Fetuses with external, internal and/or skeletal malformations and/or variations were found in all groups. The malformations and variations observed in the present study are of the types that occur spontaneously among the control rat fetuses [23–26]. At 40 mg/kg bw/day, significantly higher incidences of the total number of fetuses with external and skeletal malformations were detected, and significantly higher incidences of individual types of external and skeletal malformation were also noted. At 20 mg/kg bw/day, the incidence of the total number of fetuses with skeletal malformations was significantly higher than that of control group. Although the incidence of individual types of skeletal malformation was not significantly increased at 20 mg/kg bw/day, types of external and skeletal malformations observed at this dose were the same as those observed at 40 mg/kg bw/day. Consideration of the sum of these findings suggests that a conservative estimate of the LOAEL for the teratogenic dose of DTG is 20 mg/kg bw/day in rats when administered during the time of implantation to the term of pregnancy. DTG caused suppression of body weight gain and neurobehavioral changes in dams and abnormally morphological development and developmental delay in the offspring of rats at 20 and 40 mg/kg bw/day. Therefore, the teratogenic effects of DTG at doses without maternal toxicity, a selective teratogenicity of DTG, was not found in the current study. There are no available reports in which the developmental toxicity of DTG is assessed in any other animal species. Further studies are needed to confirm the reproductive and developmental toxicity of DTG in additional species. Developmental neurotoxicity and multigeneration studies are also required to support the conclusion of the prenatal hazard of DTG.

In conclusion, DTG caused maternal neurobehavioral changes and decreased body weight gain at 20 mg/kg bw/day and higher, embryonic/fetal deaths and lowered fetal weight at 40 mg/kg bw/day, and increased incidence of fetuses with malformations at 20 mg/kg bw/day and higher when administered during the time of implantation to the term of pregnancy in rats.

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References

[1] Scorecard. Chemical profile for 1,3-bis(*o*-tolyl)guanidine (CAS number: 97-39-2) 2005. http://www.scorecard.org/chemical-profiles/summary.tcl?edf_substance_id=+97-39-2.

[2] TOXNET. *N,N'*-bis(2-methylphenyl)guanidine 2005. http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?/temp/.

[3] Weber E, Sonders M, Quarum M, Mclean S, Pou S, Keana JFW. 1,3-Di(2-(5-^{3}H)tolyl)guanidine: a selective ligand that labels σ-type receptors for psychotomimetic opiates and antipsychotic drugs. Proc Natl Acad Sci 1986;83:8784–8.

[4] Bastianetto S, Perralt G, Sanger DJ. Pharmacological evidence for the involvement of sigma sites in DTG-induced contralateral circling in rats. Neuropharmacology 1995;34:107–14.

[5] Kest B, Mogil JS, Sternberg WF, Pechnick RN, Liebeskind JC. Antinociception following 1,3-di-*o*-tolylguanidine, a selective σ receptor ligand. Pharmacol Biochem Behav 1995;50:587–92.

[6] Bejanian M, Pechnick RN, Bova MP, George R. Effects of subcutaneous and intracerebroventricular administration of the *sigma* receptor ligand 1,3-di-*o*-tolylguanidine on body temperature in the rat: interactions with BMY 14802 and rimcazole. J Phamacol Exp Ther 1991;258:88–93.

[7] Rawls SM, Baron DA, Geller EB, Adler MW. Sigma sites mediate DTG-evoked hypothermia in rats. Pharmacol Biochem Behav 2002;73:779–86.

[8] Kest B, Mogil JS, Sternberg WF, Pechnick RN, Liebeskind JC. 1,3-Di-*o*-tolylguanidine (DTG) differentially affects acute and tonic formalin pain: antagonism by rimcazole. Phamacol Biochem Behav 1995;52:175–8.

[9] Maj J, Rogóż Z, Skuza G. Some behavioral effects of 1,3-di-o-tolylguanidine, opipramol and seritraline, the sigma site ligands. Pol J Pharmacol 1996;48:379–95.

[10] Skuza G, Rogóż Z. Effects of 1,3-di-*o*-tolylguanidine (DTG), rimcazole and EMD 57445, the σ receptor ligands, in the forced swimming test. Pol J Phamacol 1997;49:329–35.

[11] Shimizu I, Kawashima K, Ishii D, Oka M. Effects of (+)-pentazocine and 1,3-di-*o*-tolylguanidine (DTG), sigma (σ) ligands, on micturition in anaesthetized rats. Br J Pharmacol 2000;131:610–6.

[12] Skuza G, Rogóż Z. Sigma 1 receptor antagonists attenuate antidepressant-like effect induced by co-administration of 1,3-di-*o*-tolylguanidine (DTG) and memantine in the forced swimming test in rats. Pol J Phamacol 2003;55:1149–52.

[13] Clayson DB, Krewski DR. Objectives of toxicity testing. In: Arnold DL, Grice HC, Krewski DR, editors. Handbook of in vivo toxicity testing. San Diego: Academic Press; 1990. p. 3–18.

[14] RTECS (The Registry of Toxic Effects of Chemical Substances) Guanidine, 1,3-di-*o*-tolyl-; 2005. http://www.cdc.gov/niosh/rtecs/mf155cc0.html.

[15] Ema E, Kimura E, Matsumoto M, Hirose A, Kamata E. Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats. Reprod Toxicol, in press.

[16] OECD (Organisation for Economic Co-operation and Development). OECD Test Guideline for Testing of Chemicals, No. 414, Prenatal Developmental Toxicity Study. Adopted by the Council on 22nd January 2001. Paris.

[17] ME, MHLW, MITI (Ministry of Environment, Ministry of Health, Labour and Welfare, and Ministry of International Trade and Industry of Japan). Research of Designated Chemical Substances, November 2003. Tokyo.

[18] Inouye M. Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red S. Congenit Anom Kyoto 1976;16:171–3.

[19] Nishimura K. A microdissection method for detecting thoracic visceral malformations in mouse and rat fetuses. Congenit Anom Kyoto 1974;14:23–40.

[20] Wilson JG. Methods for administering agents and detecting malformations in experimental animals. In: Wilson JG, Warkany J, editors. Teratology: principles and techniques. Chicago: The University of Chicago Press; 1965. p. 262–77.

[21] Goldstein SR, Matsumoto RR, Thompson TL, Patrick RL, Bowen WD, Walker JM. Motor effects of two sigma ligands mediated by nigrostriatal dopamine neurons. Synapse 1989;4:254–8.

[22] Bastianetto S, Rouquier L, Perralt G, Sanger DJ. DTG-induced circling behaviour in rats may involve the interaction between σ sites and nigro-striatal dopaminergic pathways. Neuropharmacology 1995;34:281–7.

[23] Kameyama Y, Tanimura T, Yasuda M, editors. Spontaneous malformations in laboratory animals-photographic atlas and reference data. Congenit Anom Kyoto 1980;20:25–106.

[24] Morita H, Ariyuki F, Inomata N, Nishimura K, Hasegawa Y, Miyamoto M, Watanabe T. Spontaneous malformations in laboratory animals: frequency

of external, internal and skeletal malformations in rats, rabbits and mice. Congenit Anom Kyoto 1987;27:147–206.

[25] Nakatsuka T, Horimoto M, Ito M, Matsubara Y, Akaike M, Ariyuki F. Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. Congenit Anom Kyoto 1997;37:47–138.

[26] Barnett Jr JF, Lewis D, Tappen A, Hoberman AM, Christian MS. Reproductive indices, fetal gross, visceral and skeletal alterations, sexual maturation, passive avoidance and water maze data, a comparison of results in CD(SD)IGS rats and CD(SD) rats. In: Matsuzawa T, Inoue H, editors. Biological reference data on CD(SD)IGS rats-2000, CD(SD)IGS study group. Yokohama: c/o Charles River Japan, Inc.; 2000. p. 159–73.

