Table 1. Hematological findings

	Group	Main group 0 mg/kg/day	Main group 0.1 mg/kg/day	Main group 0.3 mg/kg/day	Main group 1.0 mg/kg/day	Satellite group 0 mg/kg/day	Satellite group 1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
WBC	(10^2/μl)	121.2 ± 31.4	94.8 ± 21.1	127.6 ± 35.4	129.8 ± 23.5	73.4 ± 26.8	111.6 ± 19.5*
RBC	(10^4/μl)	830 ± 40	846 ± 25	852 ± 20	869 ± 23	894 ± 34	886 ± 47
HGB	(g/dl)	15.6 ± 0.4	15.7 ± 0.6	15.4 ± 0.4	15.6 ± 0.7	16.0 ± 0.4	15.3 ± 0.9
MCV	fl	52.5 ± 1.8	51.4 ± 1.7	50.6 ± 0.7	50.1 ± 1.4*	50.9 ± 1.5	49.4 ± 1.9
MCH	pg	18.8 ± 0.5	18.6 ± 0.8	18.1 ± 0.4	17.9 ± 0.4*	17.9 ± 0.3	17.3 ± 0.7
Platelet	(10^4/μl)	98.7 ± 3.7	121.4 ± 5.2**	109.2 ± 8.8	111.2 ± 8.8*	107.8 ± 12.4	122.7 ± 18.6
APTT	(sec)	22 ± 4.1	19.2 ± 1.9	20.8 ± 4.2	16.6 ± 0.7*	20.4 ± 1.7	17.2 ± 2.6*
Fibrinogen	mg/dl	294 ± 20	273 ± 35	283 ± 31	200 ± 23**	304 ± 35	245 ± 22*
Females							
Number of animals		5	5	5	5	5	5
WBC	(10^2/μl)	143.4 ± 43.8	128.7 ± 25.4	151.8 ± 33.5	159.2 ± 45.1	58.6 ± 14.9	65.1 ± 13.6
RBC	(10^4/μl)	702 ± 46	680 ± 67	692 ± 50	645 ± 51	830 ± 30	846 ± 56
HGB	(g/dl)	13.1 ± 1.0	13.5 ± 1.0	13.5 ± 1.1	13.2 ± 0.8	15.4 ± 0.4	15.4 ± 1.0
MCV	fl	52.7 ± 1.3	56.7 ± 4.5	55.0 ± 1.2	58.0 ± 3.1*	51.4 ± 1.4	50.1 ± 1.2
MCH	pg	18.6 ± 0.5	20.0 ± 1.6	19.5 ± 0.6	20.5 ± 1.1*	18.6 ± 0.6	18.2 ± 0.6
Platelet	(10^4/μl)	159.4 ± 27.4	141.0 ± 22.7	164.8 ± 19.6	161.8 ± 30.9	130.6 ± 13.7	125.7 ± 18.1
APTT	(sec)	17.6 ± 1.8	17.5 ± 2.4	17.9 ± 2.3	15.2 ± 3.3	17.9 ± 2.3	17 ± 2.9
Fibrinogen	mg/dl	335 ± 53	319 ± 95	282 ± 49	228 ± 42*	207 ± 10	176 ± 31

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

mg/kg/day group. In rodents, it is clear that the hepatic response to exposure to many perfluoroalkyl compounds is initiated by the activation of the nuclear hormone receptor, PPARα (ATSDR, 2009), and PFUA activates mouse PPARα *in vitro* (Wolf *et al.*, 2012). The hepatic proliferative responses, including an increase in the liver weight and centrilobular hypertrophy of hepatocytes, observed in the present study might have been initiated by the activation of PPARα, although there is a scientific consensus that compounds which are peroxisome proliferators in rodents have little or no effect on human liver (IARC, 1995). Regarding the toxicity of PFAAs, the involvement of mechanisms other than PPARα has been suggested (Peters and Gonzalez, 2011), so further research on the toxicity mechanism of PFUA is desired.

Effects on the body weight of adult males/females and pups were observed only at 1.0 mg/kg/day. In adult animals, suppression of body weight gain was observed in males/females in the administration and/or recovery periods, although not in females in the premating and gestation periods. It is considered that these body weight changes were a direct effect of PFUA because they were not related to food consumption. There is a possibility of maternal-fetal/infant transfer of PFUA, because maternal-fetal transfer and maternal-infant transfer of PFOA through breast milk have been observed in rats (Hinderliter *et al.*, 2005). Because there was no difference in the length of the gestation period in dams dosed at 1.0 mg/kg/day compared to the controls, and because sup-

Table 2. Blood biochemical findings

		Main group				Satellite group	
Group		0 mg/kg /day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	0 mg/kg/day	1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
AST	(IU/l)	67 ± 9	70 ± 4	73 ± 17	77 ± 6	62 ± 9	73 ± 12
ALT	(IU/l)	31 ± 3	32 ± 3	34 ± 3	39 ± 7*	31 ± 5	37 ± 5
ALP	(IU/l)	427 ± 12.6	461 ± 85	514 ± 96	1021 ± 179**	379 ± 95	707 ± 152**
Total cholesterol	(mg/dl)	56 ± 14	47 ± 8	34 ± 6**	46 ± 11	55 ± 18	53 ± 13
Triglyceride	(mg/dl)	48 ± 10	70 ± 42	41 ± 9	46 ± 16	52 ± 17	45 ± 27
Phospholipid	(mg/dl)	90 ± 13	82 ± 14	65 ± 9*	87 ± 11	87 ± 19	92 ± 21
BUN	(mg/dl)	13 ± 2	14 ± 3	15 ± 1	21 ± 4**	17 ± 2	23 ± 5*
Na	(mmol/l)	147 ± 2	146 ± 2	147 ± 1	145 ± 1	145 ± 1	143 ± 1**
Cl	(mmol/l)	108 ± 2	108 ± 1	109 ± 1	109 ± 3	107 ± 1	108 ± 1
Ca	(mg/dl)	10.1 ± 0.2	10.0 ± 0.3	10.0 ± 0.3	9.7 ± 0.2*	9.9 ± 0.3	9.5 ± 0.3
Total protein	(g/dl)	6.2 ± 0.2	6.0 ± 0.3	6.1 ± 0.1	5.5 ± 0.3**	6.3 ± 0.1	5.8 ± 0.5
Albumin	(g/dl)	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.0	2.6 ± 0.1*	2.7 ± 0.1	2.8 ± 0.2
A/G		0.80 ± 0.07	0.86 ± 0.03	0.93 ± 0.05**	0.88 ± 0.06	0.77 ± 0.04	0.93 ± 0.09**
Females							
Number of animals		5	5	5	5	5	5
AST	(IU/l)	84 ± 21	92 ± 12	86 ± 15	81 ± 12	59 ± 4	68 ± 11
ALT	(IU/l)	53 ± 9	55 ± 12	50 ± 18	49 ± 1	26 ± 4	28 ± 4
ALP	(IU/l)	219 ± 72	242 ± 42	286 ± 176	263 ± 18	158 ± 28	289 ± 54**
Total cholesterol	(mg/dl)	60 ± 11	52 ± 13	41 ± 13*	49 ± 8	78 ± 16	64 ± 14
Triglyceride	(mg/dl)	54 ± 11	38 ± 12	41 ± 18	60 ± 25	28 ± 11	20 ± 3
Phospholipid	(mg/dl)	112 ± 13	94 ± 18	80 ± 20*	98 ± 11	141 ± 20	108 ± 15*
BUN	(mg/dl)	13 ± 2	13 ± 4	16 ± 3	19 ± 2**	20 ± 3	29 ± 7*
Na	(mmol/l)	141 ± 1	141 ± 2	143 ± 1	142 ± 1	143 ± 1	143 ± 1
Cl	(mmol/l)	106 ± 1	107 ± 2	108 ± 2	108 ± 2*	109 ± 1	112 ± 2
Ca	(mg/dl)	10.3 ± 0.2	10.2 ± 0.4	10.3 ± 0.1	10.0 ± 0.3	10.2 ± 0.3	9.9 ± 0.2
Total protein	(g/dl)	6.2 ± 0.2	5.8 ± 0.3*	6.0 ± 0.1	5.6 ± 0.2**	6.7 ± 0.2	5.8 ± 0.3**
Albumin	(g/dl)	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.1	2.7 ± 0.2	3.1 ± 0.2	2.9 ± 0.3
A/G		0.85 ± 0.05	0.92 ± 0.05	0.89 ± 0.07	0.91 ± 0.11	0.87 ± 0.04	1.01 ± 0.09*

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

pression of body weight gain in females during pregnancy was not observed, the lowered body weight on PND 0 was considered a direct effect of PFUA due to intrauterine exposure. Also in other PFCAs, low values of body weight of pups at birth without effects on the body weight of dams in the gestation period were observed (Butenhoff *et al.*, 2004; Loveless *et al.*, 2009). The lowered body weight on PND 4 was considered to be a direct effect of PFUA by ingestion of breast milk, as well as a secondary effect of PFUA caused by the lowered body weight in

