Table 6
Pregnancy and litter data of rats given 3-cyanopyridine by gavage.

	Dose (mg/kg/day)			
	0	5	30	180
No. of pregnant females	12	12	12	10
No. of corpora lutea[a]	16.2 ± 2.0	16.2 ± 2.0	15.7 ± 2.0	11.9 ± 3.8**
Implantation index[a,b]	93.6 ± 16.8	98.8 ± 2.7	99.2 ± 2.9	93.4 ± 11.7
No. of implantation sites[a]	15.3 ± 3.6	16.0 ± 2.2	15.6 ± 2.3	11.4 ± 4.0*
No. of litters (=no. of females completing the delivery)	12	12	12	3
Delivery index[a,c]	91.1 ± 6.0	94.2 ± 7.9	95.5 ± 5.1	57.4 ± 37.8 (3)
Total no. of pups born[a]	13.9 ± 3.4	15.1 ± 2.5	14.9 ± 2.4	7.7 ± 5.8 (3)
Live birth index[a,d]	98.8 ± 2.7	97.1 ± 4.4	97.8 ± 4.3	60.6 ± 53.3 (3)
No. of live pups on PND 0[a]	13.8 ± 3.4	14.7 ± 2.6	14.6 ± 2.4	3.3 ± 4.9 (3)
No. of dead pups on PND 0[a]	0.2 ± 0.4	0.4 ± 0.7	0.3 ± 0.7	4.3 ± 5.9 (3)
Sex ratio of live pups[e]	0.494 ± 0.098	0.438 ± 0.119	0.521 ± 0.174	0.720 ± 0.396 (2)
Viability index on PND 4[a,f]	100.0 ± 0.0	100.0 ± 0.0	99.0 ± 2.4	0.0 ± 0.0 (2)
No. of live pups on PND 4[a]	13.8 ± 3.4	14.7 ± 2.6	14.4 ± 2.3	0.0 ± 0.0 (2)
Body weight of live pups on PND 0 (g)[a]				
Male	6.84 ± 0.34	6.73 ± 0.49	6.89 ± 0.68	6.85 ± 0.21 (2)
Female	6.53 ± 0.37	6.43 ± 0.51	6.47 ± 0.71	6.70 (1)
Body weight of live pups on PND 1 (g)[a]				
Male	7.67 ± 0.52	7.38 ± 0.66	7.54 ± 0.90	– (0)
Female	7.29 ± 0.52	7.03 ± 0.65	7.12 ± 0.86	– (0)
Body weight of live pups on PND 4 (g)[a]				
Male	11.50 ± 1.29	10.69 ± 1.06	10.98 ± 1.47	– (0)
Female	10.87 ± 1.36	10.19 ± 0.97	10.30 ± 1.36	– (0)

Values in parentheses are the number of animals examined. Values in the 180 mg/kg/day group except for no. of corpora lutea, implantation index and no. of implantation sites are excluded from statistical evaluation because of insufficient sample numbers. (–) Blank.

[a] Values are given as the mean ± S.D.
[b] Implantation index (%) = no. of implantation sites/no. of corpora lutea × 100.
[c] Delivery index (%) = total no. of pups born/no. of implantation sites × 100.
[d] Live birth index (%) = no. of live pups on PND 0/total no. of pups born × 100.
[e] Sex ratio of live pups = no. of live males/total no. of live pups.
[f] Viability index on PND 4 (%) = no. of live pups on PND 4/no. of live pups on PND 0 × 100.
* Significantly different from the control group ($P < 0.05$).
** Significantly different from the control group ($P < 0.01$).

Table 7
Gross findings of pups in rats given 3-cyanopyridine by gavage.

Dose (mg/kg/day)	Male pups				Female pups			
	0	5	30	180	0	5	30	180
Findings of dead pups during lactation days 0–4								
Number of pups examined	1	4	2	19[a,b]	1	1	3	11[c]
No abnormal findings	1	4	2	3	1	1	3	0
Organ: findings								
External: generalized edema	0	0	0	16	0	0	0	11
omphalocele	0	0	0	0	0	0	0	1
Thoracic cavity: hydrothorax	0	0	0	2	0	0	0	0
Lung: pale discoloration	0	0	0	1	0	0	0	0
Heart: pale discoloration	0	0	0	1	0	0	0	0
Abdominal cavity: ascites	0	0	0	7	0	0	0	4
Liver: grayish green discoloration	0	0	0	1	0	0	0	0
deformity	0	0	0	1	0	0	0	0
Findings of pups euthanized on lactation day 4								
Number of pups examined	83	77	91	0	82	99	82	0
No abnormal findings	83	76	91	–	80	98	82	–
Organ: findings								
Liver: yellowish brown discoloration	0	0	0	–	1	0	0	–
grayish green patch, lateral left lobe	0	0	0	–	1	0	0	–
Kidney: dilatation, renal pelvis	0	0	0	–	0	1	0	–
small	0	1	0	–	0	0	0	–
dark red discoloration	0	1	0	–	0	0	0	–
Tail: lost	0	0	0	–	1	0	0	–

Values are the number of pups with findings. (–) Blank.

[a] Including one pup whose sex was not distinguished because of maternal cannibalism.
[b] Including 4 pups from dams which were found dead or were euthanized during delivery.
[c] Including 3 pups from dams which were found dead or were euthanized during delivery.

considered to be an adaptive change as a result of 3-cyanopyridine treatment because of no other pathological changes in the liver, and consistent with the previous 28-day study [10]. Changes in organ weight were also observed in the kidney and adrenal gland. The weights of the kidney and adrenal gland were increased in males at 180 mg/kg/day, and these findings were also consistent with the 28-day study [10], in which increased weights of the kidney and adrenal gland were observed in males at 180 mg/kg/day, and increased weight of the kidney and a tendency toward an increased weight of the adrenal gland were observed in females at 30 and/or 180 mg/kg/day.