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Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Twelve male and female rats per group were exposed to the rubber accelerator 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 8, 20 or 50 mg/kg bw/day. Males were dosed for a total of 49 days beginning 14 days before mating. Females were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. At 50 mg/kg bw/day, deaths were observed in two males and three females. Lowered body weight gain and food consumption were noted in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day. Mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation were observed in males and females at 20 and 50 mg/kg bw/day. No effects of DTG were found on the estrous cyclicity, precoital interval, copulation, fertility and gestational indices, numbers of corpora lutea and implantations, or gestation length. A significant decrease in the number, body weight and viability of offspring and increase in the incidence of fetuses with external malformations were found at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were observed. These data suggest that DTG may be teratogenic. The NOAELs of DTG for general and developmental toxicity in rats are 8 and 20 mg/kg bw/day, respectively.

Keywords: Di-*o*-tolylguanidine; Rubber accelerator; Sigma ligand; Reproductive and developmental toxicity; Teratogenicity; Malformation; Rat

1. Introduction

The basic rubber accelerator 1,3-di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the United States [1,2]. DTG is known as a selective sigma ligand [3]. In this context, many pharmacological studies of DTG were performed [3–12]. Ligands that interact with sigma sites have been shown to produce hypothermia [4–6]. Hypothermia induced by DTG was detected following subcutaneous or intracerebroventricle injection in rats [5,6] and intraperitoneal injection in mice [4]. The intraperitoneal injection of DTG potently reduced the pain behavior in the acute but increased pain behavior in the tonic phase in the formalin test in mice [7]. Intraperitoneal injection of DTG produced significant but short-lived increases in the withdrawal latencies in mice [4]. Bastianetto et al. [8] showed that unilateral intranigral injection caused circulating behavior in rats and suggested that sigma sites play a role in movement and posture through their association with brainstem and forebrain motor control circuits. Decreased locomotor activity induced by intraperitoneal injection [9,10], increased bladder capacity induced by intravenous injection in the anaesthetized condition [11] and no change in immobility time in open field after intraperitoneal injection [12] were also reported in rats given DTG. Toxicological studies on DTG have given little information on acute animal toxicity [13]: intraperitoneal LD50 was 25 mg/kg bw in mice; oral LD50 was 500 mg/kg bw in rats; lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. At the present time, no information is available for the reproductive and developmental toxicity of DTG. It is generally assumed that the results of animal test on chemical toxicity are relevant to human health [14]. As such, the testing for reproductive and developmental toxicity

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in animal models is an important part of the overall toxicology. The present study was conducted to obtain information on the effects of DTG on reproductive and developmental parameters in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15] and in accordance with the principles for Good Laboratory Practice [16,17] and "Guidance for Animal Care and Use" of Panapharm Laboratories Co., Ltd.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxic studies, including reproductive and developmental toxicity studies, and historical control data are available. Males and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared using wooden chips as bedding (White Flake; Charles River Japan, Inc.).

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water ad libitum and maintained in an air-conditioned room at 24 ± 2 °C, with a relative humidity of 55 ± 10%, a 12-h light/12-h dark cycle and ventilation with 13–15 air charges per hour.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform and very soluble in ether, and its melting point is 179 °C, specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot No. 30J08) used in this study was 99.6% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 8, 20 or 50 mg/kg bw. The dosage levels were determined based on the results of our previous dose-finding study, the 14-day repeated dose toxicity study in rats given DTG by gavage at 0, 10, 20, 40 or 80 mg/kg bw/day, in which deaths were found at 80 mg/kg bw/day, decreased locomotor activity, mydriasis, tremor and salivation were observed at 40 and 80 mg/kg bw/day, and no adverse effects were detected at 10 and 20 mg/kg bw/day (data not shown). DTG was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. Males (12 rats/group) were dosed for a total of 49 days beginning 14 days before mating. Females (12 rats/group) were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 ml/kg body weight based on the latest body weight during the re-mating and mating period in males and females or the body weight on day 0 of pregnancy in females after copulation. Control rats were given 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and the target concentration was 96.5 to 101.4%.

2.3. Observations

All rats were observed daily for clinical signs of toxicity. The body weight was recorded twice a week in males, and twice a week during the pre-mating and mating periods, on days 0, 7, 14 and 21 of pregnancy and on days 0 and 4 of lactation in females. Food consumption was recorded twice weekly during the pre-mating period in males, and twice weekly during the pre-mating period, on days 1, 7, 14 and 21 of pregnancy and on days 1 and 4 of lactation in females. The rats were euthanized by exsanguination under anesthesia on the next day of the last administration in males and on day 4 of lactation in females. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testes and epididymides were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovaries were stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin–eosin-stained tissue sections of these organs.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the pre-mating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period, 2 weeks, had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before noon from day 20 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 12:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded. Gender was determined on live pups examined grossly and individually weighed on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross internal examinations were performed.

2.4. Data analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, body weight gain, food consumption, length of estrous cycles, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups and weight of live pups were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous the data were analyzed using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the DTG-treated groups were compared with that of the control group with Steel's multiple comparison test. The implantation, delivery and viability indexes, and incidence of pups with anomalies and individual anomalies were analyzed with Wilcoxon's rank sum test. The mortality, copulation, fertility and gestation indexes, and sex ratio of pups were analyzed with Fisher's exact test. The 5% level of probability was used as the criterion for significant.

3. Results

Table 1 shows the findings in male rats given DTG. At 50 mg/kg bw/day, one male died after six administrations and one male died after seven administrations. These dead rats showed mydriasis, decreased locomotor activity, bradypnea, a prone position and tremor 10–20 min after the administration of DTG. In surviving males, mydriasis, decreased locomotor activity, bradypnea and prone position on days 1–9 of the administration period, tremor during the whole period of administration and salivation on days 22–49 of the administration period were also observed at 50 mg/kg bw/day. Salivation was noted on days 28–49 of the administration period at 20 mg/kg bw/day. A significant decrease in the body weight gain was found on days 1–8 (81% decrease) and days 15–22 (48% decrease) of the administration period at 50 mg/kg bw/day. At this dose, significantly lower food consumption on days 7–8 (20% decrease) and days 14–15 (7% decrease) of the administration period was also observed.

Table 1
Findings in male rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of male rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	30 ± 7	33 ± 7	25 ± 7	6 ± 9**
Days 8–15	29 ± 5	32 ± 5	32 ± 7	24 ± 7
Days 15–22	23 ± 6	25 ± 8	23 ± 7	12 ± 11**
Days 22–29	19 ± 9	22 ± 7	25 ± 8	19 ± 5
Days 29–36	22 ± 6	22 ± 6	23 ± 7	18 ± 8
Days 36–43	15 ± 8	12 ± 9	13 ± 5	14 ± 7
Days 43–50	19 ± 8	19 ± 7	13 ± 4	13 ± 11
Food consumption (g/day/rat)[a]				
Days 7–8	25 ± 3	26 ± 3	26 ± 2	20 ± 3**
Days 14–15	29 ± 2	30 ± 2	29 ± 3	27 ± 3*
Days 29–30	27 ± 2	27 ± 3	28 ± 3	25 ± 2
Days 35–36	28 ± 2	29 ± 2	29 ± 2	27 ± 2
Days 42–43	26 ± 3	25 ± 3	27 ± 4	27 ± 3
Days 49–50	28 ± 4	29 ± 3	28 ± 2	28 ± 3

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 2 presents the findings in female rats given DTG. At 50 mg/kg bw/day, two females died after the first administration and one female died after normal delivery of her pups on day 22 of pregnancy. Mydriasis, decreased locomotor activity, bradypnea, prone position, and tremor and salivation 10–20 min after the administration of DTG were observed in females died after the first administration. These clinical signs and salivation were found during pregnancy and on day of parturition in a female which died after parturition. In surviving females, mydriasis, decreased locomotor activity, bradypnea and prone position on day 1 of the administration period to day 0 of lactation, tremor on day 1 of the administration period to day 5 of pregnancy and salivation on day 4 of pregnancy to day 3 of lactation were observed at 50 mg/kg bw/day. Mydriasis, decreased locomotor