Table 3. Organ weights

		Main Group				Satellite Group	
Dose (mg/kg/day)		0 (control)	0.1	0.3	1.0	0 (control)	1.0
Males							
No. of animals examined		5	5	5	5	5	5
Brain	(g)	2.18 ± 0.08	2.18 ± 0.09	2.15 ± 0.08	2.17 ± 0.08	2.09 ± 0.04	2.14 ± 0.13
	(%)[a]	0.42 ± 0.03	0.4 ± 0.03	0.41 ± 0.01	0.44 ± 0.05	0.39 ± 0.03	0.44 ± 0.03**
Throid[b]	(mg)	22.4 ± 1.8	25.7 ± 2.5	21.1 ± 2.9	22.3 ± 3.5	23.5 ± 3.8	18.7 ± 2.9
	(%)[a]	4.3 ± 0.4	4.7 ± 0.4	4.1 ± 0.6	4.5 ± 0.7	4.4 ± 0.5	3.8 ± 0.4
Thymus	(mg)	297 ± 90	432 ± 173	342 ± 106	260 ± 61	250 ± 80	251 ± 67
	(%)[a]	57 ± 19	79 ± 27	66 ± 21	53 ± 16	47 ± 17	51 ± 11
Heart	(g)	1.52 ± 0.1	1.5 ± 0.2	1.51 ± 0.03	1.38 ± 0.17	1.46 ± 0.17	1.29 ± 0.19
	(%)[a]	0.29 ± 0.02	0.28 ± 0.04	0.29 ± 0	0.28 ± 0.02	0.28 ± 0.02	0.27 ± 0.02
Liver	(g)	15.12 ± 2.14	16.45 ± 2.06	17.54 ± 0.73	20.95 ± 2.56**	14.19 ± 1.56	19.85 ± 3.03**
	(%)[a]	2.88 ± 0.27	3.02 ± 0.19	3.39 ± 0.16**	4.18 ± 0.19**	2.67 ± 0.22	4.07 ± 0.36**
Spleen	(g)	0.84 ± 0.16	0.76 ± 0.09	0.79 ± 0.05	0.65 ± 0.09*	0.72 ± 0.11	0.72 ± 0.04
	(%)[a]	0.16 ± 0.03	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01*	0.14 ± 0.02	0.15 ± 0.01
Kidney[b]	(g)	3.43 ± 0.31	3.44 ± 0.38	3.51 ± 0.08	3.4 ± 0.17	3.51 ± 0.31	3.32 ± 0.43
	(%)[a]	0.65 ± 0.06	0.63 ± 0.04	0.68 ± 0.03	0.68 ± 0.06	0.66 ± 0.03	0.68 ± 0.04
Adrenal[b]	(mg)	64 ± 13	70 ± 8	68 ± 3	58 ± 9	61 ± 9	46 ± 8*
	(%)[a]	12 ± 2	13 ± 1	13 ± 1	12 ± 1	12 ± 2	9 ± 1
Testis[b,c]	(g)	3.34 ± 0.21	3.57 ± 0.26	3.48 ± 0.28	2.98 ± 0.86	3.49 ± 0.26	3.57 ± 0.35
	(%)[a]	0.63 ± 0.07	0.67 ± 0.05	0.68 ± 0.06	0.62 ± 0.17	0.66 ± 0.03	0.74 ± 0.07*
Epididymis[b,c]	(mg)	1339 ± 84	1420 ± 112	1368 ± 199	1578 ± 950	1337 ± 51	1388 ± 87
	(%)[a]	252 ± 21	265 ± 25	268 ± 36	335 ± 220	252 ± 11	288 ± 34
Females							
No. of animals examined		5	5	5	5	5	5
Brain	(g)	1.99 ± 0.05	1.97 ± 0.08	1.98 ± 0.09	2 ± 0.04	1.96 ± 0.09	1.86 ± 0.06
	(%)[a]	0.64 ± 0.04	0.66 ± 0.03	0.65 ± 0.05	0.67 ± 0.06	0.68 ± 0.08	0.78 ± 0.02**
Throid[b]	(mg)	17.2 ± 1.8	19.2 ± 3.2	17.5 ± 3	16.9 ± 0.7	17.2 ± 2.7	14.7 ± 1
	(%)[a]	5.5 ± 0.8	6.5 ± 1.1	5.8 ± 1.1	5.6 ± 0.6	6 ± 1	6.2 ± 0.3
Thymus	(mg)	192 ± 16	170 ± 102	243 ± 82	249 ± 58	245 ± 98	147 ± 59
	(%)[a]	61 ± 4	56 ± 32	79 ± 24	82 ± 14	85 ± 39	62 ± 23
Heart	(g)	1.02 ± 0.08	0.96 ± 0.06	0.92 ± 0.04	0.94 ± 0.11	0.86 ± 0.05	0.73 ± 0.03**
	(%)[a]	0.33 ± 0.03	0.32 ± 0.01	0.3 ± 0.01	0.31 ± 0.02	0.29 ± 0.02	0.31 ± 0.01
Liver	(g)	10.56 ± 0.68	10.61 ± 0.48	10.55 ± 1.48	12.76 ± 1.00**	7.22 ± 0.38	8.63 ± 1.04*
	(%)[a]	3.37 ± 0.12	3.57 ± 0.13	3.46 ± 0.36	4.21 ± 0.15**	2.48 ± 0.14	3.64 ± 0.47**
Spleen	(g)	0.62 ± 0.06	0.65 ± 0.16	0.65 ± 0.1	0.66 ± 0.15	0.49 ± 0.05	0.43 ± 0.05
	(%)[a]	0.2 ± 0.02	0.22 ± 0.05	0.22 ± 0.02	0.21 ± 0.03	0.17 ± 0.02	0.18 ± 0.01
Kidney[b]	(g)	2.24 ± 0.42	1.96 ± 0.18	2.06 ± 0.19	2.05 ± 0.09	1.89 ± 0.14	1.93 ± 0.17
	(%)[a]	0.72 ± 0.14	0.66 ± 0.06	0.68 ± 0.07	0.68 ± 0.04	0.64 ± 0.02	0.81 ± 0.07**
Adrenal[b]	(mg)	82 ± 4	84 ± 10	89 ± 14	80 ± 13	70 ± 8	49 ± 5**
	(%)[a]	26 ± 2	28 ± 5	30 ± 5	26 ± 3	25 ± 5	21 ± 1

Values are given as the mean ± S.D.

[a]: Ratio of organ weight to body weight (relative organ weight). [b]: Values are represented as the total weights of the organs on both sides. [c]: Organ weight was measured for all animals (number of examined animals: 7 at 0 and 1.0 mg/kg/day and 12 at 0.1 and 0.3 mg/kg/day in the main group, and 5 at 0 and 1.0 mg/kg/day in the recovery group.)

*: Significantly different from the control, $p \leq 0.05$. **: Significantly different from the control, $p \leq 0.01$.

Table 4. Histopathological findings

	Males						Females					
	Main				Satellite		Main				Satellite	
Dose (mg/kg/day)	0	0.1	0.3	1.0	0	1.0	0	0.1	0.3	1.0	0	1.0
Heart												
Number examined	5	0	0	5			5	0	0	5		
Cardiomyopathy (minimal)	1			1			0			0		
Kidney												
Number examined	5	0	1	5			5	0	0	5		1
Dilatation, pelvic	0		1	0			1			0		1
(minimal)			1									1
(moderate)							1					
Regeneration, tubular	4		1	1			1			1		0
(minimal)	3		1	1			1			1		
(mild)	1											
Liver												
Number examined	7	12	12	7	5	5	12	12	12	12	5	5
Vacuolation, hepatocytes, diffuse	0	0	0	3	0	1	0	0	0	0	0	0
(minimal)				2		1						
(mild)				1								
Necrosis, focal (minimal)	0	0	0	2	0	0	0	0	0	2	0	0
Cell infiltration, Glisson's sheath (mild)	0	0	0	0	0	0	0	0	0	0	0	2
Microgranuloma	4	3	1	2	3	3	1	1	0	2	4	4
(minimal)	4	3	1	2	3	3	1	1		2	4	2
(mild)												2
Degeneration, hepatocytes, centrilobular (minimal)	0	0	0	0	0	3	0	0	0	0	0	3
Hypertrophy, hepatocytes, centrilobular	0	0	3	7	0	5	0	0	1	11	0	5
(minimal)			2						1	8		
(mild)			1	2		3				3		2
(moderate)				5		2						3
Spleen												
Number examined	5	0	0	5			5	0	0	5		
Hematopoiesis, extramedullary (minimal)	4			1			5			4		
Stomach												
Number examined	7	12	12	7	5	5	6	0	0	5		
Erosion, glandular stomach (minimal)	0	0	0	3	0	0	2			0		
Thymus												
Number examined	5	0	0	5			5	1	0	5		
Atrophy, lymphoid (mild)	0			0			0	1		0		
Thyroid												
Number examined	5	0	0	5			5	0	0	5		
Ectopic thymus (minimal)	0			0			0			1		
Cyst, ultimobranchial (minimal)	1			2			2			0		
Testis												
Number examined	5	0	0	5								
Not remarkable	5			5								
Epididymis												
Number examined	5	1	0	5								
Granuloma, spermatic	1	1		1								
(minimal)		1		1								
(mild)	1											
Uterus												
Number examined							5	1	0	5		
Dilatation, lumina (minimal)							0	1		0		