The following findings were considered to be secondary effects of hemolytic anemia or inflammation caused by 3-cyanopyridine. Extramedullary hematopoiesis in the liver of two females was observed at 180 mg/kg/day although there was no statistical significance. Extramedullary hematopoiesis in the liver is known as one of the reactions against anemia [14]. In the 28-day study [10], hemolytic anemia-related findings, including decreased red blood cell counts, increased reticulocytes, and hemosiderin deposit of red pulp and extramedullary hematopoiesis in the spleen, were observed in both sexes at 180 mg/kg/day; therefore, hemolytic anemia could have occurred at 180 mg/kg/day also in the current study. Slight or moderate spermatic granuloma in the epididymis was observed at 5 mg/kg/day or more, although there was no statistical significance. Spermatic granuloma can arise as an inflammatory response [15]. Increased adrenal weight observed at 180 mg/kg/day may also be a reaction to inflammation. In the 28-day study [10], possible inflammatory responses were observed at 180 mg/kg/day, as follows: increased white blood cell counts, increased neutrophils, neutrophil cellular infiltration in the kidney pelvis, bladder inflammation, increased adrenal weight, and slight hypertrophy of the zona fasciculata in the adrenal cortex.

In the current study, the emaciation of pregnant females was marked at 180 mg/kg/day, and the hard or abnormal labor in pregnant females at that dose was considered to be due to aggravation of the general condition. General edema in almost all pups and ascites of the abdominal cavity and hydrothorax of thoracic cavity in some pups were observed at 180 mg/kg/day at necropsy of stillborn and dead pups, and these findings were considered to be due to circulatory problems associated with such a difficult delivery. As for fetuses, dead, mainly macerated, fetuses were observed in the uterus at necropsy for all pregnant females that did not deliver pups in the 180 mg/kg/day treatment group. The intrinsic tendency of anemia in pregnant females [16] may be enhanced by 3-cyanopyridine treatment at 180 mg/kg/day. However, there were only two cases of extramedullary hematopoiesis in the liver in the females at 180 mg/kg/day, and no other direct evidence of anemia in the current study. Besides maternal anemia, there must be the other unknown mechanism for fetal toxicity.

The following findings were not considered 3-cyanopyridine-treated effects. For parental toxicity, alopecia in females observed at 180 mg/kg/day was also observed in the female control group, at necropsy, the swelling of the left hindlimb in one male observed at 180 mg/kg/day was due to bone fracture or dislocation, and changes in the ileum were only few. In developmental findings, the number of dead or missing pups between PNDs 0 and 4 observed at 5 or 30 mg/kg/day was not significantly different from that of the control group. At necropsy of stillborn or dead pups, omphalocele in one female pup and pale discoloration of the lung and heart and grayish-green discoloration and deformity of the liver in one male pup were also observed at 180 mg/kg/day; however, these were observed in only one male and female pup and were considered spontaneous.

Regarding the female reproductive system in the current study, a prolonged estrous cycle, decreased numbers of corpora lutea and implantations and two unsuccessfully mated females were observed at 180 mg/kg/day, and there were no toxicological necropsy findings in the ovary at 180 mg/kg/day. Also in the 28-day study [10], there were no changes in the ovary weight and no histopathological findings in the ovary. The decreased number of corpora lutea suggested that dosing with 3-cyanopyridine affects ovulation. The decreased number of implantations was reflected by a decreased number of corpora lutea because there were no changes in the implantation index and fertility index at 180 mg/kg/day. The female reproductive function is regulated by the hypothalamic-pituitary-ovary axis, and female reproductive toxicity is generally caused indirectly by an imbalance of the hypothalamic-pituitary-ovary axis [17]. In the current study, the effects on the estrous cycle, corpora lutea, implantations and mating at 180 mg/kg/day may have been caused indirectly by an imbalance of the hypothalamic-pituitary-ovary axis.

Regarding the male reproductive organs, Sertoli cell vacuolation and spermatid necrosis/decrease in the testis, and spermatozoa decrease and lumen cell debris in the epididymis were observed at 180 mg/kg/day. These findings were considered to be caused by 3-cyanopyridine treatment, although decreased spermatozoa and lumen cell debris in the epididymis were associated with changes in the testis. These histopathological findings were consistent with the 28-day study [10]. The findings in the stages of spermatogenesis observed at 30 mg/kg/day or more were also considered to be caused by 3-cyanopyridine treatment. For the number of germ cells at every stage of spermatogenesis, reduction or a decreasing trend was observed at 180 mg/kg/day, and therefore, cell- and stage-specific influences on germ cells were not observed. Superficially regarded, these testicular toxicity in the current study was not caused indirectly by endocrine disruption because the changes in spermatogenesis associated with endocrine disruption are often subtle, cell- and stage-specific [18], and was caused directly by 3-cyanopyridine treatment because Sertoli cell or germ cell injury of the testis would be induced by direct action on the germinal epithelium [17]. On the other hand, the testicular toxicity by dosing with 3-cyanopyridine may also be caused indirectly *via* the hypothalamic-pituitary-gonadal axis, the same as for possible female reproductive toxicity. Further studies are needed to clarify the mechanism of 3-cyanopyridine-induced testicular toxicity. Despite such histopathological changes in the testes with decreased spermatozoa in the epididymides, no effects of 3-cyanopyridine on male reproductive ability, *i.e.* the fertility index, were observed at any doses in the current study; then, the testicular toxicity in the current study is dealt with as general toxicity. This would be because mating was performed after 14 days of dosing in this screening test before effects on germ cells could arise. In addition, rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility, particularly as evaluated in reproductive studies that allow multiple mating [19].