Table 2
Findings in female rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of female rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
No. of deaths during pregnancy	0	0	0	1
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	19 ± 8	17 ± 7	11 ± 6*	−1 ± 9**
Days 8–15	10 ± 7	15 ± 8	20 ± 5**	15 ± 10
Days 0–7 of pregnancy	34 ± 6	31 ± 6	33 ± 4	28 ± 8
Days 7–14 of pregnancy	34 ± 5	34 ± 4	36 ± 3	30 ± 10
Days 14–21 of pregnancy	85 ± 17	100 ± 14	105 ± 9*	42 ± 21**
Days 0–4 of lactation	20 ± 19	14 ± 16	22 ± 9	16 ± 13
Food consumption (g/day/rat)[a]				
Days 7–8	22 ± 3	21 ± 2	19 ± 2**	13 ± 3**
Days 14–15	20 ± 4	22 ± 3	22 ± 2	20 ± 2
Days 6–7 of pregnancy	22 ± 3	23 ± 2	23 ± 3	17 ± 3**
Days 13–14 of pregnancy	23 ± 2	24 ± 3	25 ± 2	22 ± 5
Days 20–21 of pregnancy	24 ± 4	26 ± 3	29 ± 3*	21 ± 5
Days 3–4 of lactation	41 ± 5	41 ± 3	46 ± 4*	32 ± 6**

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 3
Reproductive findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of pairs	12	12	12	10
Length of estrous cycles (day)[a]	4.0 ± 0.2	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2
Precoital interval (day)[a]	3.0 ± 1.0	2.7 ± 1.0	2.4 ± 1.1	2.2 ± 1.0
Copulation index (%)[b]				
Male	100	91.7	100	100
Female	100	91.7	100	100
Fertility index (%)[c]	100	100	91.7	100
Gestation index (%)[d]	100	100	100	90.0
Gestation length (day)[a]	22.6 ± 0.5	22.3 ± 0.5	22.5 ± 0.5	22.6 ± 0.5
Weight of testes (g)[a]	3.24 ± 0.34	3.34 ± 0.19	3.31 ± 0.28	3.30 ± 0.24
Relative weight of testes[a,e]	0.60 ± 0.05	0.62 ± 0.07	0.63 ± 0.07	0.68 ± 0.07*
Weight of epididymides (g)[a]	1.16 ± 0.10	1.21 ± 0.06	1.21 ± 0.12	1.23 ± 0.07
Relative weight of epididymides[a,e]	0.22 ± 0.02	0.22 ± 0.02	0.23 ± 0.03	0.25 ± 0.02**
Weight of ovaries (mg)[a]	101 ± 8	106 ± 6	101 ± 11	102 ± 10
Relative weight of ovaries[a,e]	30 ± 2	31 ± 2	28 ± 3	32 ± 2

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of rats copulated/no. of pairs) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females copulated) × 100.
[d] Gestation index (%) = (no. of females with parturition/no. of females copulated) × 100.
[e] Relative weight = organ weight/100 g of body weight.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

activity, bradypnea and prone position on days 2–3 of the administration period, and salivation on day 14 of pregnancy to day 3 of lactation were observed at 20 mg/kg bw/day. Body weight gain was significantly lowered on days 1–8 of the pre-mating period at 20 mg/kg bw/day (42% decrease) and on days 1–8 of the pre-mating period (105% decrease) and days 14–21 of pregnancy (49% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significantly higher body weight gain was observed on days 8–15 of the pre-mating period and days 14–21 of pregnancy. Food consumption was significantly reduced on days 7–8 of the pre-mating period at 20 mg/kg bw/day (14% decrease) and on days 7–8 of the pre-mating period (41% decrease) and days 3–4 of lactation (24% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significant increase in the food consumption was observed on days 20–21 of pregnancy and days 3–4 of lactation.

The reproductive findings in rats given DTG are presented in Table 3. No effects of DTG were observed on the length of estrous cycles, precoital interval and gestation length. One pair did not copulate at 8 mg/kg bw/day, one female did not become impregnated at 20 mg/kg bw/day and one female did not deliver any pups at 50 mg/kg bw/day; however, no significant differences were noted in the copulation, fertility or gestation index between the control and DTG-treated groups. The weights of the testes and epididymides, and absolute weight and relative weight of the ovaries in the DTG-treated groups did not differ from the control group. The relative weights of the testes (13% increase) and epididymides (14% increase) were significantly higher at 50 mg/kg bw/day.

The developmental findings in rats given DTG are shown in Table 4. There was no significant difference in the numbers of corpora lutea, implantations and stillborns, implantation index, sex ratio of live pups, viability index on day 0 of lactation and body weight of live pups on day 4 of lactation between the control and DTG-treated groups. The numbers of pups delivered (45% decrease) and live pups delivered (45% decrease) and delivery index (43% decrease) were significantly lowered at 50 mg/kg bw/day. At this dose, the viability index on day 4 of lactation (34% decrease) and body weight of live male (16% decrease) and female (19% decrease) pups on day 0 of lactation were also significantly decreased. Two dams with totally litter loss were observed. No poor maternal behavior or nursing was observed in dams at 50 mg/kg bw/day. No histopathological changes were found in the testes, epididymides and ovaries in the DTG-treated groups. External anomalies in pups of rats given DTG are also presented in Table 4. No fetuses with external malformations were observed in the control and groups given DTG at 8 and 20 mg/kg bw/day. At 50 mg/kg bw/day, fetuses with external malformations were found in 10 out of the 65 fetuses and in 3 out of the 9 litters. Oligodactyly was observed in four pups in two litters. A kinked tail was found in six pups in one litter and a short tail and anal atresia was observed in one pup in each letter. Although there was no significant difference in the incidence of fetuses with individual malformations between the control and 50 mg/kg bw/day groups, a significantly higher incidence of total number of fetuses with external malformations was noted at this dose.

Table 4
Developmental findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of litters	12	11	11	9
No. of implantations[a]	14.3 ± 2.6	16.2 ± 1.9	15.9 ± 1.4	14.2 ± 3.6
Implantation index (%)[b]	92.2	94.7	97.6	90.9
No. of pups delivered[a]	13.0 ± 2.4	15.2 ± 2.0	14.7 ± 1.4	7.2 ± 4.1**
No. of live pups delivered[a]	13.0 ± 2.4	15.1 ± 1.9	14.7 ± 1.4	7.2 ± 4.1**
No. of stillborns	0	0.1 ± 0.3	0	0
Delivery index (%)[c]	91.0	93.3	92.2	51.7**
Sex ratio of live pups (males/females)	71/85	84/82	80/82	31/34
Viability index (%)[d,e]				
Day 0 of lactation	100	99.5	100	100
Day 4 of lactation	99.4	99.4	100	65.4**
Body weight of male pups during lactation (g)[a]				
Day 0	7.4 ± 0.7	6.9 ± 0.6	7.3 ± 0.6	6.2 ± 1.0**
Day 4	11.9 ± 1.3	11.1 ± 1.0	11.7 ± 1.0	11.0 ± 2.3
Body weight of female pups during lactation (g)[a]				
Day 0	7.0 ± 0.7	6.6 ± 0.6	6.8 ± 0.7	5.7 ± 0.8**
Day 4	11.4 ± 1.3	10.5 ± 1.0	11.0 ± 0.9	10.5 ± 2.0
External examination of pups				
No. of pups (litters) with malformations	0	0	0	10 (3)*
Oligodactyly	0	0	0	4 (2)
Kinky tail	0	0	0	6 (1)
Short tail	0	0	0	1
Anal atresia	0	0	0	1

[a] Values are given as the mean ± S.D.
[b] Implantation index (%) = (no. of implantations/no. of corpora lutea) × 100.
[c] Delivery index (%) = (no. of live pups delivered/no. of implantations) × 100.
[d] Viability index on day 0 of lactation (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
[e] Viability index on day 4 of lactation (%) = (no. of live pups on day 4 of lactation/no. of live pups delivered) × 100.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

4. Discussion

The present study was conducted to obtain initial information on the possible effects of DTG on reproduction and development in rats. The data show that DTG exerts developmental toxicity and suggest that DTG possesses teratogenic potential.