Table 5. Reproductive and developmental parameters

	0 mg/kg/day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day
Number of animals (males/females)	12/12	12/12	12/12	12/12
Copulation index (males/females) (%)	100/100	100/100	100/100	100/100
Fertility index (%)	100	91.7	100	100
Gestation index (%)	100	100	100	100
Number of pregnant animals	12	11	12	12
Gestation length (days)	22.0 ± 0.3	22.1 ± 0.5	22.1 ± 0.5	21.7 ± 0.2
Number of corpora lutea	15.8 ± 1.9	16.8 ± 1.8	16.2 ± 1.9	16.2 ± 1.5
Number of implantation sites	14.6 ± 2.0	15.5 ± 3.3	15.0 ± 1.9	15.3 ± 1.6
Implantation index (%)	92.0 ± 5.5	91.0 ± 15.4	92.8 ± 6.0	94.8 ± 4.4
Number of litters	12	11	12	12
Number of live pups on PND 0	13.9 ± 2.2	14.5 ± 3.4	13.1 ± 3.1	13.5 ± 2.2
Live birth index (%)	98.9 ± 2.6	97.3 ± 5.3	93.2 ± 18.2	97.9 ± 4.1
Sex ratio	0.51	0.47	0.55	0.52
Number of live pups on PND 4	13.7 ± 1.9	14.0 ± 3.2	12.8 ± 3.1	13.4 ± 2.2
Viability index (%)	98.5 ± 2.8	97.1 ± 3.3	97.7 ± 5.9	99.4 ± 2.2
Body weight of male pups (g)				
on PND 0	6.7 ± 0.3	6.7 ± 0.6	6.4 ± 0.5	5.8 ± 0.3**
on PND 4	10.5 ± 0.5	10.1 ± 1.8	10.2 ± 1.2	8.5 ± 0.7**
Body weight of female pups (g)				
on PND 0	6.4 ± 0.4	6.3 ± 0.6	6.1 ± 0.6	5.6 ± 0.2**
on PND 4	9.9 ± 0.6	9.7 ± 1.7	9.5 ± 0.8	8.3 ± 0.7**

Values are given as the mean ± S.D.
**: Significantly different from the control, $p \leq 0.01$.

dams. In the PFOA oral dose study (Abbott *et al.*, 2007), the reduction of postnatal weight gain appeared to depend on PPARα expression.

The elimination rate of PFOA in female rats is approximately 40 times faster than in male rats (ATSDR, 2009). Organic anion transport proteins play a key role in PFCAs (C4 to C10) renal tubular reabsorption (Han *et al.*, 2012), and the slower elimination of PFOA in male rats compared to female rats has been attributed to sex hormone modulation of organic anion transporters in the kidney (ATSDR, 2009). In the present study, there were slight gender differences in the hepatotoxicity of PFUA: liver weight increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day, and histopathological findings observed in the 1.0 mg/kg/day groups were more numerous and severer in males than in females. The gender differences in hepatotoxicity observed in the present study are considered to be attributable to faster elimination in female rats, as with other PFCAs.

Increased liver weight and hepatocellular hypertrophy, induced by activation of PPARα, were generally observed in previous studies on PFAAs. Significant peroxisome proliferative activity seems to require a carbon length more than 7 (ATSDR, 2009). In gavage studies of PFAAs in male rats, which are more sensitive than females, the following results were observed; for PFOA (C8), increased liver weight and hepatocellular hypertrophy at 5 mg/kg/day for 28 days (Cui *et al.*, 2009); for perfluorononanoic acid (C9), increased liver weight at 1 mg/kg/day for 14 days (Fang *et al.*, 2012); for perfluorododecanoic acid (C12), increased liver weight at 0.02 mg/kg/day for 110 days (Ding *et al.*, 2009). In the current study of PFUA (C11), increased liver weight and centrilobular hypertrophy of hepatocytes were observed from 0.3 mg/kg/day for 42 days. In consideration of differences in the administration period or doses in these studies, the intensity of the liver toxicity of PFUA (C11) was estimated to be between C9 and C12, suggesting that the toxic potency of PFAAs (C8-C12) increases by lengthening their carbon chain. This is because hydrophobicity, which increases as carbon length increases, seems to favor biliary enterohepatic recirculation, resulting in more protracted toxicity (ATSDR, 2009). In contrast, 42-day administration of PFOdA (C18) increased liver

weight at 200 mg/kg/day but not at 40 mg/kg/day in male rats (Hirata-Koizumi *et al.*, 2012). In comparison with other PFAAs (C8-C12), including PFUA (C11), PFOdA induced liver toxicity at higher doses, and this may be due to the low absorption of PFOdA into the body.

At 1.0 mg/kg/day in the main group, the following effects on hematological and blood biochemical parameters were observed; a decrease in fibrinogen was observed in males and females, but increases in APTT and PT were not observed, suggesting that there would be no toxicologically significant effects on the blood coagulation system; decreases in fibrinogen, total protein and albumin observed in males and/or females may be due to reduced synthesis in the damaged liver; the increase in BUN observed in males and females could be due to increased hepatic protein catabolism, because urinalysis parameters and the gross and microscopic appearance of the kidneys were not changed; and the increase of ALP in males was related to the histopathological findings in the liver. These effects except for the decrease in fibrinogen in females were observed also at the end of the recovery period, and the increase of ALP was observed in females only after the recovery period. Moreover, in histopathological findings, centrilobular degeneration of hepatocytes in both sexes and Glisson's sheath cell infiltration in females were observed only at the end of the recovery period, and in females, centrilobular hypertrophy of hepatocytes was more serious at the end of the recovery period. These results suggest that the whole body elimination of PFUA in rats, as well as other PFCAs, is slow. There are some reports indicating that PFCAs are secreted in bile and undergo extensive reabsorption from the gastrointestinal tract (Kudo *et al.*, 2001; Vanden Heuvel *et al.*, 1991a, 1991b; reviewed in ATSDR, 2009). In general, PFCAs with longer carbon chains (C4-C10) have a longer half-life (Hirata-Koizumi *et al.*, 2012). Although the elimination half-life of PFUA is unknown, the half-life after intravenous injection of perfluorodecanoic acid (PFDeA, C10) in rats was about 40 to 60 days (Ohmori *et al.*, 2003), It is estimated that the half-life of PFUA is longer than the recovery period, 14 days, and it is reasonable that some effects of PFUA appear after the recovery period. The above findings may be effects of PFUA caused by enterohepatic recirculation, which lasted through the dosing and recovery periods. The decrease in grip strength of the forefoot observed in males and females at 1.0 mg/kg/day in the satellite group was considered a secondary effect related to suppression of body weight gain.

In conclusion, the NOAEL for repeated dose toxicity is considered to be 0.1 mg/kg/day based on the observed centrilobular hypertrophy of hepatocytes in both sexes at 0.3 mg/kg/day, and the NOAEL for reproductive/developmental toxicity is considered to be 0.3 mg/kg/day based on the lowered body weight of pups at birth and body weight gain at 4 days after birth inhibited at 1.0 mg/kg/day.

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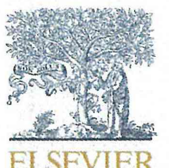

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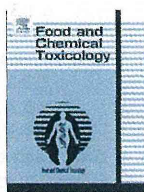

Review

The JFFMA assessment of flavoring substances structurally related to menthol and uniquely used in Japan

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ABSTRACT

Using the procedure devised by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), we performed safety evaluations on four flavoring substances structurally related to menthol (L-menthyl 2-methylbutyrate, DL-menthyl octanoate, DL-menthyl palmitate, and DL-menthyl stearate) uniquely used in Japan. While no genotoxicity study data were available in the literature, all four substances had no chemical structural alerts predictive of genotoxicity. Moreover, they all four are esters consisting of menthol and simple carboxylic acids that were assumed to be immediately hydrolyzed after ingestion and metabolized into innocuous substances for excretion. As menthol and carboxylic acids have no known genotoxicity, it was judged that the JECFA procedure could be applied to these four substances. According to Cramer's classification, these substances were categorized as class I based on their chemical structures. The estimated daily intakes for all four substances were within the range of 1.54–4.71 μg/person/day and 60–1250 μg/person/day, using the methods of Maximized Survey-Derived Intake and Single Portion Exposure Technique, respectively, based on the annual usage data of 2001, 2005, and 2010 in Japan. As the daily intakes of these substances were below the threshold of concern applied to class I substances viz., 1800 μg/person/day, it was concluded that all four substances raise no safety concerns when used for flavoring foods under the currently estimated intake levels.

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Abbreviations: ADI, acceptable daily intake; EFSA, European Food Safety Authority; FEMA, Flavor and Extract Manufacturers Association; MSDI, Maximized Survey-Derived Intake; NOEL, no observed effect level; JECFA, Joint FAO/WHO Expert Committee on Food Additives; JFFMA, Japan Flavor and Fragrance Materials Association; JASREC, The JFFMA Safety Re-evaluation Committee; SAR, Structure Activity Relationship analyses; SPET, Single Portion Exposure Technique; WHO, World Health Organization.

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1. Introduction

1.1. Flavoring substances in Japan

There is a wide variety of flavoring substances that have no nutritional properties but are used to improve the taste and aroma of food. This wide variety enables the food industry to satisfy its requirement to mimic the flavor of foods. According to the annual usage survey by the Japan Flavor and Fragrance Materials Association (JFFMA), approximately 3230 flavoring substances have been used in Japan (Someya, 2012). Among these, 128 substances are currently approved as designated additives by the Ministry of Health, Labour and Welfare, Japan. The remaining 3102 substances have been classified into 18 chemical structure groups and approved for use in Japan under the Food Sanitation Act without any safety evaluation.

1.2. Safety evaluation procedures

In 1996, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) adopted a novel safety evaluation procedure for flavoring substances (WHO, 1996). According to this procedure, the safety evaluation of many flavoring substances can be efficiently conducted using the safety data for substances that have similar chemical structures or metabolic pathways, even if the substances under investigation have no safety data. Further evaluation methods similar to the JECFA procedure are used in the United States and the European Union. In addition, the results of safety evaluations by JECFA, Flavor and Extract Manufacturers Association (FEMA) and the European Food Safety Authority (EFSA) are now widely accepted in more than 70 countries (Someya, 2012; Konishi et al., in press). Since 2003, the Food Safety Commission of Japan has performed safety evaluations of 50 selected flavoring substances and their results are consistent with the group evaluation by the JECFA procedure (Someya, 2012).