In conclusion, the results of the current study provide a more comprehensive toxicity profile of 3-cyanopyridine than has been previously reported. The NOAEL for reproductive/developmental toxicity was concluded to be 30 mg/kg/day mainly based on prolonged estrous cycle, delayed initiation of delivery, decreased gestation index, and decreased number of corpora lutea observed at 180 mg/kg/day, while the weight of the liver was slightly increased in females at 5 mg/kg/day and was increased in both sexes at 30 mg/kg/day for general toxicity.

Acknowledgement

This study was supported by the Ministry of Health, Labour and Welfare, Japan.

References

[1] 14504 Chemical Commodities. Tokyo: The Chemical Daily Co., Ltd.; 2004.
[2] METI (Ministry of Economy, Trade and Industry, Japan). Research results on the actual production and import volume of chemicals in 2011 [in Japanese]. Available at: http://www.meti.go.jp/policy/chemical_management/kasinhou/files/release/h22/2kan-shusei.pdf [accessed in March 2012].
[3] HSDB (Hazardous Substances Data Bank). 3-Pyridinecarbonitrile CAS 100-54-9. Available at: http://toxnet.nlm.nih.gov/ [accessed in March 2012].
[4] Degussa AG. Unpublished report. Degussa AG-US-IT-Nr: 74-0005-DKT. In: IUCLID Dataset; 2000.
[5] European Commission. 100-54-9. IUCLID Dataset; 2000.
[6] Majka J, Knobloch K, Szendzikowski S. Evaluation of acute toxic effect of 3-cyanopyridine. Medycyna Pracy 1979;30:109–13 [in Polish].
[7] Schafer EW, Bowles WA. Acute oral toxicity and repellency of 933 chemicals to house and deer mice. Archives of Environment Contamination and Toxicology 1985;14:111–29.
[8] Degussa AG. Unpublished report. Degussa AG-US-IT-Nr: 85-0067-DKT. In: IUCLID Dataset; 2000.
[9] Degussa AG. Unpublished report. Degussa AG-US-IT-Nr: 85-0066-DKT. In: IUCLID Dataset; 2000.
[10] MHLW (Ministry of Health, Labour, Welfare, Japan). 3-Cyanopyridine [in Japanese]. Available at: Japan Existing Chemical Data Base (JECDB), http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp. Abstract is in English.
[11] OECD (Organisation for Economic Co-operation and Development). Guideline 421, Reproduction/Developmental Toxicity Screening Test [adopted on July 27, 1995]. OECD Guidelines for the Testing of Chemicals, section 5.
[12] MOE, METI, MHLW. Standard concerning testing laboratories implementing tests for new chemical substances etc. Joint notification by director generals of Environmental Policy Bureau, Japan, Ministry of the Environment (MOE) (Kanpokihatsu No. 031121004) and Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry (METI) (Seikyokuhatsu No. 3), dated November 17, 2003 and by director general of Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare (MHLW) (Yakusyokuhatsu No. 1121003), dated November 21; 2003.
[13] MOE, METI, MHLW. Partial amendments of the standard concerning testing laboratories implementing tests for new chemical substances etc. Joint notification by director generals of Environmental Policy Bureau, Japan, Ministry of the Environment (MOE) (Kanpokihatsu No. 080704001) and Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry (METI) (Seikyokuhatsu No. 2), dated June 30, 2008 and by director general of Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare (MHLW) (Yakusyokuhatsu No. 0704001), dated July 4; 2008.
[14] Harada T, Maita K. Liver and gallbladder [Chapter 5]. In: Maekawa, et al., editors. Textbook of toxicologic histopathology [in Japanese]. Tokyo: The Japanese Society of Toxicologic Pathology; 2000. p. 179–213.
[15] Kandori H, Chatani F, Miyajima H. Male reproductive organs. The Japanese society of toxicologic pathology. In: Toxicologic Histopathology [in Japanese]. Tokyo: International Press Editing Centre Incorporation; 2000. p. 283–314.
[16] Kawada K. Experimental studies on iron deficiency anemia in pregnant rat and its feto-maternal influence. Acta Obstetrica et Gynaecologica Japonica 1982;34:75–82.
[17] Kihara T, Tanimura T. 6.5 reproductive/developmental toxicity. In: Ohno Y, et al., editors. Toxicology [new edition]. Tokyo: Asakura Publishing; 2009. p. 182–201.
[18] Russell LD, Clermont Y. Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. The Anatomical Record 1977;187:347–65.
[19] Parker RM. Testing for reproductive toxicity. Florida: CRC Press, Taylor & Francis Group; 2006.