DTG was given to males during the pre-mating and mating periods and to females during the pre-mating, mating, pregnancy and shortly after parturition. The dosage used in the present study was sufficiently high such that it should be expected to induce general toxic and neurobehavioral effects. As expected, general toxicity, such as decreases in body weight gain and food consumption, was found at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females. Decreases in the body weight gain and food consumption during the early administration period, and thereafter, significant increases in body weight gain and food consumption were observed in females at 20 mg/kg bw/day. One possible explanation for increased body weight gain during late pregnancy at 20 mg/kg bw/day may be higher number of pups and higher net weight gain during pregnancy at this dose compared with the controls. Such recovery did not occur at the highest dose. Neurobehavioral effects, such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and salivation, were also observed at 20 and 50 mg/kg bw/day. DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,18,19]. It was reported that systemic injection of DTG caused neurobehavioral changes in rats [5,6,9,10]. The present study shows that the oral administration of DTG also induces neurobehavioral changes, and it is neurobehaviorally toxic at 20 and 50 mg/kg bw/day in rats.

Higher relative weights, but not the absolute weight, of the testes and epididymides were observed at 50 mg/kg bw/day. Body weights of male rats on the day of scheduled sacrifice were 537 and 485 g in the control and 50 mg/kg bw/day groups, respectively. It seems likely that the higher relative weights of the testes and epididymides at the highest dose were due to secondarily lowered body weight but not due to the direct effects of DTG on the male reproductive organs. Other male reproductive parameters were not significantly changed, even at the highest dose. These findings suggest that DTG is not reproductively toxic to male rats. It seems unlikely that DTG exerts reproductive toxicity to female rats when administered during the pre-mating, mating, pregnancy and early lactation period, because no adverse effects on the maternal reproductive parameters, including estrous cyclicity, precoital interval, copulation

index, fertility index, gestation index, gestation length and ovarian weight, were caused by the administration of DTG in females.

As for the developmental indexes, decreases in the numbers of total pups and live pups delivered, delivery index, viability on PND 4 and body weight of live pups on PND 0 were detected at 50 mg/kg bw/day. These findings indicate that DTG is toxic to the survival and growth of offspring and exerts developmental toxicity at 50 mg/kg bw/day in rats.

In the present study, the teratogenic effect of DTG is strongly suggested by the external examinations of pups. At 50 mg/kg bw/day, a significant increase in the total number of fetuses with external malformations was noted; however, incidences of fetuses with individual types of external malformations at this dose were not significantly different from those in the control group. The external malformations observed in the present study are of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]. In the present study, only external examination in the newborn rats was performed, and no internal or skeletal examinations were performed. Even animals not ordinarily carnivorous, including nonhuman primates, are likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [24]. To accurately evaluate the prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals [24,25]. The present study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15], and this screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. In order to further evaluate the developmental toxicity, including teratogenicity, of DTG in rats, a prenatal developmental toxicity study is currently in progress.

In conclusion, DTG caused decreased body weight gain and food consumption at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females, neurobehavioral changes at 20 and 50 mg/kg bw/day in both sexes, and changes in developmental parameters at 50 mg/kg bw/day. DTG is suggested to be teratogenic. The NOAELs of DTG for general and developmental toxicity were 8 and 20 mg/kg bw/day, respectively, in rats.

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References

[1] Scorecard. Chemical profile for 1,3-bis(*o*-tolyl)guanidine (CAS number: 97-39-2); 2005 [http://www.scorecard.org/chemical-profiles/summary.tcl?edf_substance_id=+97-39-2].

[2] TOXNET. *N,N'*-Bis(2-methylphenyl)guanidine; 2005 [http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?/temp].

[3] Weber E, Sonders M, Quarum M, Mclean S, Pou S, Keana JFW. 1,3-Di (2-(5-^{3}H)tolyl)guanidine: a selective ligand that labels σ-type receptors for psychotomimetic opiates and antipsychotic drugs. Proc Natl Acad Sci 1986;83:8784–8.

[4] Kest B, Mogil JS, Sternberg WF, Pechnick RN, Liebeskind JC. Antinociception following 1,3-di-*o*-tolylguanidine, a selective σ receptor ligand. Pharmacol Biochem Behav 1995;50:587–92.

[5] Bejanian M, Pechnick RN, Bova MP, George R. Effects of subcutaneous and intracerebroventricular administration of the *sigma* receptor ligand 1,3-di-*o*-tolylguanidine on body temperature in the rat: interactions with BMY 14802 and rimcazole. J Pharmacol Exp Ther 1991;258:88–93.

[6] Rawls SM, Baron DA, Geller EB, Adler MW. Sigma sites mediate DTG-evoked hypothermia in rats. Pharmacol Biochem Behav 2002;73:779–86.

[7] Kest B, Mogil JS, Sternberg WF, Pechnick RN, Liebeskind JC. 1,3-Di-*o*-tolylguanidine (DTG) differentially affects acute and tonic formalin pain: antagonism by rimcazole. Pharmacol Biochem Behav 1995;52:175–8.

[8] Bastianetto S, Perralt G, Sanger DJ. Pharmacological evidence for the involvement of sigma sites in DTG-induced contralateral circling in rats. Neuropharmacology 1995;34:107–14.

[9] Maj J, Rogóż Z, Skuza G. Some behavioral effects of 1,3-di-*o*-tolylguanidine, opipramol and seritraline, the sigma site ligands. Pol J Pharmacol 1996;48:379–95.

[10] Skuza G, Rogóż Z. Effects of 1,3-di-*o*-tolylguanidine (DTG), rimcazole and EMD 57445, the σ receptor ligands, in the forced swimming test. Pol J Pharmacol 1997;49:329–35.

[11] Shimizu I, Kawashima K, Ishii D, Oka M. Effects of (+)-pentazocine and 1,3-di-*o*-tolylguanidine (DTG), sigma (σ) ligands, on micturition in anaesthetized rats. Brit J Pharmacol 2000;131:610–6.

[12] Skuza G, Rogóż Z. Sigma receptor antagonists attenuate antidepressant-like effect induced by co-administration of 1,3di-*o*-tolylguanidine (DTG) and memantine in the forced swimming test in rats. Pol J Pharmacol 2003;55:1149–52.

[13] RTECS (The Registry of Toxic Effects of Chemical Substances). Guanidine, 1,3-di-*o*-tolyl; 2005 [http://www.cdc.gov/niosh/rtecs/mf155cc0.html].

[14] Clayson DB, Krewski DR. Objectives of toxicity testing. In: Arnold DL, Grice HC, Krewski DR, editors. Handbook of in vivo toxicity testing. San Diego: Academic Press; 1990. p. 3–18.

[15] OECD (Organization for Economic Co-operation and Development). OECD Test Guideline for Testing of Chemicals, No. 421, Reproduction/Developmental Toxicity Screening Test. Adopted by the Council on 27th July 1995. Paris; 1995.

[16] OECD (Organization for Economic Co-operation and Development). OECD Principles on Good Laboratory Practice (as revised in 1997). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1. Paris; 1998.

