	34.4±7.1
	Females	3.84±1.71	7.25±1.50	47.1±15.7
After the last administration of HDBB				
0.5 mg/kg/day	Males	0.214±0.054	6.50±1.73	2.49±0.62
	Females	0.154±0.009	8.00±0.00	1.98±0.15
2.5 mg/kg/day	Males	1.14±0.42	5.75±1.50	13.6±5.0
	Females	0.636±0.221	7.25±1.50	8.89±3.25
12.5 mg/kg/day	Males	4.27±0.96	5.75±1.50	54.0±11.4
	Females	3.80±0.89	8.00±0.00	50.1±9.8

Values are expressed as the mean ± SD.

In vitro metabolism reaction

After a 60-minute incubation using the liver S-9 fraction prepared from male or female rats with the NADPH-generating system, the concentration of HDBB in the incubation mixture was hardly changed, as shown in Figure 3. The mean residual ratio of HDBB was 98.1% with male rat hepatic microsomes and 91.4% with female rat hepatic microsomes. On the other hand, when incubated with male and female hepatic microsomes, HDBB concentration in the incubation mixture decreased to 73.4 and 76.1% of the control, respectively. In either male or female microsomes, another peak was found around a retention time of 1–2 minutes.

Figure 4 represents residual ratios of HDBB after a 60-minute incubation with microsomes containing cDNA-expressed individual rat CYP enzymes in the presence of the NADPH generating system. Among the 14 types of CYP isoforms tested here, CYP1A1 exhibited the greatest metabolic activity of HDBB (mean residual HDBB ratio: 61.8%). CYP1A2, 2A2, 2B1, 2C6, 2C11, and 2D2 also metabolized 10–20% of HDBB. Other CYP isoforms, CYP2A1, 2C12, 2C13, 2D1, 2E1, 3A1, and 3A2, showed no significant metabolism of the chemical (mean residual ratio of HDBB: >95%). After incubation with CYP1A1, 1A2, 2A2, 2C6, 2C11, 2D2, 3A1, or 3A2, some peaks other than HDBB were detected.

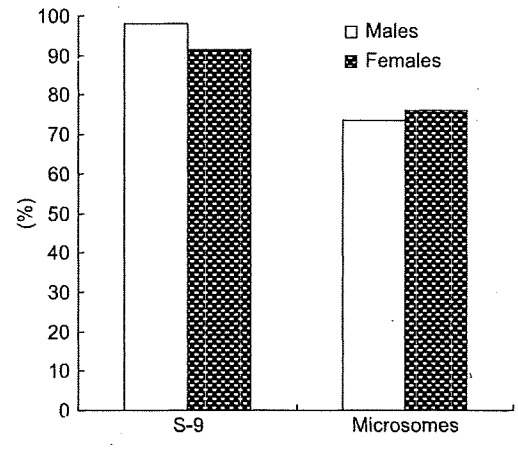

Figure 3. Residual ratios of HDBB after incubation with male and female rat liver S-9 and microsomes in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after a 60-minute incubation by that of the control, in which the incubation system was inactivated by the addition of 1 mL of acetonitrile prior to incubation.

Effect of HDBB on hepatic CYP activity (Table 2)

While microsomal protein content showed no significant differences between HDBB-treated and control groups, the total CYP content was significantly increased in males of the 2.5 and 12.5 mg/kg groups. In these groups, aminopyrine *N*-demethylase activity, and testosterone 2α- and 16α-hydroxylase activity, decreased significantly. EROD activity showed a significant decrease in males in all HDBB-treated groups. These changes were not detected in females. Lauric acid 12-hydroxylase activity was significantly increased at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. No significant changes were found in ECOD activity or testosterone 6β-hydroxylase activity in either sex.

Discussion

The current study was conducted to clarify the mechanism of marked gender-related differences in HDBB

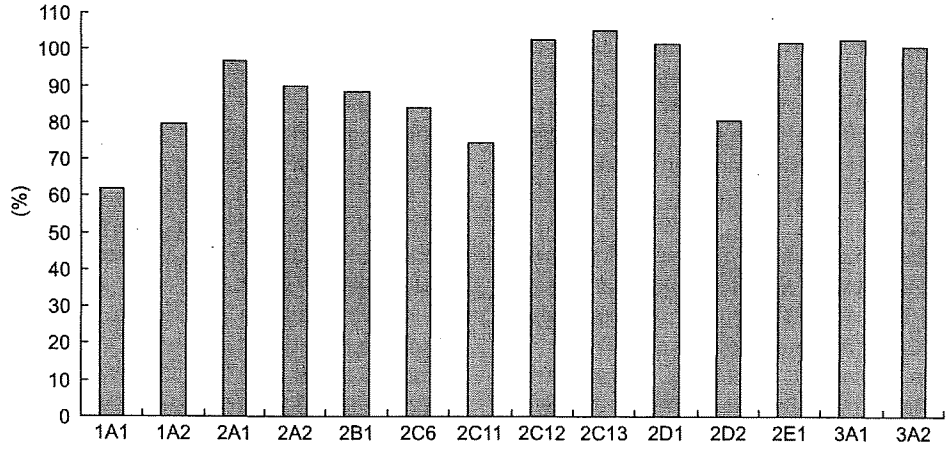

Figure 4. Residual ratios of HDBB after incubation with recombinant rat CYP isoforms in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after incubation with microsomes containing cDNA-expressed individual rat CYPs by that after incubation with control microsomes containing negligible amounts of CYP.

Table 2. Protein content, total CYP contents, and enzyme activities in hepatic microsomes of male and female rats given HDBB by gavage for 28 days.

	Dose (mg/kg/day)			
	0 (control)	0.5	2.5	12.5
No. of males	5	5	5	5
Microsomal protein content (mg/g liver)	27.2±5.3	21.6±2.3	22.0±6.0	23.5±4.3
Total CYP content (nmol/mg protein)	0.670±0.119	0.783±0.075	0.885±0.052#	0.738±0.119#
Aminopyrine *N*-demethylase activity (nmol/min/mg protein)	6.700±0.443	6.942±0.812	4.902±0.484#	4.713±0.743#
ECOD activity (nmol/min/mg protein)	1.420±0.140	1.713±0.354	1.528±0.253	1.213±0.202
EROD activity (nmol/min/mg protein)	0.0627±0.0083	0.0459±0.0045#	0.0233±0.0054#	0.0237±0.0020#
Testosterone 6β-hydroxylase activity (nmol/min/mg protein)	2.67±0.44	3.18±0.96	2.89±0.41	2.53±0.36
Testosterone 2α-hydroxylase activity (nmol/min/mg protein)	1.562±0.170	1.385±0.495	0.179±0.207#	0.000±0.000#
Testosterone 16α-hydroxylase activity (nmol/min/mg protein)	2.165±0.439	1.714±0.451	0.432±0.278#	0.119±0.137#
Lauric acid 12-hydroxylase activity (nmol/min/mg protein)	1.60±0.47	7.80±2.14$	9.99±0.58$	11.09±2.26$
No. of females	5	5	5	5
Microsomal protein content (mg/g liver)	11.4±3.5	11.7±3.5	16.2±6.7	16.5±3.0
Total CYP content (nmol/mg protein)	0.637±0.132	0.674±0.168	0.646±0.131	0.600±0.076
Aminopyrine *N*-demethylase activity (nmol/min/mg protein)	4.157±0.534	4.362±0.630	4.403±1.007	5.133±0.680
ECOD activity (nmol/min/mg protein)	0.657±0.105	0.649±0.099	0.647±0.128	0.693±0.095
EROD activity (nmol/min/mg protein)	0.0869±0.0266	0.0882±0.0145	0.0904±0.0144	0.1142±0.0237
Testosterone 6β-hydroxylase activity (nmol/min/mg protein)	0.121±0.023	0.138±0.019	0.150±0.040	0.159±0.047
Testosterone 2α-hydroxylase activity (nmol/min/mg protein)	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Testosterone 16α-hydroxylase activity (nmol/min/mg protein)	0.066±0.123	0.018±0.025	0.054±0.076	0.083±0.073
Lauric acid 12-hydroxylase activity (nmol/min/mg protein)	1.37±0.15	1.40±0.23	1.51±0.38	3.20±3.01$

Values are expressed as the mean ± SD.
#Significantly different from the control by the Williams test, $P < 0.05$.
$Significantly different from the control by the Shirley-Williams test, $P < 0.05$.

toxicity. Following 28-day HDBB administration, macroscopic changes in the liver and/or increased liver weight were found at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. This showed about a 25 times higher susceptibility of male rats to the hepatotoxicity of HDBB and demonstrated the reproducibility of our previous 28-day study (Hirata-Koizumi et al., 2007).