Food and Chemical Toxicology 56 (2013) 290–296

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

An antioxidant, N,N′-diphenyl-p-phenylenediamine (DPPD), affects labor and delivery in rats: A 28-day repeated dose test and reproduction/developmental toxicity test

Mariko Matsumoto [a,d], Makiko Yamaguchi [b], Yuka Yoshida [c], Mika Senuma [c], Hiromasa Takashima [c,1], Tomoko Kawamura [a], Hina Kato [a], Mika Takahashi [a], Mutsuko Hirata-Koizumi [a], Atsushi Ono [a], Kazuhito Yokoyama [d], Akihiko Hirose [a,*]

[a] Division of Risk Assessment, Biological Safety Center, National Institute of Health Sciences, Tokyo 158-8501, Japan
[b] Research Institue for Animal Science in Biochemistry & Toxicology, Kanagawa 252-0132, Japan
[c] Hatano Research Institute, Food and Drug Safety Center, Kanagawa 257-8523, Japan
[d] Department of Epidemiology and Environmental Health, Juntendo University Faculty of Medicine, Tokyo 113-8421, Japan

ARTICLE INFO

Article history:
Received 22 November 2012
Accepted 18 February 2013
Available online 26 February 2013

Keywords:
N,N′-diphenyl-p-phenylenediamine
Prostaglandin
Repeated dose toxicity
Reproductive toxicity
Gestation period

ABSTRACT

A 28-day repeated dose toxicity test and reproduction/developmental toxicity test for N,N′-diphenyl-p-phenylenediamine (DPPD) were conducted in [Crl:CD(SD)] SPF rats. Male and female rats were dosed with DPPD by gavage for 28 days at 0, 100, 300, or 1000 mg/kg bw/day or for a total of 42–46 days at 0, 8, 50, or 300 mg/kg bw/day. No significant adverse effects were observed in the repeated dose toxicity study up to 1000 mg/kg bw/day in both sexes. In the reproduction/developmental toxicity study, two females showed piloerection, hypothermia, and pale skin; one died and the other showed dystocia on day 23 of pregnancy at 300 mg/kg bw/day. Another female delivered only three live pups at 300 mg/kg bw/day. A significantly prolonged gestation period was observed at 50 and 300 mg/kg bw/day. The NOAELs of repeated dose toxicity and reproduction/developmental toxicity were considered to be 1000 and 8 mg/kg bw/day, respectively.

1. Introduction

N,N′-diphenyl-p-phenylenediamine (DPPD; CAS: 74-31-7), a gray or dark gray powder, is used as a polymerization inhibitor and antioxidant (HSDB, 2012). The antioxidative activity of DPPD is implemented by the donation of a hydrogen to a radical derivative and breaking the autocatalytic cycle (Chemicalland21, 2012). DPPD is widely used in rubber, oils, and feedstuffs, especially for tires in the rubber industry due to its color and stability (Chemicalland21, 2012; HSDB, 2012). Occupational exposure to DPPD may occur through inhalation and dermal contact with this compound at workplaces where DPPD is produced or used (HSDB, 2012). DPPD was detected at a high rate in leachate samples from landfills containing plastic and rubber waste at concentrations of 0.1–13 ng/L (Hasegawa and Suzuki, 2005) and was found in air samples taken from one location at 0.002–0.009 ng/m^3 (MOE, 2005) in Japan. Therefore, exposure to DPPD via the environment is also anticipated.

The oral acute toxicity of DPPD is low with LD_{50} values of 2370 mg/kg bw in rats (Marhold, 1986) and 18,000 mg/kg bw in mice (Labor Hygiene and Occupational Diseases, 1966). A long-term feeding study also showed the relatively low toxicity of DPPD in rats (Hasegawa et al., 1989). Rats were fed a diet containing 0.5% or 2% of DPPD (194 or 857 mg/kg bw/day in males; 259 or 1024 mg/kg bw/day in females) for 104 weeks, and a dose dependent reduction in body weight gain (not associated with decreased food consumption) and a significant decrease in relative weight of the liver were observed in both sexes. Calcium deposition in the kidney in males was the only significant histopathological change. Erythrocyte count, hemoglobin, and hematocrit were significantly increased in the female treatment groups while they were dose dependently decreased in males. In this study, an autopsy was carried out 8 weeks after the cessation of DPPD administration; therefore, some difficulty exists in interpreting study results.

As for reproductive and developmental effects, a study in the 1950s showed that feeding doses of commercial grade of DPPD at 0.025%, 0.10%, 0.40%, and 1.60% prolonged the gestation period in

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; COX, cyclooxygenase; DPPD, N,N′-diphenyl-p-phenylenediamine; HPV, high production volume; NSAID, non-steroidal anti-inflammatory drug; OECD, Organisation for Economic Co-operation and Development.

* Corresponding author. Address: Division of Risk Assessment, Biological Safety Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: hirose@nihs.go.jp (A. Hirose).
[1] Present address: Ina Research, Nagano 399-4501, Japan.

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.02.029

all treatment groups in rats (Oser and Oser, 1956). In this study, female rats were fed DPPD from 2 weeks before mating (a total administration period was not specified). Although fertility was not affected by the DPPD treatment, mortality of pups at birth was increased. In a later study by Marois (1998), daily doses of 20–40 mg of DPPD/animal from the 14th day of pregnancy prolonged the gestation period and caused stillbirths in rats (Marois, 1998). In these comparable studies, the fertility effects of DPPD in males were not assessed, and detailed study methods were not fully described.

DPPD is a high production volume (HPV) chemical with production or importation exceeding 1000 tonnes per year in Organisation for Economic Co-operation and Development (OECD) member countries and is listed in the most recent OECD HPV list for investigation of its environment and human health effects under the OECD Cooperative Chemical Assessment Programme (OECD, 2012). Although some early studies briefly showed DPPD toxicity, further reliable information was necessary to assess the human health effects of DPPD. Therefore, DPPD was selected as a target substance for the Safety Examination of Existing Chemicals in Japan. The present paper reports the results of the repeated dose toxicity screening test and reproductive/developmental toxicity screening test of DPPD in rats.