[17] EA, MHW and MITI (Environment Agency, Ministry of Health and Welfare, and Ministry of International Trade and Industry of Japan). Research of Designated Chemical Substances, Planning and Coordination Bureau, Environment Agency, No. 39, Environmental Health Bureau, Ministry of Health and Welfare, No. 229, Basic Industries Bureau, Ministry of International Trade and Industry, No. 85, March 31, 1984, and amendments, November 18, 1988. Tokyo; 1988.

[18] Goldstein SR, Matsumoto RR, Thompson TL, Patrick RL, Bowen WD, Walker JM. Motor effects of two sigma ligands mediated by nigrostriatal dopamine neurons. Synapse 1989;4:254–8.

[19] Bastianetto S, Rouquier L, Perralt G, Sanger DJ. DTG-induced circling behaviour in rats may involve the interaction between σ sites and nigrostriatal dopaminergic pathways. Neuropharmacology 1995;34:281–7.

[20] Kameyama Y, Tanimura T, Yasuda M, editors. Spontaneous malformations in laboratory animals—photographic atlas and reference data. Cong Anom 1980;20:25–106.

[21] Morita H, Ariyuki F, Inomata N, Nishimura K, Hasegawa Y, Miyamoto M, et al. Spontaneous malformations in laboratory animals: frequency of external, internal and skeletal malformations in rats, rabbits and mice. Cong Anom 1987;27:147–206.

[22] Nakatsuka T, Horimoto M, Ito M, Matsubara Y, Akaike M, Ariyuki F. Japan Pharmaceutical Manufacturers Association (JPMA) survey on

background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. Cong Anom 1997;37:47–138.

[23] Barnett Jr JF, Lewis D, Tappen A, Hoberman AM, Christian MS. Reproductive indices, fetal gross, visceral and skeletal alterations, sexual maturation, passive avoidance and water maze data, a comparison of results in CD(SD)IGS rats and CD(SD) rats. In: Matsuzawa T, Inoue H, editors. Biological reference data on CD(SD)IGS rats-2000, CD(SD)IGS study group. Yokohama: c/o Charles River Japan, Inc.; 2000. p. 159–73.

[24] Wilson JG. Collection and interpretation of results. In: Wilson JG, editor. Environment and birth defects. New York: Academic Press; 1973. p. 173–93.

[25] Wilson JG. Methods for administering agents and detecting malformations in experimental animals. In: Wilson JG, Warkany J, editors. Teratology: principles and techniques. Chicago: The University of Chicago Press; 1965. p. 262–77.

PACAP Receptor (PAC1-R) Expression in Rat and Rhesus Monkey Thymus

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ABSTRACT: **The expression of PACAP receptor (PAC1-R) was investigated in the thymus of rats and rhesus monkeys. In the rat thymus, PAC1-R positive cells were found in the intermediate type of thymic epithelial cells of the medulla. PAC1-R-positive cells were also seen in the thymic medulla of the rhesus monkey. The thymus showed unusual structures in some rhesus monkey dams (F0) and offspring (F1) exposed to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). Additionally, in these rhesus monkeys, PAC1-R expression was different from that in the control thymus.**

KEYWORDS: **rat thymus; rhesus monkey thymus; TCDD**

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional and pleiotropic signal molecule[1] with important activities related to immunity. We previously reported that the PACAP receptor (PAC1-R) is strongly expressed in stromal cells in the medulla of the rat thymus and that its expression is affected by irradiation.[2] In the present article we present a detailed

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FIGURE 1. (A) Immunohistochemical analysis of PAC1-R in the rat thymus (x40). (B) Immunoelectron microscopy analysis of PAC1-R. Arrows indicate PAC1-R-immunoreactivity in an intermediate type of thymic epithelial cell magnification (16,000x).

investigation of PAC1-R-positive cells in the rat thymus and examine thymus cells of rhesus monkeys exposed to tetrachlorodibenzo-*p*-dioxin (TCDD), a chemical known to impair thymus and T cells.[3]

MATERIALS AND METHODS

The cell type expressing PAC1-R in the thymus of young-adult Dark Agouti (DA) rats was determined immunohistochemically. TCDD (300 ng/kg) was administered subcutaneously to pregnant rhesus monkeys. Five percent of the

FIGURE 2. Immunohistochemical analysis of PAC1-R in the rhesus monkey thymus (x100). (A) normal. (B, C) TCDD-exposed (B: dam, C: offspring). In TCDD-exposed rhesus thymuses (B, C), discrete cortex and medulla were not seen.


cortex
medulla
(A)
(B)
(C)

initial dose was then administered every 30 days until day 90 after delivery.[4] Thymuses from dams (F0) and offspring (F1) exposed to TCDD were analyzed immunohistochemically using the antibody G51 to detect the intercellular region of PAC1-R.[2,5]

RESULTS

PAC1-R expression in the thymus was similar in rats and rhesus monkeys (FIGS. 1A and 2A). In the rat, immunoelectron microscopic analysis detected PAC1-R in the intermediate type of thymic epithelial cells of the thymic medulla (FIG. 1B). Thymocytes and other types of stromal cells were not positive for PAC1-R. Similarly, in the rhesus monkeys, PAC1-R was strongly expressed in the epithelial cells of the medulla (FIG. 2A). However, in some thymuses from TCDD-exposed F0 and F1 rhesus monkeys, a discrete cortex and medulla was not seen. In these instances fewer PAC1-R-positive cells were seen than in the control thymuses (FIGS. 2B and 2C).

DISCUSSION

PACAP is thought to regulate stromal cells in the central nervous system.[5] In the rat thymus, PAC1-R was detected in the intermediate type of thymic epithelial cells (FIGS. 1A and 1B). Our results suggest that PACAP may have an important role in the regulation of thymocyte maturation and/or proliferation.

Similarly, PAC1-R-positive cells were also seen in the rhesus monkey thymuses (FIG. 2A), and PACAP may also work as a regulator of thymocyte functions in rhesus monkeys. PAC1-R expression in TCDD-exposed thymuses appeared to be different from those of controls (FIG. 2B), suggesting that TCDD affects the thymus. Furthermore, TCDD may affect offspring thymuses via the placenta and/or through milk (FIG. 2C).

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REFERENCES

1. VAUDRY, D., B.J. GONZALEZ, M. BASILLE, *et al.* 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to function. Pharmacol. Rev. **52:** 269–324.

2. TOKUDA, N. *et al.* 2004. Expression of PAC1 receptor in rat thymus after irradiation. Regul. Pept. **123:** 167–172.
3. CAMACHO, I.A *et al.* 2004. Evidence for induction of apoptosis in T cells from murine fetal thymus following perinatal exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicol. Sci. **78:** 96–106.
4. YASUDA, I. *et al.* 2005. In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affects tooth development in rhesus monkeys. Reprod. Toxicol. **20:** 21–30.
5. SHIODA, S. *et al.* 2003. PACAP Receptor signaling. *In* Pituitary Adenylate Cyclase-Activating Polypeptide. H. Vaudry & A. Arimura, Eds.: 95–124. Kluwer Academic Publishers. Norwell, MA.

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"Per cell" normalization method for mRNA measurement by quantitative PCR and microarrays

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Abstract

Background: Transcriptome data from quantitative PCR (Q-PCR) and DNA microarrays are typically obtained from a fixed amount of RNA collected per sample. Therefore, variations in tissue cellularity and RNA yield across samples in an experimental series compromise accurate determination of the absolute level of each mRNA species per cell in any sample. Since mRNAs are copied from genomic DNA, the simplest way to express mRNA level would be as copy number per template DNA, or more practically, as copy number per cell.