Unexpectedly, we could not find sexual variation in plasma HDBB levels and toxicokinetic parameters (i.e., C_{max}, T_{max}, and $AUC_{0\text{-}24h}$) in rats orally given HDBB for 28 days. No metabolites of HDBB were detected in the plasma of either sex. Although the *in vitro* study using hepatic microsomal preparation from male and female rats showed evidence of some HDBB metabolism, no sexual differences were found in the residual HDBB ratio after a 60-minute incubation with an NADPH-generation system. *In vitro* results using recombinant CYP enzymes suggest the contribution of multiple CYP isozymes (i.e., CYP1A1, 1A2, 2A2, 2C6, 2C11, and 2D2) to the overall metabolism of HDBB in rat liver microsomes. Among these isozymes, gender-related difference was reported in CYP2C11, which is known to be a male-specific isoform (Waxman and Chang, 2005). However, considering our present result that 28-day HDBB administration markedly reduced CYP2C11-dependent testosterone 2α- and 16α-hydroxylation in the liver, male-specific metabolism catalyzed by this enzyme is unlikely to contribute to the higher susceptibility of male rats to HDBB toxicity. These findings show that gender-related differences in HDBB toxicity do not come from the variation in plasma concentration of causative substances (i.e., HDBB or its metabolites) and hepatic metabolism.

HDBB exerted sexually different effects on hepatic metabolic activities. Of particular note is the change in CYP4A-specific activity and lauric acid 12-hydroxylation, which increased at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. The dose responsiveness was consistent with that of liver-weight change and macroscopic findings. Hepatic CYP4A expression is known to be highly inducible by a diverse group of compounds referred to as peroxisome proliferators, which include the widely prescribed lipid-lowering drug of the fibrate class, phthalate ester plasticizer, the endogenous steroid, dehydroepiandrosterone, and chlorinated phenoxy and benzoic acid herbicides (Bacher and Gibson, 1988; Espandiari et al., 1995; Okita et al., 1993; Sundseth and Waxman, 1992; Wu et al., 1989). In the previous 52-week repeated dose toxicity study of HDBB, we observed the centrilobular hypertrophy of hepatocytes with eosinophilic granular cytoplasm (Hirata-Koizumi et al., 2008a), which is known to be a characteristic change found in rodents administered with peroxisome proliferators (Cattley and Popp, 2002). Other hepatic changes observed in the previous study, such as altered hepatocellular foci and lipofuscin deposition in hepatocytes, could be also induced by peroxisome proliferators (Hirata-Koizumi et al., 2008a; IARC, 1995). The present result provided additional evidence that peroxisome proliferation was involved in the mechanism of the hepatotoxicity of HDBB.

Other changes in hepatic metabolic activities included marked decreases in the above-mentioned male-specific CYP2C11 activity (testosterone 2α- and 16α-hydroxylation) and in CYP1A1-dependent EROD activity. These changes in metabolic enzyme activities would lead to little metabolism of HDBB *in vivo* despite significant metabolism by these enzymes *in vitro*. These changes in CYP2C11 and 1A1 activities *in vivo* might have resulted from the peroxisome proliferative effects of HDBB because it is reported that well-known peroxisome proliferators, WY-14643, clofibrate, gemfibrozil, and/or di-n-butyl phthalate, downregulated hepatic CYP2C11 and 1A1 expressions (Corton et al., 1998; Shaban et al., 2004). HDBB-induced hepatic changes in aminopyrine *N*-demethylase activity, which is known to be catalyzed by multiple CYP isoforms (Guengerich et al., 1982; Imaoka et al., 1988), and total CYP content are considered to be attributed to changes in the expression of various CYP isozymes, including CYP4A, 2C11, and 1A1.

Peroxisome proliferators are considered to exert biological effects via activation of a nuclear receptor, peroxisome proliferator-activated receptor-alpha (PPARα) (Green, 1995). This is strongly supported by the findings that various biological effects of peroxisome proliferators were not observed in mice that lack a functional PPARα gene (Lee et al., 1995; Ward et al., 1998). Recently, Sparatore et al. (2006) investigated the capacity of various [4-(2H-1,2,3-benzotriazol-2-yl) phenoxy]alkanoic acids to activate PPAR-modulated transcription, using transiently transfected mammalian cells (Hep G2) with a modified variant of the transactivation assay, named the Gal 4-PPAR transactivation assay. They showed that some of these compounds displayed 56–96% maximum activity of the reference drug, Wy-14643, on PPARα. The structural similarity with these compounds suggested the possible agonistic action of HDBB on PPARα. Further, in the above-mentioned Gal 4-PPAR transactivation assay, the introduction of chlorine substituent to the benzotriazole nucleus markedly decreased the activity on PPARα (Sparatore et al., 2006). This is consistent with our previous findings on the toxicity of DBHCB; this structural analog of HDBB with a chlorine substituent exerted much less of an effect on the liver than

HDBB (Ema et al., 2008). In order to further clarify the mechanism of HDBB hepatotoxicity, we are planning a PPARα transactivation assay of HDBB. In this assay, we will also determine the activity of various other benzotriazole UV absorbers, including DBHCB.

Several studies have reported that, in rats, males are more responsive than females to various effects of peroxisome proliferators, including increased liver weight, peroxisome proliferation, and peroxisomal β-oxidation, as well as changes in various enzyme activities (Amacher et al., 1997; Gray and de la Iglesia, 1984; Kawashima et al., 1989a, 1989b; Yamada et al., 1991; Svoboda et al., 1969). Male rats have higher levels of hepatic PPARα mRNA and protein than female rats (Jalouli et al., 2003), which is considered to explain the sex differences in the effects of peroxisome proliferators, at least in part. Previously, we showed that gender-related differences in HDBB toxicity were markedly reduced by castration of male and female rats (Hirata-Koizumi et al., 2008b). Similar phenomena were reported in the hepatic PPARα mRNA expression in rats (Jalouli et al., 2003); therefore, gender-related differences in HDBB toxicity might also come from such a variation in hepatic PPARα expression. In the previous study, we also showed that the gender-related difference in the toxicity of HDBB was not observed in preweaning rats (Hirata-Koizumi et al., 2008c). PPARα expression in the liver is known to be developmentally regulated; it was first detected on embryonic day 13.5 and increased during the suckling period, followed by a decrease postsuckling (Balasubramaniyan et al., 2005; Braissant and Wahli, 1998; Panadero et al., 2000). However, unfortunately, these data are based on a study conducted without separating males and females, and therefore, it has yet to be revealed when and how gender-related differences develop in rats. In order to clarify the role of hepatic PPARα expression in gender-related differences in HDBB toxicity, there is a need to investigate the ontogeny of hepatic PPARα expression with the sexes separated.

In our previous repeated-dose toxicity studies of HDBB, gender-related differences were observed not only in hepatic changes, but also in the inhibition of body-weight gain, anemia, and histopathological changes in the heart, thyroid, spleen, or kidneys (Hirata-Koizumi et al., 2007, 2008a). Since most changes were found at higher doses than the exerted hepatic effects, they could be considered to be secondary effects due to hepatic changes caused by peroxisome proliferative effects of HDBB. On the other hand, in our previous studies, HDBB caused cystic/vacuolar degeneration of hepatocytes, focal necrosis, and bile duct proliferation in the liver, which are not considered to be necessarily associated with the mechanism of peroxisome proliferation (Hirata-Koizumi et al., 2007, 2008a); therefore, the possible involvement of other mechanisms could not be ruled out. In the above-mentioned Gal 4-PPAR transactivation assay, some structural analogs of HDBB exhibited moderate activity on either PPARγ or -δ (Sparatore et al., 2006), suggesting the possible involvement of these PPAR isoforms in the development of HDBB toxicity. Investigating HDBB agonistic activity to various nuclear receptors, including PPARγ and -δ, might provide useful information for understanding the mechanism of HDBB toxicity.

Finally, we selected rats as study animals exclusively in our series of toxicity experiments on HDBB because it is most commonly used in toxicity studies. Although the current result suggested that HDBB exerts toxicity via peroxisome proliferation, it has been reported that rodents are much more sensitive to peroxisome proliferators than primates (Elcombe and Mitchell, 1986; Blaauboer et al., 1990). In order to clarify the toxicity of HDBB, it would be important to conduct studies using primates or PPARα knockout mice. Such studies would clarify the possible involvement of other mechanisms in the HDBB toxicity.

Conclusion

The current results showed no sexual variation in the plasma concentration of HDBB or its metabolites in rats orally given HDBB and in the *in vitro* hepatic metabolism of HDBB. HDBB increased hepatic CYP4A activity more markedly in male rats than in females and decreased hepatic CYP1A1 and 2C11 activity only in males. These results suggest that HDBB exerts toxicity via peroxisome proliferation, and the difference in susceptibility of male and female rats to this effect might lead to marked gender-related differences in HDBB toxicity.