2. Materials and methods

The 28-day repeated dose study was performed at the Research Institute for Animal Science (RIAS) in Biochemistry & Toxicology (Kanagawa, Japan) in compliance with "the notice on the test method concerning new chemical substances (November 21, 2003, No. 1121002, Pharmaceutical and Food Safety Bureau, MHLW; No.2, Manufacturing Industries Bureau, METI; No. 031121002, Environmental Policy Bureau, MOE)" and "the standard for the test facility conducting tests concerning new chemical substances, etc. (November 21, 2003, No. 1121003 Pharmaceutical and Food Safety Bureau, MHLW; No. 3 Manufacturing Industries Bureau, METI; No. 031121004 Environmental Policy Bureau, MOE)". Animals were treated in accordance with "the regulations for animal experimentation in RIAS" and the test was conducted with the approval of "the Animal Care and Use Committee of RIAS".

The reproduction/developmental toxicity study was performed at the Food and Drug Safety Center, Hatano Research Institute (Kanagawa, Japan) in compliance with OECD Guideline 421 Reproduction/Developmental Toxicity Screening Test, along with the above described notice and standard. Animals were treated in accordance with "the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973)", "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of Environment, dated April 28, 2006)", "Guidelines for Proper Conduct of Animal Experiments (June 1, 2006)", and "the Guideline for Animal Experiment in Hatano Research Institute, Food and Drug Safety Center".

2.1. Animals

2.1.1. The 28-day repeated dose study

Male and female [Crl:CD(SD)] SPF rats were purchased from Atsugi Breeding Center, Charles River Japan, Inc., (Kanagawa, Japan). Five-week-old male and female rats (male: 152–172 g; female: 130–147 g) found to be in good health were selected for use. Male and female rats were distributed into four groups on a random basis. Animals were reared on a basal diet, Labo MR Stock; NOSAN corporation (Tokyo, Japan) and water *ad libitum* and were housed individually. Animals were maintained in an air-conditioned room at a room temperature of 21.9–23.0 °C, relative humidity of 55–61%, 12-h light/dark cycle, and 10 and more air changes per hour.

2.1.2. The reproduction/developmental study

Male and female [Crl:CD(SD)] SPF rats were purchased from Atsugi Breeding Center, Charles River Japan, Inc., (Kanagawa, Japan). Ten-week-old male and female rats (male: 370.2–446.9 g; female: 220.4–265.2 g) found to be in good health were selected for use. Vaginal smears of each female were examined, and only females showing a 4-day or 5-day estrous cycle were used. Male and female rats were distributed into four groups on a random basis. Animals were reared on a basal diet, CE-2; CLEA Japan, Inc. (Tokyo, Japan) and water *ad libitum* and were housed individually, except for mating and lactation periods. Animals were maintained in an air-conditioned room at a room temperature of 21.5–23.5 °C, relative humidity of 47–67%, 12-h light/dark cycle, and 15 air changes per hour.

2.2. Chemicals and dosing

2.2.1. The 28-day repeated dose study

DPPD (Lot No. 307605R, purity: 99.87%) was obtained from Seiko Chemical (Tokyo, Japan). Male and female rats (5 or 10 rats/sex/group) were dosed once daily by gastric intubation with DPPD at a dose of 0 (control: methylcellulose), 100, 300, or 1000 mg/kg bw for 28 days. After the dosing period, five rats per each sex at 0 and 1000 mg/kg bw/day were reared for 14 days without administration of DPPD as the recovery groups. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight.

2.2.2. The reproduction/developmental study

DPPD (Lot No. KWR0015, purity 100%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Male and female rats (13 rats/sex/group) were dosed once daily by gastric intubation with DPPD at a dose of 0 (control: sodium carboxymethyl cellulose), 8, 50, or 300 mg/kg bw. Males were dosed for a total of 42 days beginning 14 days before mating, and females were dosed for a total of 42–46 days beginning 14 days before mating to day 4 of lactation throughout mating and gestation periods. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight.

2.3. Observations

2.3.1. The 28-day repeated dose study

The first day of dosing was designated as day 1 of administration. All rats were observed daily for clinical signs of toxicity. Clinical signs in detailed observation in all animals were recorded one day before the administration period and once a week during the administration period. Sensory reactions for a sight reaction, hearing reaction, sense of touch reaction, pain reaction, pupil reflex, and righting reflex were recorded on day 27 of the administration period and on day 13 of the recovery period. Grip strength of fore and hind limbs was tested by a grip strength meter (MK-380R/FR, Muromachi Kikai Co., Ltd., Tokyo Japan) and spontaneous motor activity was recorded by an infrared-ray passive sensor system (SUPERMEX, Muromachi Kikai Co., Ltd., Tokyo Japan) on day 27 of the administration period and on day 13 of the recovery period. Body weight was recorded on days 1, 7, 14, 21, and 28 of the administration period, on days 7 and 14 of the recovery period, and on the day of necropsy. Food consumption was recorded once a week during both administration and recovery periods. Fresh urine was sampled from animals on day 22 of the administration period and on day 8 of the recovery period. Urine samples were tested for color, pH, protein, glucose, ketone bodies, bilirubin, occult blood, and urobilinogen.

Rats were euthanized by exsanguination under anesthesia 1 day after the final administration or 1 day after completion of the recovery period. External surfaces of the rats were examined. Abdomen and thoracic cavities were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta of fasted rats. Collected blood samples were examined for hematology by an automated hematology analyzer (XT-2000i, Sysmex Co., Kobe, Japan) and automatic coagulometer (KC-10A, Amelung, US). Serum biochemistry was tested by an automatic analyzer (JCA-BM8, JEOL, Tokyo, Japan) and automated electrolyte analyzer (NAKL-132, TOA electronics Ltd., Tokyo, Japan). The brain, thymus, heart, liver, spleen, kidney, adrenal gland, thyroid gland, pituitary gland, testis, epididymis, and ovary were isolated and weighed. Histopathological evaluations were performed on these organs in addition to the eye ball, spinal cord, lung, trachea, stomach, intestines, prostate, seminal vesicle, vagina, uterus, urinary bladder, sciatic nerve, lymph nodes, and bone marrow (femur) in control and highest dose groups.