From this viewpoint, regarding the approximately 3230 flavoring substances used in Japan, approximately 2400 flavoring substances have been judged safe under limited conditions of use for flavoring by JECFA, FEMA, EFSA, and the Food Safety Commission of Japan. However, this leaves 800 flavoring substances not yet subjected to safety evaluation by any governmental or international organization. For this reason, in 2009, JFFMA established a Safety Re-evaluation Committee (JASREC) comprised of expert scientists in the fields of toxicology and food chemistry as well as representative members of JFFMA. Since then, the JASREC has begun to evaluate substances uniquely used in Japan using the JECFA evaluation procedure as shown in Supplementary Fig. 1 with the order of priority determined by annual volume of production.

In this paper, we evaluate the safety of four flavoring substances structurally related to menthol, which have been uniquely used in Japan: L-menthyl 2-methylbutyrate, DL-menthyl octanoate, DL-menthyl palmitate, and DL-menthyl stearate, according to the JECFA evaluation procedure. These substances are all carboxylic acid esters of menthol having a menthol-like cool fresh flavor, and are used for manufacturing sweet foods such as chewing gum and candy.

2. Collecting information for evaluation procedure

2.1. Estimated daily intake of flavoring substances by MSDI and SPET methods

CAS No., chemical structure and molecular weight for these four flavoring substances, L-menthyl 2-methylbutyrate, DL-menthyl octanoate, DL-menthyl palmitate, and DL-menthyl stearate, are listed in Table 1. These substances have not been reported to occur naturally, and thus, all four substances are expected to be ingested only from foods containing them as flavoring agents (JFFMA, 2003, 2006, 2011). According to the annual usage data in 2001, 2005, and 2010 in Japan, the total annual estimated volumes of L-menthyl 2-methylbutyrate, DL-menthyl octanoate, DL-menthyl palmitate, and DL-menthyl stearate, are 0.11–10.34, 4.86–17.89, 0.10–5.98, and 3.10–5.83 kg, respectively (JFFMA, 2003, 2006, 2011). Based on these data, the daily intake of each substance was calculated using the methods of the Maximized Survey-Derived Intake (MSDI; Young et al., 2006) and Single Portion Exposure Technique (SPET; WHO, 2008).

MSDI method was developed based on disappearance data from periodic surveys of ingredient manufacturers using the volume of ingredients produced during the survey year (Young et al., 2006). Because the usage volume differs every year, the maximal usage volume of each substance was selected among annual usage data obtained to avoid underestimation of the intakes. The resultant daily intakes per person of the four flavoring substances were estimated to be in the range of 1.54–4.71 μg (Table 2).

By means of the SPET method (WHO, 2008), the estimated daily intakes of L-menthyl 2-methylbutyrate, DL-menthyl octanoate, DL-menthyl palmitate, and DL-menthyl stearate, were calculated to be 1250, 60, 60, and 250 μg, respectively, based on the average use levels for flavoring substance with standard portion sizes of flavoring foods (Table 2).

Because the estimated daily intakes by SPET method were higher than those by MSDI method, it was decided to conduct a safety evaluation using the calculation values of the SPET method.

2.2. Prediction of genotoxic potential by chemical structure and in silico models

We evaluated the genotoxic potential of each menthol-related substance by analyzing the biological potential of its chemical structure. While the JECFA decision tree approaches do not cover

Table 1
Chemical information on four menthol-related flavoring substances.

Name	CAS No. and chemical structure	Molecular weight
L-Menthyl 2-methylbutyrate	1309597-13-4	240.38
DL-Menthyl octanoate	1353435-52-5	282.46
DL-Menthyl palmitate	861351-16-8	394.67
DL-Menthyl stearate	93919-01-8	422.73

genotoxic carcinogenic potential in its evaluation (WHO, 1996), potential of genotoxicity is now evaluated before the steps of decision tree approaches by incorporating structural alert and other available genotoxic information of structurally-related chemicals in JECFA (WHO, 1998c, 2008, 2009, 2011). In this review, following the approaches taken by JECFA, we applied structural alerts to chemical substructure groups, as is generally done for chemicals lacking genotoxicity information (Ashby and Tennant, 1988, 1991; Tennant et al., 1990). While genotoxicity study data are not available for all four substances, there were no structural alerts in their chemical structures. In addition, we further examined the genotoxic potential of the four substances using Structure Activity Relationship (SAR; Hansch, 1969) analyses utilizing *in silico* prediction software that has been applied to the regulatory evaluation of flavoring substances (Cronin et al., 2003; Ono et al., 2012). We used three SAR software programs that have different algorithms for analysis: DEREK (version 10.0.2; Lhasa Ltd., Leeds, UK), MultiCASE (version 1.90; Multicase Inc., Cleveland, OH, USA), and ADME-WORKS (version 4.0; Fujitsu Kyusyu Systems Ltd., Fukuoka, Japan). All the *in silico* software programs used in this review provided the judgment that all four substances have no potential for genotoxicity. Furthermore, menthol and carboxylic acids, which are metabolites of menthyl esters, also do not have genotoxic potential (WHO, 1999a,b). In addition, the genotoxicity information of structurally-related substances were used for evaluation (see Section 3.3.3). Based on these results, we decided that the JECFA evaluation procedure could be applied to these four menthol-related substances.

2.3. Estimation of absorption, metabolism, and excretion

As the information on the four substances regarding absorption, metabolism, and excretion was absent from the JECFA assessment reports (WHO, 1998a,b, 1999a,b), esters structurally related to the four substances were alternatively examined to predict their metabolic fate. Among the structurally related esters, L-menthol propylene glycol carbonate and L-menthol ethylene glycol carbonate were easily hydrolyzed by incubation with rat liver homogenates (WHO, 1999b). In addition, cyclandelate, which shares a similar chemical structure with menthyl esters, was also easily hydrolyzed by incubation with rat liver microsomes (White et al., 1990). Based on these results, menthyl esters were judged to be hydrolyzed into menthol and carboxylic acids (WHO, 1999b). In rats, menthol has been shown to be excreted into urine or feces as glucuronic acid conjugates or various oxidation products (Madyastha and Srivatsan, 1988; Yamaguchi et al., 1994). Additional oxidation products identified include a primary alcohol, a triol, and hydroxy acids (Yamaguchi et al., 1994). Conversely, methylbutyric acid, octanoic acid, stearic acid, and palmitic acid, which are predicted to be generated by the hydrolysis of menthyl esters, undergo β-oxidation and are completely metabolized in the tricarboxylic acid cycle (WHO, 1998a). Therefore, based on these data, menthol and carboxylic acids generated by the hydrolysis of esters are judged to be metabolized into innocuous products (WHO, 1999a).

3. Safety evaluation of the four substances according to the JECFA evaluation procedure

3.1. The decision tree approaches

Details of the JECFA evaluation procedure are described in WHO (1996). A summary of the decision tree approach for this procedure is shown in Supplementary Fig. 1.

3.1.1. Step 1

In the first step, each flavoring substance was subjected to classification into three chemical structure classes: I, II, or III, according to Cramer's decision tree as shown in Supplementary

Table 2
Estimated daily intake data of four menthol-related flavoring substances in Japan.

Substance	Annual volume of production (kg/year)			Estimated daily intake		Name and code of the food group(s) showing the highest use level[c]
	2001	2005	2010	MSDI value[a] (μg/person/day)	SPET value[b] (μg/person/day)	
L-Menthyl 2-methylbutyrate	0.11	10.34	2.69	2.72	1250	01.7 Dairy-based desserts
DL-Menthyl octanoate	4.86	6.20	17.89	4.71	60	05.2 Confectionery (hard and soft candy)
DL-Menthyl palmitate	0.10	5.98	3.25	1.58	60	05.2 Confectionery (hard and soft candy)
DL-Menthyl stearate	5.05	5.83	3.10	1.54	250	01.7 Dairy-based desserts

Abbreviations: MSDI, Maximized Survey-Derived Intake; SPET, Single Portion Exposure Technique.
[a] Calculated by the MSDI method based on the maximal usage volume from the annual usage data of 2001, 2005 and 2010 in Japan. MSDI value (μg/person/day) = annual volume of production (kg) × 10^9 (μg/kg)/population of consumers × 0.8 × 365 days. The population of consumers was assumed to be 13 × 10^6.
[b] Calculated by the Single Portion Exposure Technique method (WHO, 2008).
[c] Name and code of the food group(s) were listed in Codex General Standard For Food Additives, Codex Stan 192-1995 (FAO, 2011).

Fig. 2 (Cramer et al., 1978). Substances of class I have a simple chemical structure with known efficient metabolic pathways that would suggest a low oral toxicity. Substances of class II are less innocuous than class I, but do not contain structural features suggestive of having toxicity like substances in class III. Substances of class III have a chemical structure that may cause significant toxicity.

According to the following judgments in the Cramer decision tree (Supplementary Fig. 2), all four substances were assigned to structural class I. In addition, menthol, which is a component of these substances, is also belonging to structural class I.