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REVIEW ARTICLE

Disappearance of gender-related difference in the toxicity of benzotriazole ultraviolet absorber in juvenile rats

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ABSTRACT **2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB) is an ultraviolet absorber used in plastic resin products, such as building materials and automobile components. In oral repeated dose toxicity studies using 5- or 6-week-old rats, this chemical induced hepatic histopathological changes, such as hypertrophy accompanied with eosinophilic granular changes and focal necrosis of hepatocytes, and male rats showed nearly 25 times higher susceptibility to the toxic effects than females. Castration at approximately 4 weeks of age markedly reduced the sex-related variation in HDBB toxicity, but some difference, less than five times, remained between male and female castrated rats. Following oral HDBB administration to male and female juvenile rats from postnatal days 4–21, such gender-related difference in toxic susceptibility was not detected; therefore, it is speculated that the determinants of susceptibility to HDBB toxicity are differentiated between sexes after weaning. In young rats given HDBB, there was no gender-related difference in plasma HDBB concentration, and no metabolites were detected in the plasma of either sex. HDBB induced lauric acid 12-hydroxylase activity in the liver and this change was more pronounced in males than in females. These findings indicate that HDBB could show hepatic peroxisome proliferation activity, and the difference in the susceptibility of male and female rats to this effect might lead to marked gender-related differences in toxicity.**

Key Words: benzotriazole ultraviolet absorber, gender-related difference, hepatotoxicity, juvenile rat, peroxisome proliferation

INTRODUCTION

Benzotriazole ultraviolet (UV) absorbers, which have a phenolic group attached to the benzotriazole structure, have excellent absorption capacity with a full spectrum of UV absorption (Toyota Tsusho Corporation and Toyotsu Plachem Corporation 2009); therefore, they are used in a variety of polymers to improve their long-term weather resistance and stability. However, as these compounds are generally poorly biodegradable and some are also highly bioaccumulative (US EPA 2008; METI, MHLW and MOE 2009), there are growing concerns about their effects on human health through the environment.

Regarding toxicity, only limited information has been published in scientific journals; however, in 1999, the Phenolic Benzotriazole Association voluntarily agreed to participate in the US High Production Volume Chemical Challenge Program, and reviewed the existing industrial data on four kinds of benzotriazole UV absorbers (US EPA 2008). In Japan, six compounds in this group were selected as object substances in an existing chemical testing program by the government, and some results of screening toxicity tests have already been released to the public (METI, MHLW and MOE 2009). These data indicate a consistent trend of the toxicity of benzotriazole UV absorbers; low acute toxicity and a lack of reproductive/developmental toxicity and genotoxicity. As for repeated dose toxicity, the main target seems to be the liver, but the severity varies in each compound. In rats, the lowest observed adverse effect levels range from 0.5 mg/kg per day to more than 1000 mg/kg per day.

2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS no. 3846-71-7; HDBB) is a benzotriazole UV absorber that has severe hepatotoxicity. The structural formula of this compound is presented in Figure 1. Recently, we showed a marked gender-related difference in the toxicity of HDBB in young rats (Hirata-Koizumi *et al.* 2007, 2008a). Interestingly, the sex-related difference was markedly reduced by castration and abolished in preweaning rats (Hirata-Koizumi *et al.* 2008b,c). In the present review, we summarize the toxicity of HDBB and its marked gender-related difference in young rats, and then describe the toxicity in castrated and preweaning rats. Finally, we discuss the possible mechanism for the gender-related difference in young rats, based on our most recent work (Hirata-Koizumi *et al.* 2009).

SUMMARY OF TOXICITY INFORMATION

In a single oral dose toxicity study, HDBB induced no mortality in male and female CD(SD)IGS rats (6 weeks of age), even at a dose of 2000 mg/kg (MHLW 2003). No clinical signs of toxicity or changes in body weight were found at this dose. These findings indicate that the acute toxicity of HDBB is very low.

However, in repeated-dose toxicity studies, severe toxicity is observed at relatively low doses. In a 28-day repeated-dose toxicity study, HDBB was administered by gavage to male and female CD(SD)IGS rats from 5 weeks of age, and toxic effects were found mainly in the liver, such as hypertrophy and vacuolar degeneration of hepatocytes, focal necrosis and bile duct proliferation (Hirata-Koizumi *et al.* 2007). Other changes included anemia, degeneration and hypertrophy of the myocardium in the heart, and hypertrophy of the tubular epithelium in the kidneys. These adverse effects were found even at the lowest dose of 0.5 mg/kg in males, but in females, they were detected only at 12.5 mg/kg and above. After a 14-day recovery period, these changes were mostly recovered in females, but not in males. In a 52-week repeated-dose toxicity study, CD(SD)IGS rats were given HDBB by gavage at 0.1, 0.5 or

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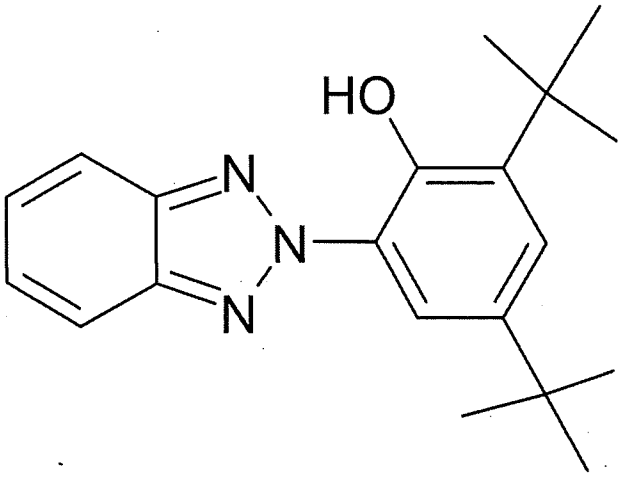

Fig. 1 Structural formula of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl) benzotriazole (HDBB).

2.5 mg/kg per day in males and at 0.5, 2.5 or 12.5 mg/kg per day in females, starting from 6 weeks of age (Hirata-Koizumi *et al.* 2008a). At the completion of administration, the liver was principally affected at 0.5 mg/kg and above in males and at 12.5 mg/kg in females, and the histopathological findings included precancerous changes (i.e. altered hepatocellular foci). Lowered body weight and anemia were also found at 0.5 mg/kg and above only in males. Based on these findings, the no observed adverse effect level (NOAEL) for repeated dose toxicity of HDBB was concluded to be 0.1 mg/kg per day in males and 2.5 mg/kg per day in females.

HDBB did not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* in the absence or presence of an exogenous metabolic activation system (MHLW 2003). As the substance also gave negative responses in the chromosomal aberration test using cultured Chinese hamster lung (CHL/IU) cells, either with or without metabolic activation, it was not considered to be genotoxic.

Although no data are available on the reproductive/developmental toxicity of HDBB, reproductive and developmental toxicity studies of its structural analogues are reported. In a combined repeated dose and reproductive/developmental toxicity screening test using rats (combined tests), 2-(2′-hydroxy-3′-*sec*-butyl-5′-*tert*-butylphenyl)benzotriazole (HBBB) exerted no effects on reproductive/developmental parameters, even at the highest dose of 12.5 mg/kg, while hepatic changes were observed in parental animals at this dose (METI 2007a). A similar result was reported in the combined test of 2-(3′,5′-di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB); hepatic effects were found at 25 mg/kg and higher, but reproductive/developmental parameters were not affected even at the highest dose of 250 mg/kg (Ema *et al.* 2008). The latter compound also did not show any adverse effects on maternal rats and embryonic/fetal development in the prenatal developmental toxicity study (Ema *et al.* 2006).

GENDER-RELATED DIFFERENCE IN TOXICITY

As mentioned above, male rats were much more susceptible to the toxicity of HDBB than females. Comparing the toxic susceptibility between sexes, the NOAEL must be a useful parameter, although it is very dependent on dose-selection. Based on the NOAEL, the difference could be considered to be approximately 25 times.

For other benzotriazole UV absorbers, while the above-mentioned structural analogues, HBBB and DBHCB, also caused male predominant hepatic effects, no clear gender-related difference was found in the hepatic effects of 2-(2′-hydroxy-5′-methylphenyl)benzotriazole in the combined tests (METI 2007a,b; Ema *et al.* 2008). Review documents of the US High Production Volume Chemical Challenge Program suggest that the toxic susceptibility of males to 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole is higher than females, but conversely, a higher susceptibility of females to the toxicity of 2-(2H-benzotriazole-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol was reported (US EPA 2008). Although the available data are limited, they indicate that the branched alkyl group attached to both meta positions of the phenolic group to the benzotriazole ring might play an important part in male predominance in the toxicity of benzotriazole UV absorbers.

Gonadal influence on gender-related differences

Gender-related differences in the toxic susceptibility of rats have been documented for many other industrial chemicals (Muraoka & Itoh 1980), environmental pollutants (Knuckles *et al.* 2004), insecticides (Carlson & DuBois 1970; Agarwal *et al.* 1982) and pharmaceuticals (McGovren *et al.* 1981; Coleman *et al.* 1990; Wang *et al.* 2001; Stern *et al.* 2007). As it is known that sex hormones play important roles in such sex-related differences in toxic responses, we examined the effects of castration on gender-related differences in the toxicity of HDBB.

Male and female castrated CD(SD) rats were given HDBB by gavage at 0.5, 2.5 or 12.5 mg/kg per day for 28 days (Hirata-Koizumi *et al.* 2008b). The rats were castrated at approximately 4 weeks of age under ether anesthesia, and were subjected to HDBB treatment at 6 weeks of age. At the completion of administration, hepatic histopathological changes, including nucleolar enlargement, increased mitosis, hypertrophy and/or focal necrosis of hepatocytes, were observed with blood biochemical changes, such as increased levels of albumin, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase, at 0.5 mg/kg and above in males, and at 2.5 mg/kg and above in females. Hepatic changes at 0.5 mg/kg in males were slightly milder than at 2.5 mg/kg in females, showing that the difference in susceptibility of male and female castrated rats was less than five times. As shown in Figure 2, comparison of the rate of changes in relative liver weight provided a clear description of nearly 25 times difference in the susceptibility of male and female intact rats to HDBB hepatotoxicity and marked reduction by castration.