2.3.2. The reproduction/developmental study

The first day of dosing was designated as day 1 of administration or day 1 of the premating period. The day of successful mating was designated as day 0 of the pregnancy period. The day on which parturition was completed by 11:00 was designated as day 0 of the lactation period. All rats were observed daily for clinical signs of toxicity. Body weight was recorded once a week during the administration period, and on the day of autopsy in males, and once a week during the pre-mating and mating periods, on days 0, 7, 14, and 21 of pregnancy, on days 0 and 4 of the lactation period and on a day of autopsy in females. Food consumption was recorded on days 1–2, 7–8, 13–14, 29–30, 35–36, and 41–42 of the administration period in males, and on days 1–2, 7–8, and 13–14 of the pre-mating period, on days 0–1, 7–8, 14–15, and 20–21 of the pregnancy period, and on days 3–4 of the lactation period in females. 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 2-week mating period had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating. Once insemination was confirmed, females were checked for signs of parturition before 11:00 from day 21 of pregnancy. Females were allowed to deliver spontaneously and nurse their pups until day 5 of the lactation period. Litter size and

numbers of live and dead pups were recorded, and live pups were sexed and individually weighed on days 0 and 4 of the lactation period. Pups were inspected for external malformations on day 0 of the lactation period.

Rats were euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 5 of the lactation period in females. External surfaces of rats were examined. Abdomen and thoracic cavities were opened, and gross internal examination was performed. The testis, epididymis, prostate, and seminal vesicle were isolated from all males, and the testis and epididymis were weighed and histopathologically examined. The ovary, uterus, vagina, and mammary gland were isolated, and the ovary was weighed and histopathologically examined. Organs were stored in 10% formalin with 0.1 M phosphate buffer. Organs that showed gross pathological changes were histopathologically examined. The numbers of corpora lutea and implantation sites were counted. On day 5 of the lactation period, pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

2.4. Data analysis

To assess the homogeneity of data, parametric data were analyzed with Bartlett's test or the *F*-test. When homogeneity was recognized, data were analyzed using a one-way analysis of variance or the Student's *t*-test. Non-homogeneous data were analyzed with Kruskal–Wallis's rank test or the Aspin–Welch *t*-test. Non-parametric data were analyzed with Kruskal–Wallis's rank test or Mann–Whitney's U test. The Dunnett test or Dunnett type test was used to assess multiple comparisons. Fisher's exact test was used to assess categorical data. Five per cent levels of probability were used as the criterion for significance. Statistical analysis of pups was carried out using the litter as the experimental unit in the reproductive/developmental study.

2.5. Evaluation of bilirubin measurements by the diazo method

In the repeated dose study, bilirubin levels significantly increased without being related to toxicological effects in males. Because both bilirubin and DPPD contain –NH substitutes, the interference of DPPD with bilirubin measurements was anticipated. The interference of DPPD with bilirubin measurements was tested as follows. Serum samples were taken from untreated male rats, and 0.2 mL of DPPD at 0.001, 0.01, 0.1, and 1 mg/mL (1:1 aceton and dimethyl sulfoxide) was added to 0.5 mL serum of rats. In addition, rat liver S9 was added to DPPD at 0.1 mg/mL to test the interference of DPPD metabolites. Bilirubin levels were measured by the diazo method, the same method as that of the repeated dose study.

3. Results

3.1. The 28-day repeated dose study

No deaths were observed in any groups. There were no effects on the clinical observation, detailed clinical observation, sensory function, motor activity, body weight, or hematological findings. Food consumption significantly decreased in the fourth week at 300 mg/kg bw/day and in the third and fourth weeks at 1000 mg/kg bw/day in males (Table 1). Table 2 presents the urinary examination in rats given DPPD at the end of the administration period. Protein levels significantly decreased in all treatment groups, but this was not dose dependent and was considered to be due to spontaneously occurring higher levels in control groups.

As shown in Table 3, no effects were found in the hematological examination in rats dosed with DPPD for 28 days. Table 4 presents serum biochemistry in rats given DPPD at the end of the administration period. Total bilirubin significantly increased in all treatment groups at the end of the treatment period in males, but it was not observed at the end of the recovery period. When DPPD was added to rat serum, bilirubin levels measured by the diazo method increased in a concentration-related manner with or without the rat S9 mix (Table 5). Therefore, increased bilirubin levels in this study were considered to be due to interference by DPPD. In females, γ-GTP significantly decreased (0.63 IU/L) at 1000 mg/kg bw/day at the end of the administration period, but it was within the background data of the facility (0.31–2.06 IU/L) and was not considered to be toxicologically significant. This change was not observed at the end of the recovery period.

Table 6 shows the incidence of histopathological findings in rats. At necropsy, slight hydrometra in the uterus was found in one female at 300 mg/kg bw/day, and dilatation of the lumen was histopathologically observed in the uterus of this female at the end of administration period; however, no gross or histopathological effects in the uterus were observed at 1000 mg/kg bw/day. Relative and absolute weights of the thyroid gland in males and absolute weight of the kidney in females significantly increased at 100 mg/kg bw/day, but histopathological changes were not significantly different in these organs at the end of the administration period. No other effects were observed in organ weights in both sexes. In the histopathological examination, no significant changes were observed in both sexes.