Results: Here we report a method (designated the "Percellome" method) for normalizing the expression of mRNA values in biological samples. It provides a "per cell" readout in mRNA copy number and is applicable to both quantitative PCR (Q-PCR) and DNA microarray studies. The genomic DNA content of each sample homogenate was measured from a small aliquot to derive the number of cells in the sample. A cocktail of five external spike RNAs admixed in a dose-graded manner (dose-graded spike cocktail; GSC) was prepared and added to each homogenate in proportion to its DNA content. In this way, the spike mRNAs represented absolute copy numbers per cell in the sample. The signals from the five spike mRNAs were used as a dose-response standard curve for each sample, enabling us to convert all the signals measured to copy numbers per cell in an expression profile-independent manner. A series of samples was measured by Q-PCR and Affymetrix GeneChip microarrays using this Percellome method, and the results showed up to 90 % concordance.

Conclusion: Percellome data can be compared directly among samples and among different studies, and between different platforms, without further normalization. Therefore, "percellome" normalization can serve as a standard method for exchanging and comparing data across different platforms and among different laboratories.

Background

Normalization of gene expression data between different samples generated in the same laboratory using a single platform, and/or generated in different geographical

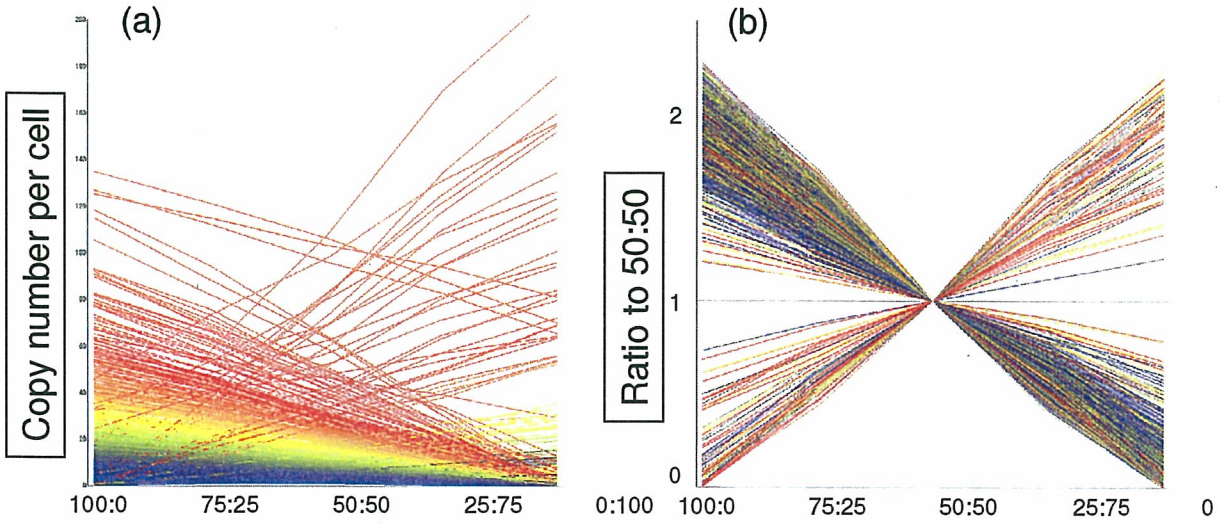

Figure 1
Dose-response linearity check by LBM. Dose-response linearity of the Affymetrix GeneChip by the LBM (liver-brain mix) sample set. Five samples, i.e. mixtures of mouse liver and brain at ratios of 100:0, 75:25, 50:50, 25:75 and 0:100, were spiked with GSC and measured by Affymetrix GeneChips Mouse430-2. Signals were normalized by the Percellome method as described in the text. Line graphs are in (a) copy numbers and (b) ratio to 50:50 sample for the top 1,000 probe sets with coefficient of correlation (R^2) closest to 1 among those having 1 copy or more per cell in the 50:50 sample (19,979 probe sets out of 45,101). The number of probe sets with $R^2 > 0.950$ was 8,655, and $R^2 > 0.900$ was 11,719.

regions using multiple platforms, is central to the establishment of a reliable reference database for toxicogenomics and pharmacogenomics. Transforming expression data into a "per cell" database is an effective way of normalizing expression data across samples and platforms. However, transcriptome data from the quantitative PCR (Q-PCR) and DNA microarray analyses currently deposited in the database are related to a fixed amount of RNA collected per sample. Variations in RNA yield across samples in an experimental series compromise accurate determination of the absolute level of each mRNA species per cell in any sample. Normalization against housekeeping genes for PCRs, and global normalization of ratiometric data for microarrays, is typically performed to account for this informational loss. Additional methods, such as the use of external mRNA spikes, reportedly improve the quality of data from microarray systems. For example, Holstege et al. [1] described a spike method against total RNA, based on their finding that the yields of total RNA from wild type and mutant cells were very similar. Hill et al. [2] reported a spike method against total RNA for normalizing hybridization data such that the sensitivities of individual arrays could be compared. Lee et al. [3] demonstrated that "housekeeping genes" cannot be used as a reference control, and van de Peppel et al. [4] described a normalization method of mRNA against total RNA using an external spike mixture. To achieve satisfactory performance they used multiple graded doses of external spikes, covering a wide range of expression, in order to align the ratiometric data by Lowess normalization [5]. Hekstra et al. [6] presented a method for calculating the final cRNA concentration in a hybridization solution. Sterrenburg et al. [7] and Dudley et al. [8] reported the use of common reference control samples for two-color microarray analyses of the human and yeast genomes, respectively. These are pools of antisense oligo sequences against all sense oligos present on the microarray. Instead of antisense oligos, Talaat et al. [9] used genomic DNA as a common reference control in studies of *E. coli*. Statistical approaches have been proposed for ratiometric data to improve inter-microarray variations, especially of non-linear relations [10]. However, because control samples may differ among studies, ratiometric data cannot easily be compared across multiple studies unless a common reference, such as a mixture of all antisense counterparts of spotted sense sequences is used [7-9]. Nevertheless, as long as the normalization is calibrated to total RNA, variations in total RNA profile cannot be effectively cancelled out. Although

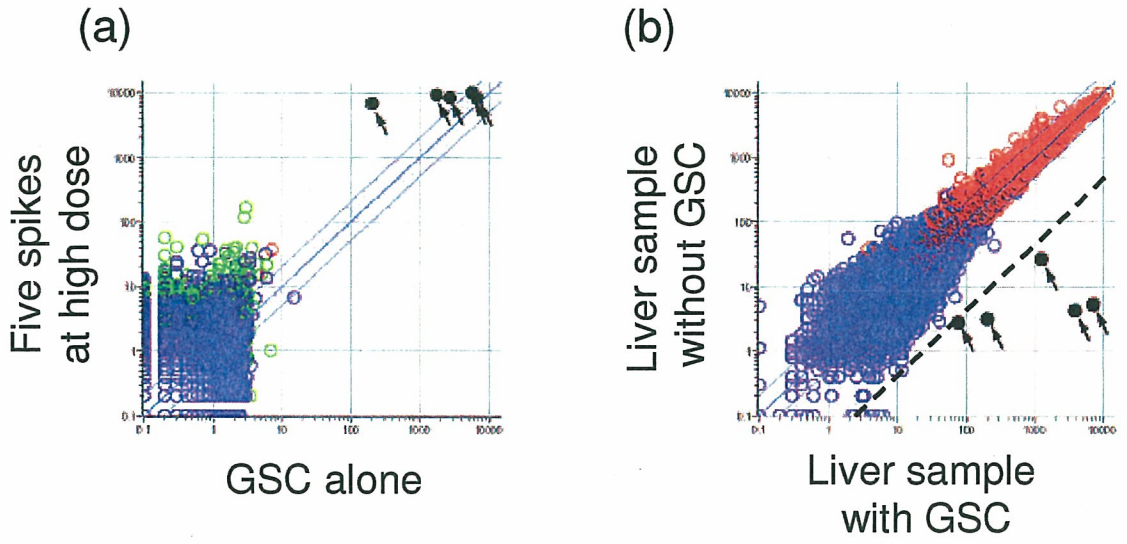

Figure 2
Cross-hybridization of GSC. Cross-hybridization of the GSC spike mRNAs to Affymetrix GeneChip. (a) A scatter plot of a blank sample with the GSC (horizontal axis) and a blank with the five spike RNAs at a high dosage (vertical axis) measured by MG-U74v2A GeneChips (raw values generated by Affymetrix MAS 5.0 software). The five spikes are indicated by black dots with arrows. Signals of the murine probe sets were below 20 on the horizontal axis, indicating negligible cross-hybridization of GSC spike mRNAs to the murine probe sets. (b) A scatter plot of a liver sample with GSC (horizontal axis) and without GSC (vertical axis) measured by MG-U74v2A GeneChips. The five spikes are again indicated by black dots with arrows. The dotted line is the 1/25 fold (4%) line. Cross-hybridization of mouse liver mRNAs to the GSC signals was considered negligible (less than 4%).

some of these reports share the idea that "absolute expression" and "transcripts per cell" should entail robust normalization, further practical development to enable universal application has been awaited.