Lack of gender-related differences in juvenile rats

Although the castration of male and female rats markedly reduced the sex-related variation in HDBB toxicity, some difference remained between male and female castrated rats. It is speculated that the determinants of susceptibility to HDBB toxicity are already differentiated between sexes by 4 weeks of age, when the castration was performed; therefore, we determined the sex-related difference in the susceptibility to HDBB toxicity in juvenile rats, which were considered to be under more limited influence of sex hormones.

Male and female CD(SD) rats were given HDBB by gavage at 0.1, 0.5, 2.5 or 12.5 mg/kg per day on postnatal days 4–21 (Hirata-Koizumi *et al.* 2008c). Blood biochemical changes, including increases in the levels of albumin, AST and ALP, were found in both sexes at 12.5 mg/kg. Histopathological examination of the liver revealed hepatocellular changes, such as nucleolar enlargement,

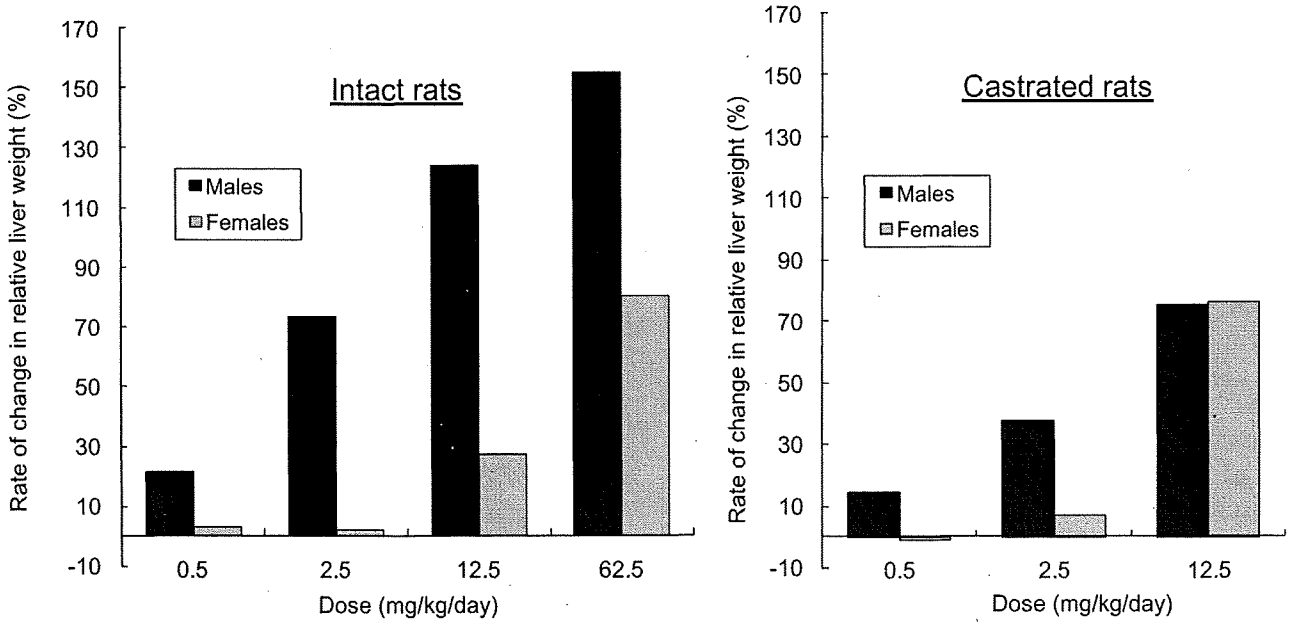

Fig. 2 Comparison of change in the relative liver weight of male and female intact and castrated rats administered 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB). Male and female intact or castrated rats were administered HDBB by gavage for 28 days, starting at 5 or 6 weeks of age (Hirata-Koizumi *et al.* 2007, 2008b). Castration was performed at approximately 4 weeks of age under ether anesthesia. Reproduced from Hirata-Koizumi *et al.* (2008b) with permission.

anisokaryosis, increased mitosis and/or hypertrophy, at 2.5 mg/kg and above, and the incidence and degree was similar in both sexes. These results indicate no gender-related differences in the susceptibility to toxic effects of HDBB in preweaning rats. The rate of changes in the relative liver weight clearly showed a lack of gender-related differences in HDBB hepatotoxicity in preweaning rats (Fig. 3).

Possible mechanism of gender-related differences in young rats

Gender-related differences in HDBB toxicity were found not only for hepatic effects, but also for the body weight reduction, anemia and histopathological changes in the heart and kidneys of young rats; therefore, we first speculated that sex-related variation in HDBB toxicity might be explained by the difference in blood concentration of their causative substances (HDBB or these metabolites). In fact, it is well-known that some metabolic enzymes and membrane transporters are expressed in a sex-dependent manner in rats (Buist *et al.* 2002, 2003; Morris *et al.* 2003; Waxman & Chang 2005). It is also reported that sex-related variation in such toxicokinetic determinants is under the control of sex hormones and is not apparent until puberty; however, following 28-day oral administration of HDBB to 5-week-old CD(SD) rats, no sex-related variation was found in plasma HDBB levels, as shown in Figure 4 (Hirata-Koizumi *et al.* 2009). No metabolites of HDBB were detected in the plasma of either sex. Although an *in vitro* study using hepatic S9 fractions and microsomes prepared from male and female SD rats showed evidence of some HDBB metabolism, no sex-related differences were found in the residual HDBB ratio after 60-min incubation with an NADPH generation system (Fig. 5). These findings suggest that toxicodynamic factors would contribute to gender-related differences in the toxicity of HDBB.

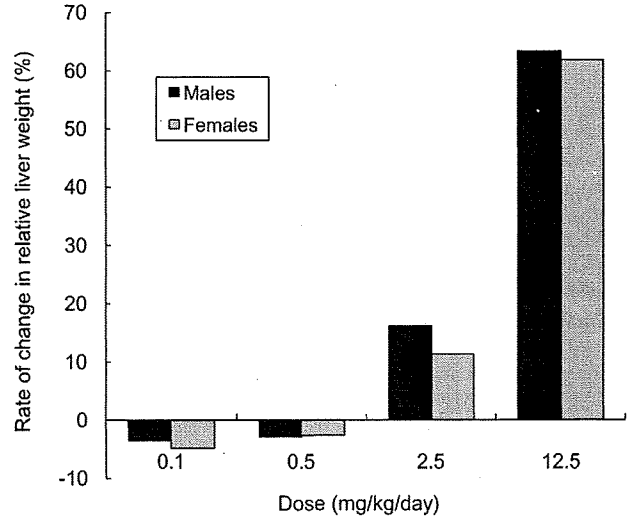

Fig. 3 Comparison of change in the relative liver weight of male and female preweaning rats administered with 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB). Male and female rats were administered HDBB by gavage from postnatal days 4 to 21 (Hirata-Koizumi *et al.* 2008c).

In repeated dose toxicity studies using young and preweaning rats, the centrilobular hypertrophy of hepatocytes caused by HDBB was accompanied with eosinophilic granular changes (Hirata-Koizumi *et al.* 2008a,c). As this is known to be a characteristic change in rodents administered peroxisome proliferators, such as

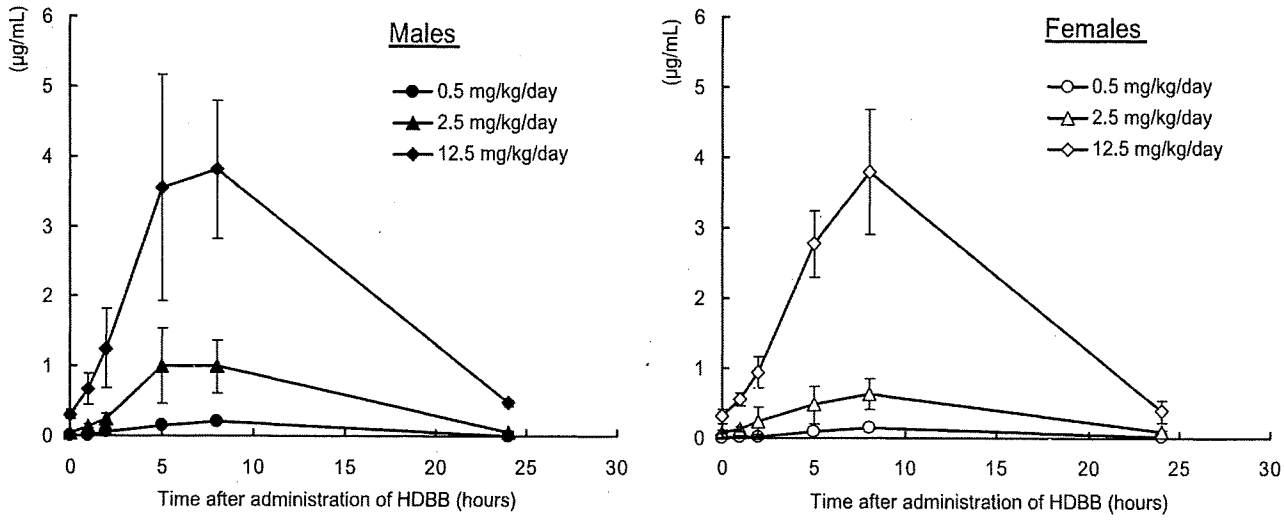

Fig. 4 Plasma 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB) concentrations against time after HDBB administration to male and female rats. HDBB was given by gavage to male and female rats for 28 days, starting at 5 weeks of age, and blood samples were collected from the jugular vein before and 1, 2, 5, 8, and 24 hours after the 28th dose (Hirata-Koizumi *et al.* 2009). Data are expressed as the mean ± SD. Reproduced from Hirata-Koizumi *et al.* (2009) with permission.