3.2. The reproduction/developmental study

There were no effects on body weight, body weight gain, and food consumption. Neither death nor clinical toxicity was observed in males. One female in the 50 mg/kg bw/day group was sacrificed on day 9 of the administration period for incorrect operation at the time of the dosage. At 300 mg/kg bw/day, two females showed piloerection, hypothermia, and pale skin on day 23 of pregnancy. One of these two females died and the other was sacrificed due to dystocia on day 23 of pregnancy. Another female showing piloerection and pale skin delivered only three live pups. Nesting and nursing were not observed in this female, and this female was sacrificed on day 1 of lactation due to total litter loss. In addition, one female showed piloerection on day 23 of gestation, and another female showed pale skin on day 22 of gestation at 300 mg/kg bw/day. However, no abnormalities were found in their delivery.

No effects were observed in the organ weights of male and female rats given DPPD. The following gross pathological findings were observed in two females who died or were sacrificed on

Table 1
Body weight and food consumption in rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)	Male				Female			
	0	100	300	1000	0	100	300	1000
Body weight (g)								
Day 1	162 ± 6	164 ± 4	163 ± 7	162 ± 6	139 ± 5	138 ± 6	139 ± 7	139 ± 6
Day 7	213 ± 12	211 ± 6	216 ± 10	211 ± 10	162 ± 8	162 ± 5	159 ± 5	161 ± 9
Day 14	272 ± 16	273 ± 8	277 ± 10	268 ± 18	182 ± 11	182 ± 13	180 ± 10	181 ± 10
Day 21	325 ± 21	329 ± 15	329 ± 15	314 ± 24	203 ± 11	213 ± 16	202 ± 13	203 ± 15
Day 28	362 ± 27	368 ± 26	363 ± 14	349 ± 28	223 ± 11	230 ± 23	220 ± 13	220 ± 19
Food consumption (g/rat/day)								
Week 1	30 ± 4	30 ± 2	31 ± 3	29 ± 2	23 ± 3	21 ± 4	23 ± 2	22 ± 3
Week 2	32 ± 3	34 ± 1	33 ± 1	31 ± 3	21 ± 3	24 ± 3	22 ± 4	22 ± 2
Week 3	34 ± 3	36 ± 2	34 ± 2	32 ± 2*	23 ± 2	24 ± 4	22 ± 4	22 ± 3
Week 4	41 ± 4	38 ± 3	35 ± 3*	31 ± 3**	24 ± 3	24 ± 5	24 ± 1	23 ± 2

* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett test).

Table 2
Urinary findings of rats treated with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
No. of animals		5	5	5	5	5	5	5	5
Color	Colorless	1	0	0	0	0	0	0	0
	Pale yellow	4	5	5	5	5	5	5	5
Cloudy	Negligible	5	5	5	5	5	5	5	5
pH	7.0	4	1	2	3	0	0	0	0
	7.5	1	4	3	1	1	2	1	2
	8.0	1	0	0	0	4	1	3	3
	8.5	0	0	0	0	0	2	1	0
Protein[a]	±	0	5**	5**	4*	1	0	1	1
	1+	4	0**	0**	0*	4	4	3	3
	2+	1	0**	0**	1*	0	1	1	1
Occult blood	Negligible	5	5	5	5	5	5	5	5
Urobilinogen	0.1 (ehrlich unit/dL)	5	5	5	5	5	5	5	5
Bilirubin	Negligible	5	5	5	5	5	5	5	5

* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett test).
[a] Protein: ± (15–30 mg/dL), 1+ (30–100 mg/dL), 2+ (100–300 mg/dL).

Table 3
Hematological findings of rats treated with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
RBC	(10^4/μL)	811 ± 37	773 ± 27	773 ± 41	800 ± 70	768 ± 28	739 ± 46	741 ± 57	777 ± 10
Hb	(g/dL)	15.6 ± 0.4	15.2 ± 0.4	15.1 ± 0.4	15.3 ± 1.0	14.9 ± 0.5	14.5 ± 0.5	14.2 ± 0.9	15.0 ± 0.3
Ht	(%)	47.4 ± 1.2	46.4 ± 1.0	46.3 ± 1.6	46.3 ± 2.9	44.4 ± 1.5	43.3 ± 1.4	42.5 ± 2.6	44.6 ± 0.4
MCV	(fL)	58.8 ± 2.4	60.0 ± 1.0	59.8 ± 2.9	58.2 ± 2.4	57.8 ± 1.9	58.6 ± 1.9	57.6 ± 1.9	57.6 ± 1.1
MCH	(pg)	19.3 ± 0.7	19.6 ± 0.3	19.6 ± 0.7	19.1 ± 0.7	19.4 ± 0.6	19.6 ± 0.7	19.2 ± 0.5	19.3 ± 0.3
MCHC	(%)	33.0 ± 0.2	32.7 ± 0.2	32.7 ± 0.5	33.0 ± 0.5	33.6 ± 0.3	33.4 ± 0.5	33.4 ± 0.4	33.6 ± 0.5
Ret.	(‰)	27.7 ± 5.4	28.5 ± 8.2	30.5 ± 6.0	32.4 ± 13.8	19.6 ± 1.8	23.6 ± 3.4	23.1 ± 4.9	19.5 ± 4.4
PT	(s)	13.1 ± 0.5	13.2 ± 0.2	13.1 ± 0.2	13.5 ± 0.4	13.3 ± 0.2	13.1 ± 0.2	13.0 ± 0.4	13.2 ± 0.4
APTT	(s)	20.9 ± 1.8	22.2 ± 1.1	20.5 ± 1.1	22.3 ± 0.6	18.4 ± 1.6	17.6 ± 1.1	17.7 ± 1.1	18.0 ± 0.8
Platelet	(10^4/μL)	141 ± 10	130 ± 8	133 ± 16	150 ± 22	121 ± 16	122 ± 10	124 ± 10	132 ± 15
WBC	(10^2/μL)	76 ± 25	76 ± 21	57 ± 12	69 ± 10	59 ± 23	39 ± 10	38 ± 9	44 ± 8

RBC: Red blood cell; Hb: Hemoglobin; Ht: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; Ret.: Reticulocyte; PT: prothrombin time; APTT: Activated partial thromboplastin time; WBC: White blood cells.