Q1. Is the substance a *normal constituent of the body* or an optical isomer of such? → No

Q2. Does the substance contain any of the following functional groups: an aliphatic secondary amine or a salt thereof, cyano, N-nitroso, diazo (e.g. CH_2N_2), triazeno ($RN{=}NNH_2$), or quaternary nitrogen, except in any of the following forms: $\rangle C{=}N^+R_2$, $\rangle C{=}N^+H_2$, or the hydrochloride or sulfate salt of a primary or tertiary amine? → No

Q3. Does the structure contain elements other than carbon, hydrogen, oxygen, nitrogen, or divalent sulfur? → No

Q5. Is it a simply branched acyclic aliphatic hydrocarbon or a common carbohydrate? → No

Q6. Is the substance a benzene derivative bearing substituents consisting only of hydrocarbon chains or 1′-hydroxy or hydroxy ester-substituted hydrocarbon chains and one or more alkoxy groups, one of which must be at *para*-position against the hydrocarbon chain? → No

Q7. Is the substance heterocyclic? → No

Q16. Is it a common terpene-hydrocarbon, -alcohol, -aldehyde, or -carboxylic acid (not a ketone)? → No

Q17. Is the substance readily hydrolyzed to a common terpene, -alcohol, -aldehyde, or -carboxylic acid. If the answer is yes, treat the hydrolyzed residues separately and proceed to 18 for the terpene moiety and to 19 for any non-terpenoid moiety. → Yes

Q18. Is the substance one of the following:

(a) A vicinal diketone; ketone, or ketal of a ketone attached to a terminal vinyl group.
(b) A secondary alcohol or ester of a secondary alcohol attached to a terminal vinyl group.
(c) Allyl alcohol or its acetal, ketal, or ester derivative.
(d) Allyl mercaptan, an allyl sulfide, an allyl thioester, or allyl amine.
(e) Acrolein, a methacrolein or their acetals.
(f) Acrylic or methacrylic acid.
(g) An acetylenic compound.
(h) An acyclic aliphatic ketone, ketal, or ketoalcohol with no other functional groups and with four or more carbons on either side of the keto group.
(i) A substance in which the functional groups are all sterically hindered.

→ No → Class I

3.1.2. Step 2

In the second step, the metabolic fate of each substance was predicted by chemical structure and applied to the decision tree approach consisting of two branches: one for substances that are metabolized into innocuous products, and the other for substances that are not. In this second step, all four substances were judged to be easily hydrolyzed into menthol and carboxylic acids by carboxylesterases or esterases, and the resultant substances are metabolized into innocuous products as mentioned above (WHO, 1999b). Then, the evaluation of all substances proceeded to the A-side of the decision tree (Supplementary Fig. 1 Step A3).

3.1.3. Step A3

In this step, estimated daily intake of each substance was compared with the threshold level of human exposure, according to Cramer's structural classes, in which the estimated threshold levels are 1800, 540, and 90 μg/person/day for structural classes I, II, and III, respectively (Munro et al., 1996, 1999).

As mentioned above, the estimated daily intakes of four flavoring substances as calculated by SPET method were higher than those by MSDI method, and were within the range of 60–1250 μg/kg/day. All these intake values were below the threshold of concern applied to structural class I.

3.2. Consideration of combined intakes from the use as flavoring agents

The estimated daily intakes of all four substances in Japan according to the MSDI method were within the range 1.54–4.71 μg/person/day (JFFMA, 2003, 2006, 2011). Because JECFA judges that evaluation of combined intake is not necessary if the highest MSDI value is less than 20 μg/day (WHO, 2011), it was decided to omit evaluation of combined intake of all four substances.

3.3. Assessing toxicological information

As the information for all four substances on *in vitro* and *in vivo* toxicity was absent from the study data accessed and tabulated by JECFA (WHO, 1976, 1998a,b, 1999a,b, 2009), the metabolites of the four substances were alternatively examined for toxicity assessment.

3.3.1. Short-term toxicity

With regard to short-term toxicity, groups of 40 male and 40 female rats of unknown strain were given diets containing L-menthol and DL-menthol at doses of 0, 100, or 200 mg/kg/day for 5.5 weeks (Herken, 1961). As a result, there were no adverse treatment-related effects on the body weight gain, excretion of glucuronate conjugate, water intake, blood electrolytes, or behavioral changes against stimuli.

Groups of 10 male and 10 female B6C3F$_1$ mice were given diets containing DL-menthol at doses of 0, 140, 280, 560, 1100, or 2300 mg/kg/day for 13 days (National Toxicology Program, 1979). Death occurred in six animals during the experimental period without any relationship to dose. The final body weight was unchanged between dosed groups except for a decrease in female mice at 2300 mg/kg. Histopathologically, a significantly increased incidence of perivascular lymphocyte infiltration and interstitial nephritis was noted in the kidneys of female mice at 1100 and 2300 mg/kg.

Groups of 10 male and 10 female rats of unknown strain were given diets containing DL-menthol at doses of 0, 200, 400, or 800 mg/kg/day for 28 days (Thorup et al., 1983a,b; Madsen et al., 1986). Statistically significant increases in absolute and relative liver weights were noted in male rats at all doses and in female rats at the middle and high doses. Hepatocellular vacuolization was noted in four animals at 200 mg/kg, five animals at 400 mg/kg, and four animals at 800 mg/kg; however, it was not judged to be related to the dose of the test substance.

Groups of 10 male and 10 female Fischer 344 rats were given diets containing DL-menthol at doses of 0, 93, 190, 380, 750, or 1500 mg/kg/day for 13 weeks (National Toxicology Program, 1979). A significantly increased incidence of interstitial nephritis was observed in the highest dose group.

Palmitic acid was administered orally to rats (the strain, number of animals, and sex were unknown) for 150 days at a dose of 5000 mg/kg/day and no toxicologically relevant effects were noted (Mori, 1953).

Stearic acid was administered orally to mice (the strain, number of animals, and sex were unknown) for 3 weeks at a dose of 1500 mg/kg/day and again no toxicologically relevant effects were observed (Tove, 1964).

3.3.2. Long-term toxicity

Long-term toxicity and carcinogenicity studies of DL-menthol have been reported for mice and rats. Groups of 50 male and 50 female B6C3F$_1$ mice were given diets containing DL-menthol at doses of 0, 300, or 600 mg/kg for 103 weeks (National Toxicology Program, 1979). A slight decrease in the body weight was noted in the treatment groups. For male mice, the survival ratio in treatment groups was similar to that for the untreated controls. For female mice, the survival ratio in the highest group was lower than that for the untreated controls; however, the survival ratio in all female groups was within the range of background data in the studies conducted under the National Toxicology Program (Haseman et al., 1985). Histopathologically, a statistically significant increased incidence of hepatocellular carcinomas was observed in male mice at the highest dose; however, there was no significant difference in the incidence when compared with background control data for mice of this age and strain. A statistically significant increased incidence of bronchioloalveolar cell adenomas was observed in female mice in the treatment groups; however, there was no statistically significant difference from the background control data (Haseman et al., 1986).

Groups of 50 male and 50 female Fischer 344 rats were given diets containing DL-menthol at doses of 0, 190, or 380 mg/kg for 103 weeks (National Toxicology Program, 1979). The body weight of treatment groups was lower than that in the untreated controls. Mortality in treatment groups was similar to that in the untreated controls. Chronic inflammation in the kidneys was observed in treated groups; however, it was not considered to be a menthol-related effect because this finding is commonly observed in aged Fischer 344 rats. There was no increased incidence of tumors related to DL-menthol in either males or females.

Based on these data, JECFA allocated an acceptable daily intake (ADI) of 4 mg/kg/day for DL-menthol on the basis of the no-observed-effect-level (NOEL) of 380 mg/kg/day in the long-term carcinogenicity study in Fischer 344 rats, by applying a safety factor of 100 (WHO, 1999a). In addition, menthol exists as two optical isomers, DL-menthol and L-menthol. JECFA also concluded that the estimated ADI could be adopted for the L-menthol based on the data providing no indication of any difference in the toxicity of DL-menthol and L-menthol (WHO, 1999a).

3.3.3. Genotoxicity

Several *in vitro* and *in vivo* genotoxicity studies of menthol-related substances have been reported (Andersen and Jennies, 1984; Blevins and Taylor, 1982; Food and Drug Administration, 1975, 1976; Hartmann and Speit, 1997; Heck et al., 1989; Ishidate et al., 1984; Ivett et al., 1989; Morimoto, 2005; Murthy et al., 1991; Myhr and Caspary, 1991; Nohmi et al., 1985; Oda et al., 1978; Shelby et al., 1993; Shimizu et al., 1985; Sofuni et al., 1985; Storer et al., 1996; Tennant et al., 1987; Yoo, 1986). These results are summarized in Tables 3–5. A negative result has been reported

Table 3
Summary of genotoxicity data for DL-menthol.

Test system	Test objects	Concentration	Judgment	Reference (year)
In vitro				
Reverse mutation	*Salmonella typhimurium* [a] (TA92, TA1535, TA100, TA1537, TA94, TA98)	⩽5 mg/plate	Negative	Ishidate et al. (1984)
Reverse mutation	*Salmonella typhimurium* [a]	666 μg/plate	Negative	Tennant et al. (1987)
Reverse mutation	*Salmonella typhimurium* [a] (TA100, TA2637, TA98)	⩽0.5 mg/plate	Negative	Nohmi et al. (1985)
Chromosomal aberration	Chinese hamster fibroblasts	⩽0.2 mg/mL	Negative	Ishidate et al. (1984)
Chromosomal aberration	Chinese hamster lung fibroblasts	⩽0.3 mg/mL	Negative	Sofuni et al. (1985)
Chromosomal aberration and sister chromatid exchange	Chinese hamster ovary cells	⩽250 μg/mL	Negative	Tennant et al. (1987)
Chromosomal aberration and sister chromatid exchange	Chinese hamster ovary cells	⩽167 μg/mL	Negative	Ivett et al. (1989)
Forward mutation	L5178Y mouse lymphoma cells	⩽200 μg/mL	Negative	Tennant et al. (1987)
Forward mutation	L5178Y mouse lymphoma cells	⩽200 μg/mL	Negative	Myhr and Caspary (1991)
In vivo				
Micronucleus formation	Male B6C3F$_1$ mouse bone marrow	⩽1 g/kg body weight	Negative	Shelby et al. (1993)

[a] With and without metabolic activation.