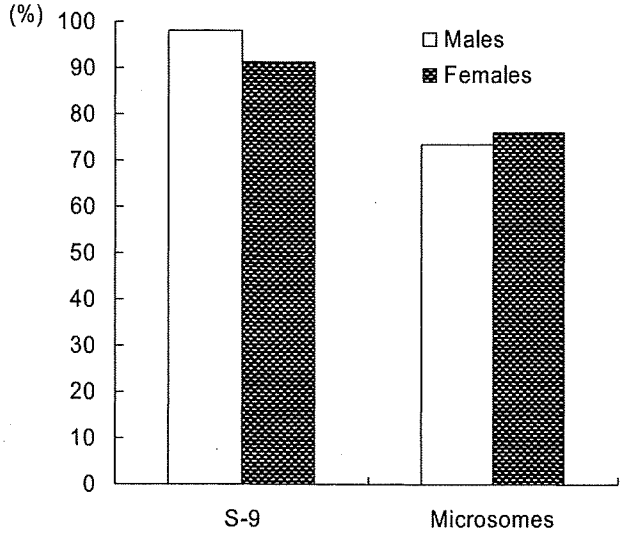

Fig. 5 Residual ratios of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl) benzotriazole (HDBB) after incubation with male and female rat liver S9 or microsomes in the presence of NADPH-generating system. HDBB was dissolved in acetonitrile and incubated with hepatic S9 fractions or microsomes prepared from male and female rats in the presence of NADPH-generating system for 60 min (Hirata-Koizumi *et al.* 2009). The residual HDBB ratio was calculated by dividing the peak area of HDBB after incubation with that of the control, in which the incubation system was inactivated by the addition of acetonitrile prior to incubation. Reproduced from Hirata-Koizumi *et al.* (2009) with permission.

fibrate hypolipidemic drugs and phthalate plasticizers (Cattley & Popp 2002), peroxisome proliferation might be involved in the mechanism of HDBB hepatotoxicity. Supporting this speculation, our recent study revealed that HDBB induced hepatic cytochrome P450 (CYP) 4A-specific activity, lauric acid 12-hydroxylation (Hirata-Koizumi *et al.* 2009), which is highly inducible by peroxisome proliferators (Bacher & Gibson 1988; Sundseth & Waxman 1992; Okita *et al.* 1993; Espandiari *et al.* 1995). The dose-responsiveness was consistent with that of liver weight change and macroscopic findings.

Several studies have reported that in rats, males are more responsive than females to various effects of peroxisome proliferators, including increased liver weight, peroxisome proliferation, and peroxisomal β-oxidation, as well as changes in various enzyme activities (Svoboda *et al.* 1969; Gray & de la Iglesia 1984; Kawashima *et al.* 1989a,b; Yamada *et al.* 1991; Amacher *et al.* 1997). Peroxisome proliferators are considered to exert biological effects through activation of a nuclear receptor, peroxisome proliferator-activated receptor (PPAR) α (Green 1995; Lee *et al.* 1995; Ward *et al.* 1998). Male rats have higher levels of hepatic PPARα mRNA and protein than females (Jalouli *et al.* 2003), which is considered to explain the sex-related differences in the effects of peroxisome proliferators, at least in part. Jalouli *et al.* (2003) reported that castration of male and female rats markedly reduced the gender difference in hepatic PPARα mRNA expression. We showed similar phenomena in HDBB toxicity (Hirata-Koizumi *et al.* 2008b), suggesting that the gender-related difference in HDBB toxicity might also come from such variation in hepatic PPARα expression. In our future studies, we plan to investigate HDBB agonistic activity with various nuclear receptors, including PPARα.

Our findings that male and female preweaning rats showed similar susceptibility to HDBB toxicity (Hirata-Koizumi *et al.* 2008c) suggests a lack of sex-related variation in hepatic PPARα expression during the early postnatal period. PPARα expression in the liver is known to be developmentally regulated (Braissant & Wahli 1998; Panadero *et al.* 2000; Balasubramaniyan *et al.* 2005); however, unfortunately, the available developmental data on PPARα expression are based on a study conducted that did not separate males and females, and therefore, it has yet to be revealed when and how gender-related differences develop in rats. To clarify the role of hepatic PPARα expression in sex-related differences in

HDBB toxicity, we are currently investigating the ontogeny of hepatic PPARα expression in each sex.

CONCLUSION

HDBB exerts male predominant hepatotoxicity in young rats. The gender-related difference was markedly reduced by castration, and completely abolished in preweaning rats. In young rats, HDBB induced lauric acid 12-hydroxylase activity in the liver and this change was more pronounced in males than females. These findings suggest that HDBB would have hepatic peroxisome proliferation activity, and the difference in the susceptibility of male and female rats to this effect might lead to marked gender-related differences in toxicity.

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Gene expression profiling in rat liver treated with compounds inducing elevation of bilirubin

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We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for the diagnosis of elevated total bilirubin (TBIL) and direct bilirubin (DBIL), we extracted 59 probe sets of rat hepatic genes from the data for seven typical drugs, gemfibrozil, phalloidin, colchicine, bendazac, rifampicin, cyclosporine A, and chlorpromazine, which induced this phenotype from 3 to 28 days of repeated administration in the present study. Principal component analysis (PCA) using these probes clearly separated dose- and time-dependent clusters in the treated groups from their controls. Eighteen more drugs in the database, reported to elevate TBIL and DBIL, were estimated by PCA using these probe sets. Of these, 12 drugs, that is methapyrilene, thioacetamide, ticlopidine, ethinyl estradiol, alpha-naphthylisothiocyanate, indomethacin, methyltestosterone, penicillamine, allyl alcohol, aspirin, iproniazid, and isoniazid were also separated from the control clusters, as were the seven typical drugs causing elevation of TBIL and DBIL. The principal component 1 (PC1) value showed high correlation with TBIL and DBIL. In the cases of colchicine, bendazac, chlorpromazine, gemfibrozil, and phalloidin, the possible elevation of TBIL and DBIL could be predicted by expression of these genes 24 h after single administration. We conclude that these identified 59 probe sets could be useful to diagnose the cause of elevation of TBIL and DBIL, and that toxicogenomics would be a promising approach for prediction of this type of toxicity.

Key words: bilirubin; liver; principal component analysis; rat; toxicogenomics

Introduction

The Toxicogenomics Project is a 5-year collaborative project by the National Institute of Biomedical Innovation, the National Institute of Health Science, and 15 pharmaceutical companies in Japan that started in 2002.[1] Its aim was to construct a large-scale toxicology database of transcriptomes to predict toxicity of new chemical entities in the early stage of drug development. Over 150 chemicals, mainly medicinal compounds, were selected and their gene expression in the liver was comprehensively measured by using Affymetrix GeneChip®, Santa Clara, California, USA. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system, and the prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by the Toxicogenomics Project, Japan). Recently, we identified a biomarker for the diagnosis of hepatic phospholipidosis, nongenotoxic hepatocarcinogenicity, glutathione depletion-responsive, and serum triglyceride-decreasing by using our database.[2–5]

Bilirubin is mainly a breakdown product of heme (part of the hemoglobin in the red blood cells). The heme is then turned into unconjugated bilirubin (indirect bilirubin) in the macrophages of the spleen. It is then bound to albumin and sent to the liver. In the liver it is conjugated with glucuronic acid (direct bilirubin, DBIL), making it soluble in water. Total bilirubin (TBIL) and DBIL levels can be measured in the blood, but indirect bilirubin is calculated from the

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total and direct bilirubin. Bilirubin levels reflect the balance between production and excretion.