Here, we report a method for normalizing expression data across samples and methods to the cell number of each sample, using the DNA content as indicator. This normalization method is independent of the gene expression profile of the sample, and may contribute to transcriptome studies as a common standard for data comparison and interchange.

Results

Dose-response linearity of the measurement system as a basis for the Percellome method

The fidelity of transcript detection is the key to this "per cell" based normalization method, which generates transcriptome data in "mRNA copy numbers per cell". The Q-PCR system was tested by serially diluting samples to confirm the linear relationship between Ct values and the log of sample mRNA concentration (data not shown). High density oligonucleotide microarrays from Affymetrix [11] were used in our experiments. We tested the linearity of the Affymetrix GeneChips using a set of five samples made of mixtures of liver and brain in ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (designated "LBM" for liver-brain mix). The results showed a linear relationship ($R^2 > 0.90$) between fluorescence intensity and input for a sufficient proportion of probe sets, i.e. about 37% of the probe sets in the older MG-U74v2 and 70% in the newest Mouse Genome 430 2.0 GeneChip were above the detection level (approximately one copy per cell) in the 50:50 sample (Figure 1) [see Additional files 1 and 2].

Dose-response linearity alone is not sufficient to generate true mRNA copy numbers. An important additional requirement is that the ratio of signal intensity to mRNA copy number should be equal among all GeneChip probe sets of mRNAs and PCR primers. The Q-PCR primer sets were designed to perform at similar amplification rates to minimize differences between amplicons. The melting

Table 1: The spike factors for various organs/tissues

Species	Organ/Tissue (adult, unless otherwise noted)	Spike Factor	total RNA/genomic DNA	SD
Mouse	Liver	0.2	211	46
Mouse	Lung	0.02	22	4
Mouse	Heart	0.05	-	-
Mouse	Thymus	0.01	8	2
Mouse	Colon Epitherium	0.05	105	30
Mouse	Kidney	0.1	-	-
Mouse	Brain	0.1	-	-
Mouse	Suprachiasmatic nucleus (SCN)	0.1	-	-
Mouse	Hypothalamus	0.1	63	4
Mouse	Pituitary	0.1	52	8
Mouse	Ovary	0.02	35	4
Mouse	Uterus	0.02	42	12
Mouse	Vagina	0.02	81	38
Mouse	Testis	0.15	56	7
Mouse	Epididymis	0.07	53	16
Mouse	Bone marrow	0.02	14	3
Mouse	Spleen	0.02	-	-
Mouse	Whole Embryo	0.15	97	36
Mouse	Fetal Telencephalon E10.5–16.5	0.1	48	9
Mouse	Neurosphere (E11.5–14.5)	0.03	42	10
Mouse	E9.5 embryo heart	0.15	58	15
Mouse	cell lines	0.2	-	-
Rat	Liver	0.2	-	-
Rat	Kidney	0.2	-	-
Rat	Uterus	0.04	56	5
Rat	Ovary	0.04	56	9
Human	Cancer Cell Lines	0.2	116	26
Xenopus	liver	0.03	-	-
Xenopus	embryo	0.15	-	-

temperature was set between 60° and 65°C with a product size of approximately 100 base pairs using an algorithm (nearest neighbor method, TAKARA BIO Inc., Japan), and the amplification co-efficiency (E) was set within the range 0.9 ± 0.1 (E = 2^{-(1/slope)}-1 on a plot of log2 (template) against Ct value). For the GeneChip system, the signal/copy performance of each probe set depended on the strategy of designing the probes to keep the hybridization constant/melting temperature within a narrow range, ensuring that the dose-response performances of the probe sets were similar (cf. http://www.affymetrix.com/technology/design/index.affx). Failing this, any differences should at least be kept constant within the same make/version of the GeneChip. Taking into consideration the biases that lead to imperfections in estimating absolute copy numbers in each gene/probe set, we developed normalization methods to set up a common scale for Q-PCR and Affymetrix GeneChip systems.

The grade-dosed spike cocktail (GSC) and the "spike factor" for the Percellome method

A set of external spike mRNAs was used to transfer the measurement of cell number in the sample (as reflected by its DNA content) to transcriptome analysis. For the spikes, we utilized five *Bacillus subtilis* mRNAs that were left open for users in the Affymetrix GeneChip series. The extent to which the *Bacillus* RNAs cross-hybridized with other probe sets was checked for the Affymetrix GeneChip system. The GSC was applied to Murine Genome U74Av2 Array (MG-U74v2) GeneChips with or without a liver sample. As shown in Figure 2, cross-hybridization between *Bacillus* RNAs and the murine gene probe sets was negligible [see Additional files 3 and 4]. Mouse Genome 430 2.0 Array (Mouse430-2), Mouse Expression Arrays 430A (MOE430A) and B (MOE430B), Rat Expression Array 230A (RAE230A), *Xenopus laevis* Genome Array and Human Genome U95Av2 (HG-U95Av2) and U133A (HG-U133A) Arrays sharing the same probe sets for these spike mRNAs showed no sign of cross-hybridization with the *Bacillus* probes (data not shown).

We prepared a cocktail containing in vitro transcribed Bacillus mRNAs in threefold concentration steps, i.e. 777.6 pM (for AFFX-ThrX-3_at), 259.4 pM (for AFFX-LysX-3_at), 86.4 pM (for AFFX-PheX-3_at), 28.8 pM (for AFFX-DapX-3_at) and 9.6 pM (for AFFX-TrpnX-3_at). By referring to the amount of DNA in a diploid cell and employing a "spike factor" determined by the ratio of

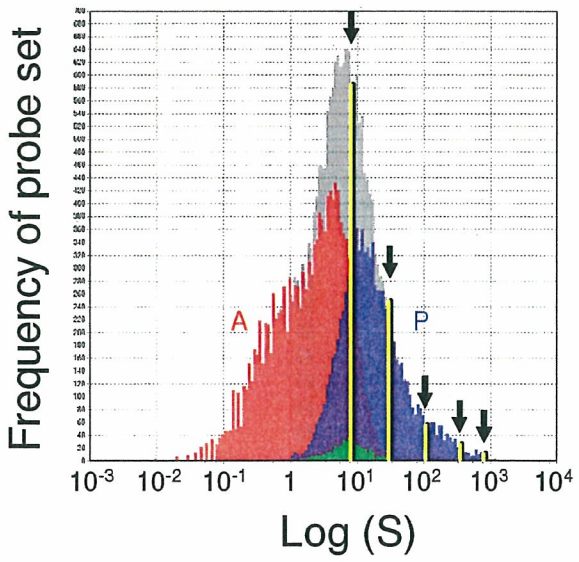

Figure 3
Positioning of GSC spike mRNAs in Affymetrix GeneChip dose-response range. A frequency histogram of the probe sets of Affymetrix GeneChip Mouse430-2 is shown. The histogram for all probe sets (gray) shows near-normal distribution. Blue columns are the "present" calls (P), red columns "absent" calls (A) and green "marginal" calls. The five yellow lines indicate the positions of the GSC spike mRNAs that are chosen to cover the "present" call range by a proper "spike factor".

total RNA to genomic DNA in a tissue type (Table 1), the spike mRNAs were calculated to correspond to 468.1, 156.0, 52.0, 17.3 and 5.8 copies per cell (diploid), respectively, for the mouse liver samples (spike factor = 0.2). The ratio of mRNAs in the cocktail is empirically chosen depending on the linear range of the measurement system and the available number of spikes. Here, we set the ratio to three to cover the "present" call probe sets of the Affymetrix GeneChip system (Figure 3).