Table 4
Summary of genotoxicity data for *l*-menthol.

Test system	Test objects	Concentration	Judgment	Reference (year)
In vitro				
Reverse mutation	*Salmonella typhimurium* [a] (TA98, TA100, TA2637)	≤0.5 mg/plate	Negative	Nohmi et al. (1985)
Reverse mutation	*Salmonella typhimurium* [a] (TA98, TA100, TA1535, TA1537)	6.4–800 μg/plate	Negative	Andersen and Jennies (1984)
Reverse mutation	*Escherichia coli* (WP2 uvrA)	0.1–0.8 mg/plate	Negative	Yoo (1986)
DNA repair	*Bacillus subtilis*	≤10 mg/disk	Negative	Yoo (1986)
Gene mutation	*Bacillus subtilis*	≤20 mg/plate	Negative	Oda et al. (1978)
Chromosomal aberration	Chinese hamster lung fibroblasts	≤0.125 mg/mL	Negative	Sofuni et al. (1985)
Chromosomal aberration	Human WI-38 embryonic lung cells	10 mg/mL	Negative	Food and Drug Administration (1975)
Chromosomal aberration and sister chromatid exchange	Human peripheral blood lymphocytes	0.1–10 mmol/L	Negative	Murthy et al. (1991)
In vivo				
Host-mediated mutagenicity	*Salmonella typhimurium* TA1530, G46/ICR mouse host	≤5000 mg/kg body weight	Negative	Food and Drug Administration (1975)
Host-mediated mutagenicity	*Saccharomyces cerevisiae* D3/ICR mouse host	≤5000 mg/kg body weight	Negative	Food and Drug Administration (1975)
Chromosomal aberration	Albino rat bone marrow	≤3 g/kg body weight	Negative	Food and Drug Administration (1975)
Dominant lethal mutation	Rat	≤3 g/kg body weight	Negative	Food and Drug Administration (1975)

[a] With and without metabolic activation.

Table 5
Summary of *in vitro* genotoxicity data for D-menthol, octanoic acid, stearic acid and L-menthyl (*R,S*)-3-hydroxybutyrate.

Substance	Test system	Test objects	Concentration	Judgment	Reference (year)
D-Menthol	DNA damage	Rat hepatocytes	0.7–1.3 mmol/L	Positive	Storer et al. (1996)
	DNA damage	Chinese hamster V79 cells	0.5–2 mmol/L	Negative	Hartmann and Speit (1997)
	DNA damage	Human blood cells	0.5–2 mmol/L	Negative	Hartmann and Speit (1997)
Octanoic acid	Plate and suspension assays	*Salmonella typhimurium*[a] (TA1535, TA1537, TA1538)	0.0000625–0.00025%	Negative	Food and Drug Administration (1976)
	Non-activation suspension test	*Saccharomyces cerevisiae* D4	0.000325–0.001300%	Negative	Food and Drug Administration (1976)
	Unscheduled DNA synthesis	Rat hepatocytes	300 μg/mL	Negative	Heck et al. (1989)
	Ames test (plate incorporation assay)	*Salmonella typhimurium*[a] (TA98, TA100, TA1538, TA1535, TA1537)	50 mg/plate	Negative	Heck et al. (1989)
Stearic acid	Modified Ames test (pre-incubation method)	*Salmonella typhimurium*[a] (TA98, TA100, TA1535, TA1537, TA1538)	1–1000 μg/plate	Negative	Shimizu et al. (1985)
	Ames test	*Salmonella typhimurium*[a] (TA98, TA100, TA1535, TA1537, TA1538)	50 μg/plate	Negative	Blevins and Taylor (1982)
L-Menthyl (*R,S*)-3-hydroxybutyrate	Reverse mutation	*Salmonella typhimurium*[a] (TA98, TA100, TA1535, TA1537, TA1538)	78, 156, 312, 625, 1250, 2500, 10,000 μL/plate	Negative	Morimoto (2005)

[a] With and without metabolic activation.

in the Ames assay of L-menthyl (R,S)-3-hydroxybutyrate (WHO, 2009). As a component of menthyl esters, one positive result was reported in an *in vitro* DNA damage test of D-menthol (Storer et al., 1996), but other reverse mutation tests showed negative results. In addition, *in vivo* genotoxicity study results using the mouse micronucleus test, mouse host-mediated mutagenicity test and rat chromosome aberration test were all negative (Food and Drug Administration, 1975; Shelby et al., 1993). For carboxylic acids, it was reported that octanoic acid and stearic acid have no genotoxicity *in vitro* (Blevins and Taylor, 1982; Food and Drug Administration, 1976; Heck et al., 1989; Shimizu et al., 1985).

4. Conclusion of the evaluation based on JECFA procedure

This review has presented the key scientific data relevant to the safety evaluation of menthol-related substances for use as a food flavor using the JECFA evaluation procedure. In the prediction of genotoxicity, all four substances had no structural alerts and it was judged that these substances had no genotoxic potential based on *in silico* software analyses. The available study reports on genotoxicity also provided negative results except for one positive response for D-menthol in a DNA damage test.

In the JECFA evaluation procedure, all four substances were classified as class I according to Cramer's decision tree, which leads to the assumption of low oral toxicity. In addition, all four substances were judged to be immediately metabolized into innocuous products, because these substances are all menthyl esters consisting of menthol and simple carboxylic acids that were assumed to be hydrolyzed after ingestion. The estimated daily intakes for all the substances were within the range of 1.54–4.71 μg/person/day and 60–1250 μg/person/day, using the MSDI and SPET methods, respectively. Even the higher levels estimated

by the SPET method were below the threshold level for human exposure of class I substances of 1800 μg/person/day. From these considerations, it is concluded that all four substances raise no safety concerns for use as flavoring substances at the currently estimated intake levels.

Several toxicological studies revealed that DL-menthol has a minimal systemic toxicity. JECFA established an ADI of 4 mg/kg/day for DL-menthol on the basis of a NOEL of 380 mg/kg/day from long-term carcinogenicity studies in rats. While there are no toxicity study data for these four flavoring substances, the estimated daily intakes of all four substances were well below the ADI level for DL-menthol. Therefore, it is judged that menthol derived from the ingestion of four substances does not exert any systemic toxicity.

5. Conclusions

In conclusion, the use of four substances structurally related to menthol, i.e., L-menthyl 2-methylbutyrate, DL-menthyl octanoate, DL-menthyl palmitate, and DL-menthyl stearate for flavoring food, poses no health risk to humans and the intake of each substance as an added food-flavoring ingredient is safe at the present levels of use.

Conflict of Interest

Y.M., H.O., K.S., F.S., S.H. and S.M. are employed by flavor manufacturers whose product lines include flavoring substances. The views and opinions expressed in this article are those of the authors and not necessarily those of their respective employers. H.A., A.O., M.N., M.D., S.O., M.S. and T.M. declare that no conflicts of interest exist.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.11.044.

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Letter

A new parameter that supports speculation on the possible mechanism of hypothyroidism induced by chemical substances in repeated-dose toxicity studies

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ABSTRACT — Hypothyroidism induced by xenobiotic treatment was analyzed for possible underlying mechanism(s) on the basis of different responses of the thyroid gland and the liver, using a newly-created database of repeated-dose toxicity of 500 chemicals. Two mechanisms are proposed: direct inhibition of thyroid hormone biosynthesis in the thyroid gland, and stimulated degradation of thyroid hormone by induction of hepatic drug-metabolizing enzymes. In the database there were 10 chemicals inducing hypertrophy/hyperplasia of follicular cells in the thyroid gland and having data on thyroid glands. On the basis of the chemical structure and information available in the literature, we judged three chemicals to be typical thioamide derivatives that act directly on the thyroid gland, and the others as non-thioamide derivatives that were unlikely to have any direct action on the thyroid gland. All these chemicals were classified into two groups using the ratios of relative weight increase rate of thyroid gland versus that of the liver. These values were at least 1.7, but 3.2 or more in the most of the cases for thioamide derivatives, and 1.2 or less for non-thioamide derivatives. This background analysis suggests the feasibility of parameter-supported speculation on the possible underlying mechanism when new repeated-dose toxicity data on hypothyroidism becomes available.

Key words: Hypothyroidism mechanism, Hypertrophy, Hyperplasia, Follicular cells, Hepatomegaly, Relative organ weights

INTRODUCTION

The enlargement of the thyroid gland due to hypertrophy and hyperplasia of follicular cells sometimes occurs due to the toxic effects of repeated doses of various chemicals. This can be often understood as hypothyroidism, a functional decline of the thyroid gland. The thyroid gland, although small, is a unique form of tissue that is crucial to the metabolism of the entire body. Thyroid follicular cells produce thyroid hormones such as thyroxine (T_4) and triiodothyronine (T_3). The biosynthesis is enhanced by thyroid-stimulating hormone (TSH). If blood T_4 and T_3 levels fall for any reason, secretion of TSH by the pituitary gland increases due to a loss of negative feedback inhibition along the hypothalamus-pituitary-thyroid axis.