Increased TBIL causes jaundice of the prehepatic, hepatic, and posthepatic types. The "prehepatic type" is derived from increased bilirubin production. This can be due to a number of causes, including hemolytic anemia and internal hemorrhage. If the DBIL is normal, then the problem is an excess of unconjugated bilirubin, and the location of the problem is upstream of bilirubin excretion. The "hepatic type" indicates problems in the liver, which are reflected as deficiencies in bilirubin metabolism (e.g., reduced hepatocyte uptake of bilirubin, impaired conjugation of bilirubin, and reduced hepatocyte secretion of bilirubin). Some examples would be drug-induced hepatic disorders (especially antipsychotic drugs, some sex hormones, and a wide range of other drugs), cirrhosis, and viral hepatitis. The "posthepatic type" is derived from obstruction of the bile ducts, which are reflected as deficiencies in bilirubin excretion. An obstruction such as gallstones or cancer can be located either within the liver or outside the liver.

In the present study, we selected elevation of TBIL and DBIL as a target phenotype and tried to identify candidate biomarkers that identify chemicals with the potential to cause this phenotype by using our database.

Materials and methods

Compounds

The compounds used for the data analysis are listed in Table 1, in which the chemical name, abbreviation, dosage, administration route, and vehicle used in the study are summarized.

Animal treatment

The experiments were carried out as previously described in the literature.[6] Male Crl:CD (SD) rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5 weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of five animals, using a computerized stratified random grouping method based on the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 h (7:00 a.m–7:00 p.m) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21–25 °C with a relative humidity

Table 1 List of compounds used in the present study

Compound name	*Abbreviation*	*Dose (dose level, mg/kg)*			*Administration route*	*Vehicle*	*Bilirubin*
		Low	*Middle*	*High*			
Gemfibrozil	GFZ	30	100	300	po	CO	A
Phalloidin	PHA	NA	NA	0.5	ip	SA	B
Colchicine	COL	1.5/0.5[a]	5/1.5[a]	15/5[a]	po	MC	C
Bendazac	BDZ	100/30[a]	300/100[a]	1000/300[a]	po	MC	C
Rifampicin	RIF	20	60	200	po	MC	C
Cyclosporine A	CSA	30/10[a]	100/30[a]	300/100[a]	po	CO	B
Chlorpromazine	CPZ	4.5	15	45 (150[b])	po	MC	C
Methapyrilene	MP	10	30	100	po	MC	B
Thioacetamide	TAA	4.5	15	45	po	MC	C
Ticlopidine	TCP	30	100	300	po	MC	D
Ethinyl estradiol	EE	1	3	10	po	CO	A
Alpha-naphthylisothiocyanate	ANIT	1.5	5	15	po	CO	D
Indomethacin	IM	0.5	1.6	5	po	MC	C
Methyltestosterone	MTS	30	100	300	po	MC	A
Penicillamine	PEN	100	300	1000	po	MC	A
Allyl alcohol	AA	3	10	30	po	CO	D
Aspirin	ASA	45	150	450	po	MC	D
Iproniazid	IPA	6	20	60	po	MC	B
Isoniazid	INAH	50	100	200	po	MC	C
Glibenclamide	GBC	100	300	1000	po	CO	D
Cyclophosphamide	CPA	1.5	5	15	po	MC	B
Nitrofurantoin	NFT	10	30	100	po	MC	D
Valproate	VPA	45	150	450	po	MC	D
Methotrexate	MTX	0.1	0.3	1	po	MC	C
Tetracycline	TC	100	300	1000	po	MC	C

po, peroral; CO, corn oil; A, bilirubin oxidase method; NA, not applicable; ip, intraperitoneal; SA, saline; B, vanadate oxidation method; MC, 0.5 wt/vol% methylcellulose; C, azobilirubin method; D, alkali azobilirubin method.
The animals were treated for 3, 7, 14, or 28 days, except PHA, which were treated for 3 or 7 days.
[a]As single dose/repeated dose.
[b]Extra-high dose (only single dose of CPZ).

of 40–70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Tokyo, Japan). Rats in each group were orally dosed various drugs suspended or dissolved either in 0.5% methylcellulose solution or corn oil according to their dispersibility, except phalloidin, which was dissolved in saline and administered intraperitoneally. For single-dose experiments the rats were sacrificed at 3, 6, 9, and 24 h after dosing. For repeated dose experiments, the animals were treated for 3, 7, 14, or 28 days, except phalloidin, which were treated for 3 or 7 days, and they were sacrificed 24 h after the last dosing. Food was not withdrawn before sacrifice and the time of autopsy was done between 9:00 a.m–11:00 a.m. for the repeated dose group and 24 h after the single dose group. Blood samples for routine biochemical analysis were collected into heparinized tubes under ether anesthesia from the abdominal aorta after which the animals were sacrificed. As the animal experiments were performed in four different contract research organizations where different automated blood chemistry analyzers were used, TBIL and DBIL were quantified by four different methods, that is bilirubin oxidase method (TBA-120FR, Toshiba Medical Systems Corporation, Tokyo, Japan), vanadate oxidation method (Hitachi H7170, Hitachi High-Technologies Corporation, Tokyo, Japan), azobilirubin method (COBAS MIRA plus, Roche Diagnostics, Indianapolis, Indiana, USA), and alkali azobilirubin method (Hitachi H7070, Hitachi High-Technologies Corporation, Tokyo, Japan). Because of this difference, the absolute values could not be compared, and the judgment of hyperbilirubinemia was done based on the difference from the control value. These methods are shown in Table 1 as A, B, C, and D in this order. The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

Microarray analysis

After collecting the blood the animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNAlater® (Ambion, Austin, Texas, USA) overnight at 4 °C, and frozen at −80 °C until use. Liver samples were homogenized with the buffer RNeasy Lysis Buffer (RLT) supplied in the RNeasy Mini Kit (Qiagen, Valencia, California, USA) and total RNA was isolated according to the manufacturer's instructions. Microarray analysis was conducted on three out of five samples, selected based on their body weight excluding the highest and the lowest, for each group by using the GeneChip® Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, California, USA), containing 31,042 probe sets. The procedure was conducted basically according to the manufacturer's instructions using the Superscript Choice System (Invitrogen, Carlsbad, California, USA) and T7-(dT)24-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, New York, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA were hybridized to a Rat Genome 230 2.0 Array for 18 h at 45 °C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using a Fluidics Station 400 (Affymetrix) and then scanned by a Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0 Affymetrix, and intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization).

Microarray data analysis

To extract probe sets related to the elevation of TBIL and DBIL, we first used the gene expression data from liver from rats treated by repeated administration for 3, 7, 14, and 28 days, with gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA) and chlorpromazine (CPZ), which are known to cause elevation of TBIL and DBIL, and that was confirmed in the present study.

After removing the probe sets with Affymetrix absent call in all the 48 sample sets ($N = 3$ for 4 time points and 4 dose levels for one drug), except PHA, which had 12 sample sets ($N = 3$ for 2 time points by repeated administration for 3 and 7 days, and 2 dose levels), genes differentially expressed by the treatment were extracted by Welch's ANOVA/*t*-test ($P < 0.05$) for the dose level at one time point. This procedure was continued for all time points, and the genes showing significant change at any point were combined as elevation of TBIL and DBIL responsive genes. In the next step, commonly mobilized genes among these seven chemicals were selected.

The individual expression value (global mean) was converted to ratio by the mean of corresponding control value, and all the values with different doses and time points of the test compounds were gathered, and they were normalized by converting them to *z*-scores for each gene (pergene normalization). Principal component analysis (PCA) was

performed using Spotfire Decision Site (Spotfire, Somerville, Massachusetts, USA).

Pathway and gene ontology (GO) analysis

The identified probe sets were subjected to GO analysis by DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://david.abcc.ncifcrf.gov/) using Fisher's exact test.[7]

Results

Blood biochemical examination

The results of TBIL and DBIL examination of seven typical compounds (GFZ, PHA, COL, BDZ, RIF, CSA, and CPZ) known to induce their elevation in rat plasma are shown in Figure 1. In most cases, both TBIL and DBIL were elevated and this tended to progress with dose and time. In the cases of COL and BDZ, DBIL was increased with a peak at 4th day and TBIL showed the same change as COL. TBIL was increased for all sampling points at high dose of BDZ. With CPZ, the DBIL was elevated at the 15th day or later, but changes in the TBIL were obscure at any sampling point.