We tested this grade-dosed spike cocktail (GSC) by Q-PCR and confirmed that the Ct values of the spike mRNAs were linearly related to the log concentrations (cf. Figure 4a), i.e. could be expressed as

$$Ct = \alpha \log C + \beta \quad \{1\}$$

The GSC was also tested by the GeneChip system and it was confirmed that the log of the spike mRNA signal intensities was linearly related to the log of their concentrations (cf. Figure 4b),

$$\log S = \gamma \log C + \delta \quad \{2\}$$

The linear relationship between the Ct values (Ct) and the log of RNA concentration (log C) was reasonable given the definition of Ct values (derived from the number of PCR cycles, i.e. doubling processes). The linear relationship between the log of GeneChip signal intensity (log S) and the log of RNA concentration (log C) was rationalized by the near-normal distribution of log S over all transcripts (cf. Figure 3).

Calculation of copy numbers of all genes/probe sets per cell

As described above, using a combination of DNA content and the spike factor of the sample, the GSC spike mRNAs become direct indicators of the copy numbers (C') per cell. When the samples were measured by Q-PCR or GeneChip analysis, the five GSC spike signals in each sample should obey function {1} for Q-PCR and function {2} for GeneChip with a good linearity. If the observed linearity was poor, a series of quality controls was performed and the measurement repeated. The coefficients of the functions were determined for each sample by the least squares method. Under the assumption that all genes/probe sets share the same signal/copy relationship, signal data for all genes/probe sets were fitted to the functions {1'} or {2'}, which are the individualized functions of {1} and {2} for each sample measurement (i).

$$Ct = \alpha i \log(C') + \beta i \quad \{1'\}$$

$$\text{Log } (S) = \gamma i \log(C') + \delta i \quad \{2'\}$$

(i = sample measurement no.)

The Q-PCR Ct values (Ct) and microarray signal values (S) of all mRNA species in the sample (i) are converted to copy numbers per cell (C') by the inverses of functions {1'} and {2'}, i.e. {3} and {4} below:

$$C' = B\text{^}((Ct-\beta i)/\alpha i) \quad \{3\}$$

for Q-PCR (Figure 4a).;

$$C' = B\text{^}((\log S-\gamma i)/\delta i) \quad \{4\}$$

for GeneChips (Figure 4b),

where B is the logarithmic base used in {1} and {2} (see Materials and Methods for details).

Real world performance of the Percellome method

The correspondence between Q-PCR and GeneChip was tested using a sample set from 2,3,7,8-tetrachlorodibenzodioxin (TCDD)-treated mice. Sixty male C57BL/6 mice

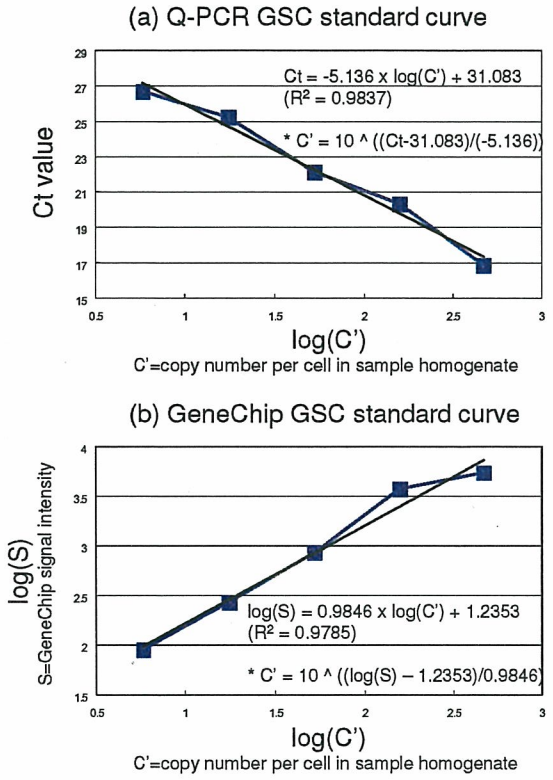

Figure 4
The dose-response linearity of the GSC spikes in Q-PCR and the Affymetrix GeneChip array system. Linear relationships are shown between (a) the Q-PCR Ct values and log of copy number (log (C')), and (b) the GeneChip log signal intensity (log(S)) and log of copy number (log (C')) of the GSC mRNAs. The regression functions were obtained by the least squares method. The inverse functions (*) were further used to generate the copy numbers of all other genes/probe sets for Percellome normalization.

were divided into 20 groups of 3 mice each. TCDD was administered once orally at doses of 0, 1, 3, 10 and 30 μg/kg, and the livers were sampled 2, 4, 8 and 24 h after administration. Nineteen primer pairs were prepared for Q-PCR and the Ct values of the liver transcriptome were measured. The same 60 liver samples were measured using the Affymetrix Mouse430-2 GeneChip [see Additional files 5 through 8 and 9 through 12]. Q-PCR and GeneChip data were normalized against cell number by functions {3} and {4}, respectively. The averages and standard deviations (sd) of each group (n = 3) were calculated and plotted as three layers of isoborograms on to 5 × 4 matrix three-dimensional graphs (Figure 5). Together with another sample set (data not shown), a total of thirty-six primer pairs were compared, and there was a correlation of up to 90% between the Q-PCR and GeneChip surfaces. It is notable that not only the average surfaces but also the +1sd and -1sd surfaces corresponded closely in shape and size. We infer that the differences resulted mainly from biological variations among the three animals in each experimental group rather than from measurement error (cf. Figure 7).

An important feature of Percellome normalization is its independence from the overall expression profile of the sample. When gene expression profiles differ among samples, Percellome normalization produces a robust transcriptome that is different from total-RNA dependent global normalization. As an example, Figure 6 shows the results of an experiment on the uterotrophic response of ovariectomized mice to estrogen treatment [12] [see Additional files 13 and 14]. The uteri of the vehicle control are atrophic because the ovaries, the source of intrinsic estrogens, are absent. The uteri of the treated groups are hypertrophic owing to estrogenic stimulus from the test compound administered. Global normalization (90 percentile) between the vehicle control group and the high-dose (1,000 mg/kg) group indicated that 4,600 of 12,000 probe sets showed 2-fold or greater increase, 470 were reduced by 0.5 or less, and 7,400 remained between these extremes. In contrast, analysis of Percellome-normalized data revealed that almost all the 12,000 probe sets showed a 2-fold or greater increase, including actin, GAPDH and other housekeeping genes. The hypertrophic tissues, consisting of cells with abundant cytoplasm, provide convincing evidence for the increases in various cellular components including housekeeping gene products.

Another important feature of Percellome normalization is the commonality of the expression scale across platforms. Batch conversion can be performed between results obtained from different platforms when the data are generated by the Percellome method. A practical strategy for such normalization is to prepare a set of samples from a target organ of interest with differences in gene expression, and measure them once by each platform. Data conversion functions with good linear dose-response relationships can be obtained individually for those genes/probe sets that are measured by both platforms (Figure 7).

Discussion

We have developed a novel method for normalizing mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration. For non-diploid or aneuploid samples, an average DNA content per cell should be determined beforehand for accurate adjustment. When there is significant DNA synthesis, a similar adjustment should be considered.