There are several mechanisms that reduce T_4 and T_3 biosynthesis in the thyroid gland, including inhibition of iodine uptake, inhibition of peroxidase, and blockade of thyroid hormone secretion (Capen, 2008). Although numerous chemicals are known to inhibit thyroid peroxidase activity, it is generally believed that thioamide derivatives (such as thiourea, thiouracil, propylthiour-

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acil, methimazole, carbimazole, and goitrin) are the key structural chemicals that inhibit thyroid peroxidase. Propylthiouracil is the most potent chemical in rats, based on observations of a reduction in iodine concentration in the thyroid gland 10 days after oral administration (Astwood *et al.*, 1945; McGinty and Bywater, 1945). There are several other chemicals which are neither structurally related to thioamide derivatives nor belong to groups of other thyroid peroxidase inhibitors (Capen, 2008). Some of them, such as phenobarbital, pregnenolone-16α-carbonitrile, and pyrethrins, induce hepatic microsomal enzymes and disrupt thyroid function in rats (Hood *et al.*, 1999; Finch *et al.*, 2006). The increased activity of uridine 5′-diphospho (UDP)-glucuronyltransferase in the liver by induction of hepatic microsomal enzymes reduces the levels of T_4 in the blood, which leads to a reduction of feedback inhibition along the hypothalamus-pituitary-thyroid axis. The induction of hepatic microsomal enzymes is closely correlated with hepatomegaly and hypertrophy of the centrilobular hepatocytes. At the same time, some increase in thyroid gland weight occurs with hypertrophy of the follicular cells.

Speculation on the mechanism of hypothyroidism caused by each chemical is not easy, since the data set of toxicity tests, obtained according to appropriate test guidelines, does not include any data on mechanisms, such as the enzyme activity of thyroid peroxidase in the thyroid gland and drug metabolism in the liver. Hood *et al.* (1999) demonstrated time-dependent changes in the relative weights of thyroid and liver after administration of phenobarbital, pregnenolone-16α-carbonitrile, and propylthiouracil, accompanied by those of blood T_4 and T_3 levels. The degree of changes in the weights of both organs induced by propylthiouracil appeared to be higher than those induced by hepatic enzyme inducers. In the case of direct action, the liver may show a certain amount of enlargement, although drastic hypertrophy of the thyroid gland is observed at an early stage. In the case of induction of hepatic drug-metabolizing enzymes, the degree of correlation between the response of the liver and the thyroid gland has not hitherto been analyzed in detail. Therefore, it would be valuable if the possible mechanism of hypothyroidism caused by other chemicals could be predicted by comparing the degree of increase in the weights of both organs, if the thyroid gland shows enlargement in repeated-dose studies. The lack of studies giving an accurate comparison of the response of the two organs prompted us to undertake this investigation.

In mid-2012, we released a repeated-dose toxicity database for 500 chemicals, the Hazard Evaluation Support System Database (HESS DB) (Hayashi and Sakuratani, 2011; Abe *et al.*, 2012). The database comprises data on histopathology and organ weight from test results, and has a search function for chemicals that cause specific histopathological changes. We used the HESS DB to select chemicals that caused hypertrophy/hyperplasia of thyroid follicular cells and to retrieve data on relative thyroid and liver weights following administration of these chemicals. We compared the degree of response of both organs, and attempted to clarify whether hypertrophy/hyperplasia of thyroid follicular cells occurs as a result of direct action of the chemicals on the thyroid gland or via induction of drug-metabolizing enzymes in the liver.

MATERIALS AND METHODS

Database

The HESS DB currently contains toxicity data on repeated-dose studies in rats for 500 chemicals. It includes test data on hematology, blood chemistry, absolute and relative organ weight, necropsy and histopathology, and testing conditions, as well as a summary of the study evaluated by experts in toxicology (Hayashi and Sakuratani, 2011; Abe *et al.*, 2012). This database has been accessible at no charge from the website of the National Institute of Technology and Evaluation (NITE), Japan, since June 2012 (http://www.safe.nite.go.jp/english/kasinn/qsar/hess-e.html). Most toxicity studies presented in the HESS DB have been performed in compliance with the standard test guidelines and Good Laboratory Practice; and other studies have been performed in conditions similar to those mentioned in the standard test guidelines and have been reported in peer-reviewed journals. This database comprises the test results for both males and females. From the results of the toxicity study performed according to OECD Guideline 422, "Combined Repeated-dose toxicity Study with a Reproductive/Developmental Toxicity Screening test" (OECD, 1996), data from male rats only were included in the HESS DB because female rats have a history of pregnancy and delivery.

Selection of chemicals that induced hypothyroidism and retrieval of data on thyroid and liver responses

The HESS DB has a search function for chemicals that induce similar histopathological changes (Abe *et al.*, 2012). The search function is based on a thesaurus of histopathological findings constructed for the DB (Nishikawa, 2010). To select chemicals that induce hypothyroidism, "hypertrophy of thyroid gland in the endocrine system" was used as a search term in the histopathology tab of the main window of HESS DB (Fig. 1A). "Hyperplasia of thyroid

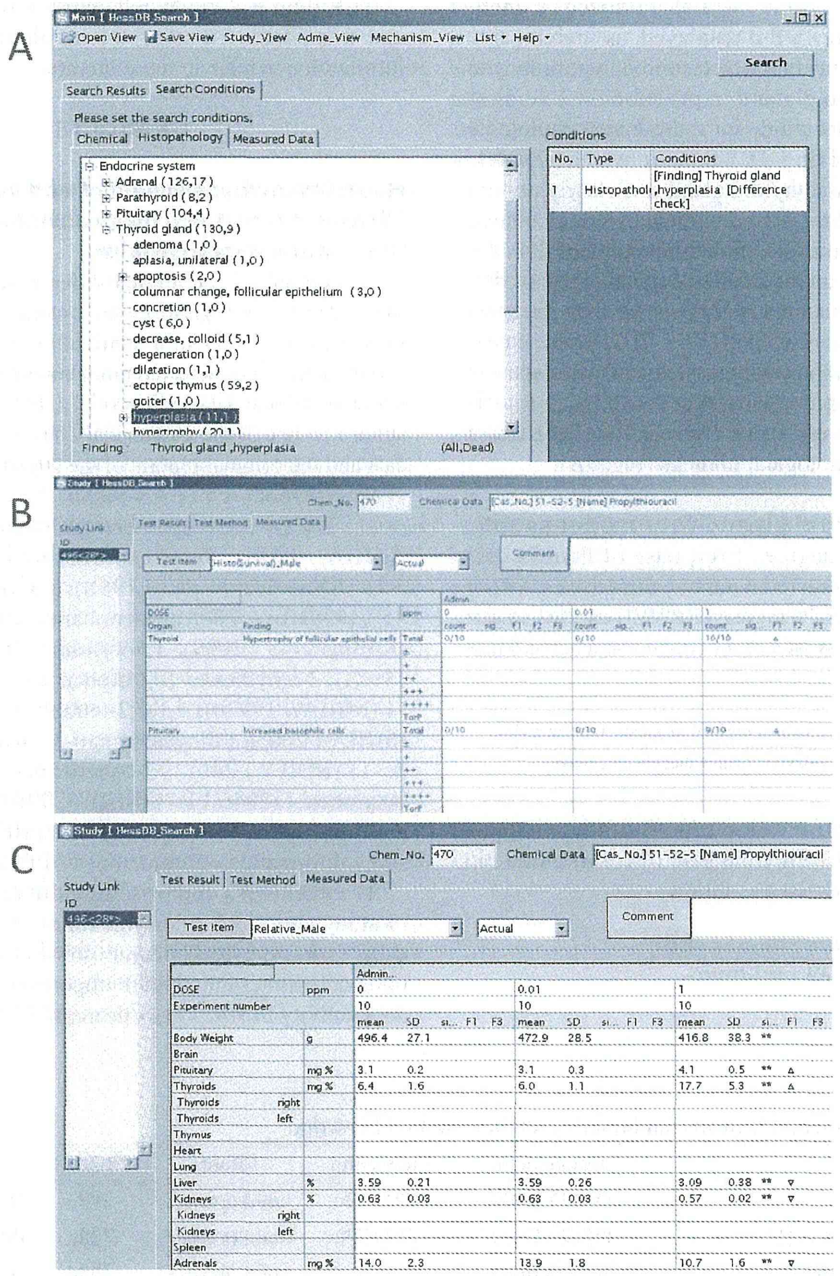

Fig. 1. Searching for chemicals that induce hypothyroidism using HESS DB and retrieval of data on thyroid gland and liver responses. (A) Screen for histopathology search. This is a screenshot showing a search for chemicals that induce hyperplasia of the thyroid gland in the endocrine system. (B) Screen showing histopathology data that reveals the incidences of observed histopathological findings at each dose shown in the toxicity test report. (C) Screen showing relative organ weight data, displaying the organ weight at each dose shown in the toxicity test report. Asterisks indicate the statistically significant differences marked in the toxicity test report (*: $p < 0.05$, **: $p < 0.01$). Open triangles represent toxic signs as defined by professional pathologists (△: increase, ▽: decrease, F1: flag defined by the experts who developed the database. F3: flag defined by the committee overseeing Japan's Chemical Substances Control Law. The details of the procedure for searching for antithyroid chemicals are described in the Materials and Methods section.

gland in endocrine system" was also utilized as another search term. The HESS DB displayed, as search results, the structures of chemicals which caused hypertrophy and hyperplasia of thyroid gland in repeated-dose studies at any dose. The results window for these histopathological findings was opened (Fig. 1B). Studies in which hypertrophy/hyperplasia of the thyroid gland was clearly observed as a toxic effect of the tested chemicals were evaluated and selected on the basis of dose-dependency. For the present analysis, data on the relative organ weights of thyroid gland and liver at all tested doses were then retrieved when such data were available (Fig. 1C). In these cases, incidences of hypertrophy/hyperplasia of follicular cells in the thyroid gland or hypertrophy/swelling of centrilobular hepatocytes were also retrieved from the Results window of histopathological findings (Fig. 1B).