Microarray data analysis

Differentially expressed genes with statistically significant differences were extracted from each of the seven representative drugs elevating TBIL and DBIL as described in the Materials and methods section. The numbers of extracted probe sets were 3690 for

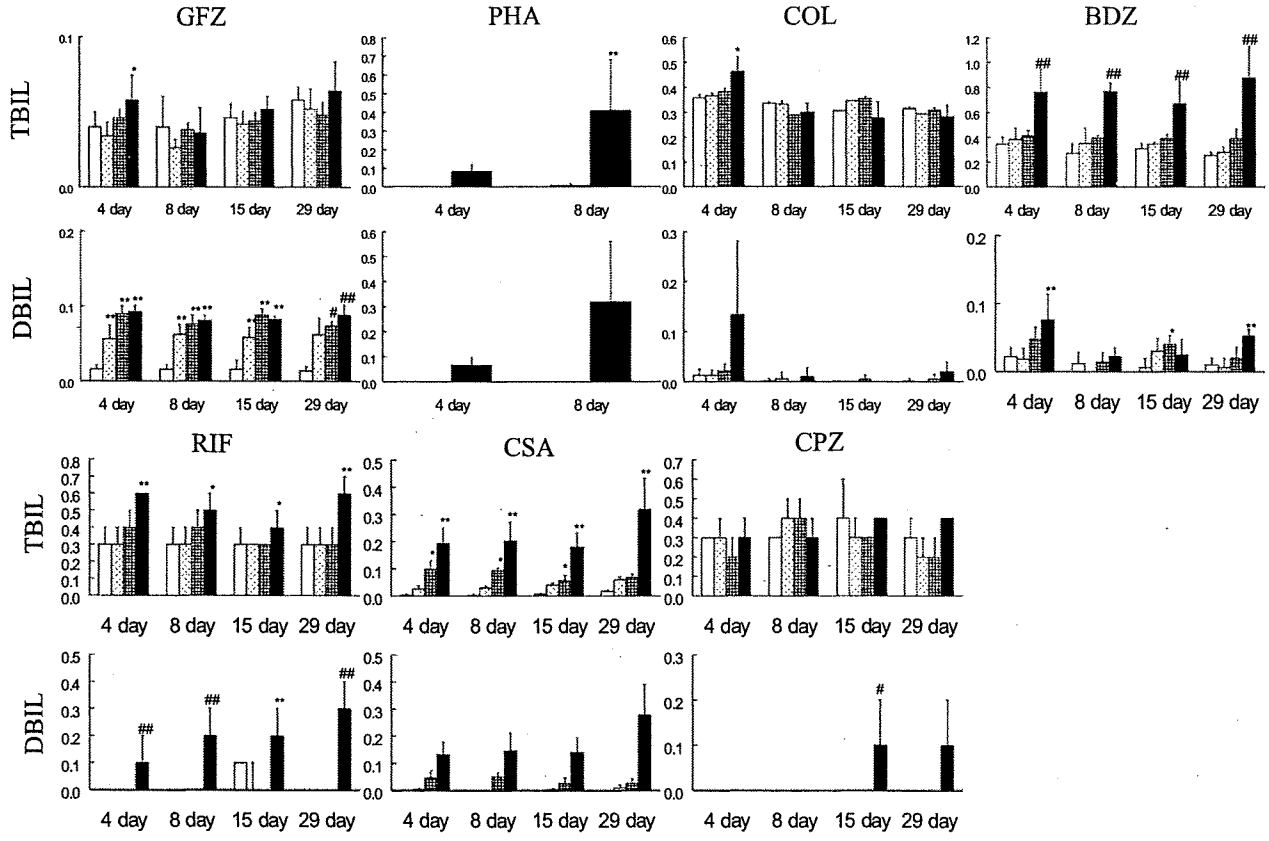

Figure 1 Plasma total bilirubin (TBIL) and direct bilirubin (DBIL) concentrations for rats treated with gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), or chlorpromazine (CPZ). Six-week-old male Sprague-Dawley rats were treated with each compound for 3, 7, 14, or 28 days, and they were sacrificed 24 h after the last dose. Blood samples were collected at sacrifice. Plasma TBIL and DBIL concentrations were estimated as described in the Materials and methods section. As the animal experiments were performed in four different contract research organizations where different automated blood chemistry analyzers were used, TBIL and DBIL were quantified by four different methods (see Table 1). Because of this difference, the absolute values could not be compared and the judgment of hyperbilirubinemia was done based on the difference from the control value. Open (control), dotted (low dose), checked (middle dose) and filled (high dose) columns represent plasma TBIL and DBIL concentrations (mg/dL). Values are expressed as mean ± SD of five rats each for each time and compound. Significant difference from the control rats: ($*P < 0.05$, $**P < 0.01$: Dunnett test, $\#P < 0.05$, $\#\#P < 0.01$: Dunnett type mean rank test).

BDZ, 5398 for COL, 3473 for CPZ, 4258 for CSA, 4656 for GFZ, 4917 for PHA, and 2403 for RIF. We then selected the probe sets that were commonly changed in all the compounds and 59 probe sets were obtained. A list of these probe sets is in Table 2, where each probe is categorized by its biological function. Figure 2 shows the expression changes of these 59 probe sets at high dose of repeated administration, as a heat map of the average of log 2 ratios. It appears that the trend of the change is to decrease, and the direction of change of the probe sets is not necessarily common with these drugs. Based on gene ontology, the contents of the genes related to cellular metabolism, biosynthesis, lipid metabolism, cellular biosynthesis, cellular physiological process, response to stress, physiological process, and serine family amino acid metabolism were significantly increased (Table 3).

Principal component analysis

Using the 59 probe sets extracted as above, PCA was performed on the seven drugs elevating TBIL and DBIL. As shown in Figure 3, the treated samples were dose-dependently separated to form clusters from the controls, mainly toward the direction of PC1 (contribution rate: 30.4%). Of the genes contributing to PC1, those with high eigenvalue are listed in Table 4. To examine the time-dependency, all the samples were aligned on a one dimensional graph of PC1 (Figure 4). It appeared that the PC1 value generally increased with time and with the dose for these drugs. In the cases of COL and BDZ, the PC1 value increased with the peak on the 4th day. In case of CPZ, time- and dose-dependency were obscure although the group treated clearly formed a cluster separated from the control cluster.

Verification of the probe sets using 18 test compounds

In a survey of the literature, in addition to the seven drugs above, we identified 18 more drugs in our database that had been reported to elevate TBIL and DBIL. The data of TBIL and DBIL of the 18 compounds are shown in Figure 5. We performed PCA using the 59 probe sets on the seven typical and 18 additional drugs (25 total), and depicted them in Figure 6 as a one-dimensional graph with PC1 (contribution rate: 35.9 %). It was revealed that 12 out of 18 drugs, that is methapyrilene, thioacetamide, ticlopidine, ethinyl estradiol, alpha-naphthylisothiocyanate, indomethacin, methyltestosterone, penicillamine, allyl alcohol, aspirin, iproniazid, and isoniazid were separated from control clusters in the same way as the seven typical drugs elevating TBIL and DBIL. Of these 12 drugs, MP, TAA, EE, ANIT, PEN, and IPA showed significant elevation of TBIL and DBIL for at least one time point during repeated administration, whereas the remaining six drugs did not show any toxicologically meaningful elevation. Glibenclamide (GBC) and cyclophosphamide did not change their position very much on the PCA, whereas their extent was roughly equivalent to that of CPZ, a positive control. The remaining four drugs, that is nitrofurantoin, valproate, methotrexate, and tetracycline stayed in the same position as their controls. Reviewing the data of TBIL and DBIL of the latter six drugs with low PC1 values, it was revealed that a statistically significant elevation was absent except for CPA, which showed an increase after 28 days of repeated administration, but its absolute value was quite low (Figure 5). In general, most drugs that had high PC1 values showed high serum concentration of TBIL and DBIL. As shown in Figure 7, the PC1 value had a high correlation with TBIL and DBIL levels.

Possibility of the distinction by samples taken at 24 h after single dose

The above results clearly suggested that the list of extracted 59 probe sets was a useful diagnostic marker for the elevation of TBIL and DBIL in rat liver. The next question is whether the list works as a prognostic marker for drugs elevating TBIL and DBIL. To examine this possibility, we analyzed the data within 24 h of a single dose of the first seven drugs. Figure 8 shows TBIL and DBIL at 3, 6, 9, and 24 h after a single dose. Although there was some significant increase in these measures, most of them were considered to be toxicologically insignificant based on their absolute values. It was thus concluded that no severe elevation of TBIL and DBIL occurred within 24 h for a single dose. Using the gene expression data at 24 h after dosing, PCA was performed using the 59 probe sets. As shown in Figure 9, all the drugs except RIF and CSA were clearly separated from the control samples in the direction of PC1(contribution rate: 33.9%). Among these, CPZ, which showed low PC1 values in repeated dosing (Figures 4 and 6), was not distant from its control by single dosing. Interestingly, however, an excellent separation was attained when extra-high dose of CPZ was added (Figure 9).