Calculation of thyroid gland-liver response ratio

To quantify the degree of response of thyroid gland and liver at the administered dose in comparison with the control, relative weight increase (ReWI) rate was calculated for respective organs at the highest and second highest doses as follows.

$$\text{ReWI rate} = \frac{\text{relative organ weight at administration dose}}{\text{relative organ weight at control}}$$

Then, to determine the ratio of ReWI of thyroid gland versus that of liver, the thyroid gland-liver response ratio (TLR ratio) was calculated as follows.

$$\text{TLR ratio} = \frac{\text{ReWI rate [thyroid gland]}}{\text{ReWI rate [liver]}}$$

TLR ratio was compared between selected chemicals, and was addressed using the toxicological mechanism information available in the literature.

RESULTS

Hypertrophy/hyperplasia of and weight response of thyroid gland and liver to chemical substances that induce hypothyroidism

We selected 22 chemicals that caused hypertrophy/hyperplasia of the thyroid gland using the HESS DB. We then searched the DB for availability of data on weight of thyroid gland. The selected numbers of chemicals and studies totaled 10 and 11, respectively. The DB contained two studies on one of these chemicals. The names of the chemicals and the original reports of the study are as follows.

Propylthiouracil (CAS number: 51-52-5) (Yamasaki *et al.*, 2002); 2-mercaptobenzimidazole (1) (583-39-1) (MHLW, 1996); 2-mercaptobenzimidazole (2) (583-39-1) (Kawasaki *et al.*, 1998); ethylenethiourea (96-45-7) (MHLW, 2005); phenobarbital sodium (57-30-7) (Kojima *et al.*, 2009); 3,5-xylidine (108-69-0) (MHLW, 1997a); 2-ethylhexan-1-yl diphenyl phosphate (1241-94-7) (MHLW, 1997b); 1,1,2,2-tetrabromoethane (79-27-6) (MHLW, 2003a); dicyclopentan-1-ylsilanediol (211495-85-1) (MHLW, 2001); 2-benzotriazol-2-yl-4,6-di-*tert*-butylphenol (3846-71-7) (MHLW, 2003b); and 4-methyl-2,4-diphenylpent-1-ene (6362-80-7) (MHLW, 2007). The study information is summarized in Table 1.

The chemicals comprise three thioamide derivatives (4 study data) and 7 non-thioamide derivatives (7 study data). It was previously demonstrated that the three thioamide derivatives inhibited thyroperoxidase or lactoperoxidase activity *in vitro*. They decreased T_4 and T_3 levels and

Table 1. Information on chemicals and toxicity studies used in this work.

Chemical	Test guideline	Rat strain	Route	Period	Reference
Propylthiouracil	OECD TG407	CD (SD)	oral (gavage)	28d	Yamasaki *et al.* 2002
2-Mercaptobenzimidazole (1)	OECD TG407	CD (SD)	oral (gavage)	28d	MHLW, 1996
2-Mercaptobenzimidazole (2)	No description	Wistar	oral (gavage)	28d	Kawasaki *et al.* 1998
Ethylenethiourea	OECD TG407	CD (SD)	oral (gavage)	28d	MHLW, 2005
Phenobarbital sodium	No description	F344	oral (gavage)	28d	Kojima *et al.* 2009
3,5-Xylidine	OECD TG407	CD (SD)	oral (gavage)	28d	MHLW, 1997a
2-Ethylhexan-1-yl diphenyl phosphate	OECD TG407	CD (SD)	oral (gavage)	28d	MHLW, 1997b
1,1,2,2-Tetrabromoethane	OECD TG407	CD (SD)	oral (gavage)	28d	MHLW, 2003a
2-Benzotriazol-2-yl-4,6-di-tert-butylphenol	OECD TG407	CD (SD)	oral (gavage)	28d	MHLW, 2003b
4-Methyl-2,4-diphenylpent-1-ene	OECD TG422	CD (SD)	oral (gavage)	42d	MHLW, 2007

increased TSH levels in the blood, and were accompanied by increased thyroid weight in rats (Doerge, 1986, 1988; Doerge and Takazawa, 1990; Freyberger and Ahr, 2006; Kawasaki *et al.*, 1998; Yamasaki *et al.*, 2002). Of the seven non-thioamide derivatives, phenobarbital was shown to induce T_4 and T_3 UDP-glucuronyltransferase activity and increase serum TSH, accompanied by increased liver weight and thyroid follicular cell proliferation (Hood *et al.*, 1999; Hood and Klaassen, 2000; Klaassen and Hood, 2001). An increase in microsomal glucuronyltransferase was observed in rats treated with xylidine isomers (Magnusson *et al.*, 1979). For the remaining five non-thioamides, no particular references were found in terms of enzyme activities or the levels of these hormones in the blood.

We then retrieved the data on incidences of hypertrophy/hyperplasia of follicular cells of the thyroid gland or hypertrophy/swelling of the centrilobular hepatocytes and the relative weights of both organs at all the tested dose from the HESS DB (Table 2). Both the histopathological changes and relative organ weight increases were more prominent in the thyroid gland than in the liver for thioamide derivatives, whereas they were almost equivalent between the two organs for non-thioamide derivatives.

Comparison of the response of thyroid gland versus that of liver for thioamides and non-thioamides

Using the data on relative weight of thyroid gland and liver, relative weight increase rate (ReWI rate) was calculated for both thyroid gland and liver at the two highest doses versus the control dose. The thyroid gland-liver response (TLR) ratio was then calculated as a ratio of ReWI rate [thyroid gland] versus ReWI rate [liver]. These values are shown in Table 3. In the case of propylthiouracil, ReWIs were 2.77 for the thyroid gland and 0.86 for the liver in males at the highest dose (1 mg/kg/day) where the thyroid responses were statistically significant. The TLR ratio was 3.21, suggesting the response of the thyroid gland to be clearly greater than that of the liver. For 2-mercaptobenzimidazole (1), 2-mercaptobenzimidazole (2) and ethylenethiourea, the TLR ratios were 4.59, 8.46 and 1.70, respectively, at the highest doses. Taken together, TLR ratios for thioamide derivatives were at least 1.7, but 3.2 or more than in most cases.

For the seven non-thioamide chemicals (phenobarbital sodium, 3,5-xylidine, 2-ethylhexan-1-yl diphenyl phosphate, 1,1,2,2-tetrabromoethane, dicyclopentan-1-ylsilanediol, 2-benzotriazol-2-yl-4,6-di-tert-butylphenol, and 4-methyl-2,4-diphenylpent-1-ene), the TLR ratios were 0.75, 1.15, 0.86, 0.77, 1.03, 0.55, and 0.87, respectively, at the highest doses. At the second highest dose, hepatic responses generally occurred, but the thyroid gland did not always respond. Taken together, TLR ratios for non-thioamide derivatives were below 1.2.

DISCUSSION

Increased organ weights and hypertrophy/hyperplasia of the follicular cells in the thyroid gland are sometimes observed as toxic effects of repeated administration of chemical substances. In this study, we searched for chemical substances that cause the toxicity, and retrieved the data on relative organ weights of the thyroid gland and liver using a newly-developed repeated-dose toxicity database, the HESS DB (Hayashi and Sakuratani, 2011; Abe *et al.*, 2012). Based on the chemical structure and ratio of relative weight increase of thyroid gland versus liver (TLR ratio), we found a clear difference in the ratio between thioamides, which directly act on thyroid gland, and non-thioamides, which appear to be hepatic drug-metabolizing enzyme inducers.

Pathological changes such as hypertrophy, hyperplasia, and swelling are subtle and often dependent on the judgment of the pathologist. We therefore utilized the relative weights of the thyroid gland and the liver as a quantitative indicator to compare the degree of response of both organs. The final comparison was performed with TLR ratio at doses that usually induced a greater than 10% increase in the weight of the thyroid gland. Our results showed the TLR ratios for thioamide derivatives were at least 1.7, but 3.2 or more in most cases, whereas the ratios were less than 1.2 for non-thioamide derivatives. Given that phenobarbital and xylidine isomers induce hepatic glucuronyltransferase, and that the remaining non-thioamides were not structurally similar to any chemicals known to act directly on the thyroid gland, we conclude that the non-thioamide derivatives cause hypothyroidism by inducing hepatic drug-metabolizing enzymes. The toxicity studies used in our analysis varied according to strain and age of rat, administration route, period and dose, making it difficult to set a single absolute TLR threshold ratio for classification of antithyroid chemicals according to whether they act directly or indirectly. From the analysis in this study, we propose that direct action is highly likely at more than 1.7, indirect mechanisms are probable at less than 1.2, and both mechanisms are possible at 1.2 - 1.7. To achieve a more precise criterion, it will be necessary to look at histopathological data and to determine the inhibitory effect of thyroperoxidase or lactoperoxidase activities *in vitro*, or the biosynthetic activities of thyroid hormones *in vivo*. However, this parame-