Discussion

Hepatotoxic adverse effects, often indicated by cholestasis, are a main concern in drug development and severe hepatotoxicity may cause a drug to be

Table 2 List of 59 probe sets changed in seven compounds elevating of TBIL and DBIL

Probe ID	*Accession No.*	*Gene title*	*Gene symbol*
Lipid metabolic process			
1368520_at	NM_012737	Apolipoprotein A-IV	Apoa4
1370150_a_at	NM_012703	Thyroid hormone responsive protein	Thrsp
1371615_at	BI279069	Diacylglycerol O-acyltransferase homolog 2 (mouse)	Dgat2
1374440_at	BE098506	Dehydrogenase/reductase (SDR family) member 8	Dhrs8
1387139_at	NM_032082	Hydroxyacid oxidase 2 (long chain)	Hao2
1387508_at	NM_017300	Bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
1390549_at	AA859796	Adiponectin receptor 2	Adipor2
1394112_at	AA945123	Hydroxyacid oxidase 1	Hao1
Transporter			
1368621_at	NM_022960	Aquaporin 9	Aqp9
1369074_at	NM_130748	Solute carrier family 38, member 4	Slc38a4
1379592_at	AI045151	Similar to citrin (predicted)	RGD1565889_predicted
1386960_at	NM_031589	Solute carrier family 37 (glucose-6-phosphate transporter), member 4	Slc37a4
1390591_at	AI169163	Solute carrier family 17 (sodium phosphate), member 3	Slc17a3
1393216_at	BI282044	Solute carrier family 33 (acetyl-CoA transporter), member 1	Slc33a1
1398249_at	NM_053965	Solute carrier family 25 (mitochondrial carnitine/ acylcarnitine translocase), member 20	Slc25a20
Ubiquitin-Proteasome			
1383073_at	BG666028	Ubiquitin specific protease 14	Usp14
1398831_at	NM_031629	Proteasome (prosome, macropain) subunit, beta type 4	Psmb4
Mitochondrial function			
1388931_at	AA799440	Mitochondrial ribosomal protein L13	Mrpl13
1367941_at	NM_031326	Transcription factor A, mitochondrial	Tfam
1370918_a_at	BI275939	ATP synthase, H^+ transporting, mitochondrial F1 complex, γpolypeptide 1	Atp5c1
1372080_at	BI287936	Inner membrane protein, mitochondrial	Immt
1398326_at	BI282332	Similar to Nur77 downstream protein 2	MGC105647
Cell proliferation/Cell cycle			
1367764_at	NM_012923	Cyclin G1	Ccng1
1367927_at	BI282863	Prohibitin	Phb
1369738_s_at	NM_017334	cAMP responsive element modulator	Crem
1387714_at	AB031423	cAMP responsive element modulator	Crem
1372437_at	BM390921	S-phase kinase-associated protein 1A	Skp1a
1367512_at	AA998435	Chromatin modifying protein 5	Chmp5
1374591_at	AI409042	similar to protein tyrosine phosphatase, receptor type, D (predicted)	RGD1561090_predicted
1388469_at	AA945615	Insulin-like growth factor I mRNA, 3' end of mRNA	—
Translation			
1367610_at	NM_031103	Ribosomal protein L19	Rpl19
1388244_s_at	BG153272	Ribosomal protein SA	Rpsa
1371973_at	AI237620	Eukaryotic translation initiation factor 3, subunit 6	Eif3s6
Metabolism			
1371076_at	AI454613	Cytochrome P450, family 2, subfamily b, polypeptide 15 /// Cytochrome P450, family 2, subfamily b, polypeptide 2	Cyp2b15 /// Cyp2b2
1368905_at	NM_133586	Carboxylesterase 2 (intestine, liver)	Ces2
1369558_at	NM_022614	Inhibin beta C	Inhbc
1387022_at	NM_022407	Aldehyde dehydrogenase family 1, member A1	Aldh1a1
1387034_at	NM_012619	Phenylalanine hydroxylase	Pah
1388788_at	BG664131	Glutaryl-Coenzyme A dehydrogenase (predicted)	Gcdh_predicted
1398286_at	M64755	Cysteine sulfinic acid decarboxylase	Csad
Response to oxidative stress			
1367896_at	AB030829	Carbonic anhydrase 3	Ca3
1370064_at	AB004454	Presenilin 2	Psen2
Inflammatory response			
1367804_at	NM_017170	Serum amyloid P-component	Apcs
Blood coagulation			
1388330_at	BM384958	Vitamin K epoxide reductase complex, subunit 1	Vkorc1
1374765_at	BI288055	Transcribed locus, moderately similar to XP_001090810.1 fibrinogen gamma chain isoform 9 [Macaca mulatta]	—
Other			
1372479_at	AI175666	Transcribed locus	—
1373313_at	BM391570	Transcribed locus	—
1374943_at	AI170809	Transcribed locus	—
1375845_at	BI290029	Similar to Aig1 protein (predicted)	RGD1562920_predicted
1377048_at	H31813	Similar to cDNA sequence BC021917	RGD1311026

(continued)

Table 2 *(continued)*

Probe ID	*Accession No.*	*Gene title*	*Gene symbol*
1377686_at	AA859337	Transcribed locus	—
1381574_at	BF403907	Similar to putative protein, with at least 6 transmembrane domains, of ancient origin (58.5 kD) (3N884) (predicted)	RGD1312038_predicted
1383732_at	AA819810	Similar to hypothetical protein MGC37914 (predicted)	RGD1307603_predicted
1387856_at	BI274457	Calponin 3, acidic	Cnn3
1388119_at	BM392140	Similar to heterogeneous nuclear ribonucleoprotein A3 /// similar to heterogeneous nuclear ribonucleoprotein A3 (predicted) /// similar to regulator of G-protein signalling like 1	LOC364506 /// LOC684137 /// RGD1566284_predicted
1390326_at	BF564217	Angiogenin, ribonuclease A family, member 1	Ang1
1392172_at	AI169984	Chemokine (C-C motif) ligand 9	Ccl9
1393123_at	BM392153	Complement component 8, gamma polypeptide (predicted)	C8g_predicted
1398409_at	AA850428	Transcribed locus	—

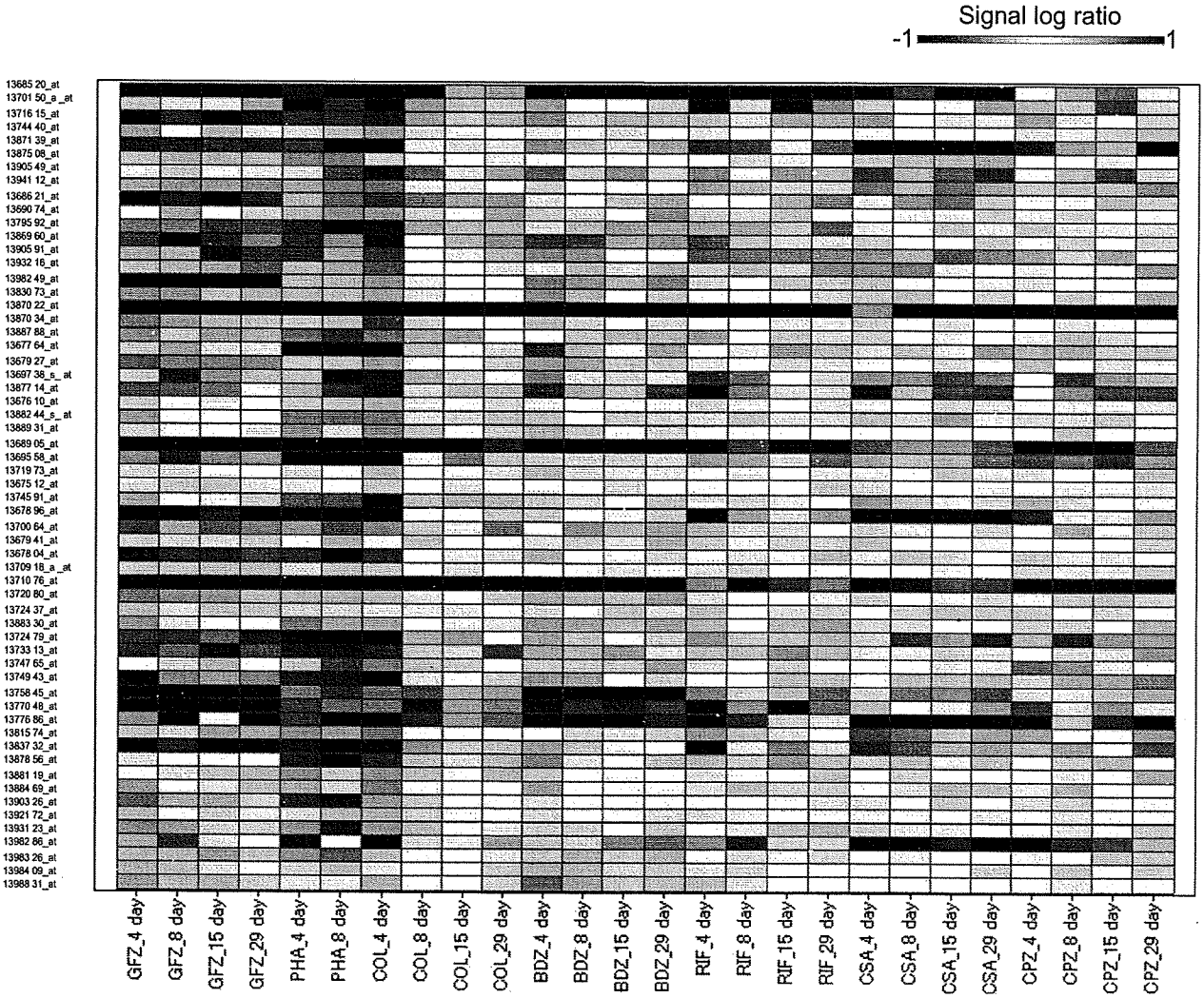

Figure 2 Heat map of the gene expression profiles of gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ) that induced elevation of total bilirubin (TBIL) and direct bilirubin (DBIL) in the present study using the commonly mobilized 59 probe sets. Values are expressed as average log 2 ratio, for each time point at high dosage.