Table 4
Serum biochemistry in rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
No. of animals		5	5	5	5	5	5	5	5
LDH	(IU/L)	358 ± 153	289 ± 92	335 ± 123	349 ± 132	458 ± 119	341 ± 125	463 ± 233	406 ± 120
AST	(IU/L)	71 ± 8	84 ± 23	75 ± 4	72 ± 6	78 ± 8	67 ± 5	77 ± 13	94 ± 57
ALT	(IU/L)	34 ± 1	45 ± 20	38 ± 5	35 ± 2	28 ± 4	29 ± 4	30 ± 4	32 ± 13
ALP	(IU/L)	808 ± 78	819 ± 136	774 ± 52	818 ± 188	579 ± 48	426 ± 62	460 ± 152	452 ± 93
γ-GTP	(IU/L)	0.61 ± 0.27	0.40 ± 0.23	2.34 ± 4.08	0.42 ± 0.10	1.32 ± 0.48	0.81 ± 0.39	1.35 ± 0.08	0.63 ± 0.25*
T. protein	(g/dL)	5.82 ± 0.30	5.76 ± 0.29	5.82 ± 0.21	5.79 ± 0.11	5.96 ± 0.27	5.85 ± 0.10	5.88 ± 0.26	5.90 ± 0.18
Albumin	(g/dL)	2.90 ± 0.30	2.73 ± 0.23	2.86 ± 0.22	2.99 ± 0.17	3.12 ± 0.29	3.00 ± 0.09	3.06 ± 0.28	3.14 ± 0.18
Albumin/Globulin		0.99 ± 0.10	0.90 ± 0.06	0.97 ± 0.09	1.07 ± 0.09	1.11 ± 0.14	1.05 ± 0.06	1.09 ± 0.12	1.13 ± 0.07
T. cholesterol	(mg/dL)	62 ± 10	79 ± 19	81 ± 7	64 ± 13	79 ± 7	91 ± 15	75 ± 14	74 ± 10
Triglycerides	(mg/dL)	68 ± 26	64 ± 8	51 ± 17	56 ± 12	27 ± 11	27 ± 7	24 ± 10	18 ± 3
Glucose	(mg/dL)	152 ± 14	155 ± 11	145 ± 8	146 ± 7	128 ± 8	139 ± 7	133 ± 9	138 ± 22
BUN	(mg/dL)	14.4 ± 1.4	13.3 ± 1.8	12.3 ± 2.3	13.0 ± 1.2	15.5 ± 2.1	13.9 ± 2.2	14.0 ± 2.3	15.6 ± 3.2
Creatinine	(mg/dL)	0.38 ± 0.02	0.37 ± 0.04	0.40 ± 0.02	0.41 ± 0.03	0.46 ± 0.06	0.39 ± 0.05	0.39 ± 0.04	0.41 ± 0.06
T. bilirubin[a]	(mg/dL)	0.33 ± 0.05	0.53 ± 0.05**	0.60 ± 0.10**	0.61 ± 0.09**	0.26 ± 0.05	0.26 ± 0.01	0.29 ± 0.03	0.29 ± 0.04
Calcium	(mg/dL)	9.9 ± 0.5	9.8 ± 0.1	9.8 ± 0.2	9.8 ± 0.3	9.6 ± 0.4	9.5 ± 0.3	9.3 ± 0.2	9.5 ± 0.3
Phosphorus	(mg/dL)	8.2 ± 0.5	8.6 ± 0.4	8.4 ± 0.6	8.5 ± 0.6	7.0 ± 0.9	7.3 ± 0.5	6.8 ± 0.7	7.0 ± 0.5
Sodium	(mEq/L)	146 ± 1	147 ± 1	147 ± 0	146 ± 1	146 ± 1	147 ± 1	148 ± 2	147 ± 2
Potassium	(mEq/L)	4.93 ± 0.46	4.97 ± 0.16	5.15 ± 0.28	5.37 ± 0.41	5.04 ± 0.38	4.96 ± 0.52	4.94 ± 0.33	4.90 ± 0.32
Chloride	(mEq/L)	104 ± 2	105 ± 1	104 ± 2	105 ± 1	108 ± 1	108 ± 2	108 ± 3	108 ± 0

LDH: lactate dehydrogenase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.
* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett type test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett type test).
[a] Interference of DPPD with bilirubin measurements in male rats is described in Sections 3 and 4 (see Table 5 also).

Table 5
Total bilirubin levels in male rat serum with or without the S9 mix measured by the diazo method.

DPPD (mg/mL)	Total bilirubin (mg/dL)	Total bilirubin (mg/dL)
	Without the S9 mix	With the S9 mix[a]
0 (serum)	0.28	0.34
0 (serum and vehicle[b])	0.33	0.35
0.001	0.32	
0.01	0.37	
0.1	1.03	1.04
1	1.17	

[a] Rat liver S9.
[b] Vehicle: acetone and dimethyl sulfoxide (1:1).

day 23 of pregnancy: hemorrhage in the lumen of the uterus, incomplete retention and red color in the lung, and dark red medulla and hardness on the kidney in both animals; hydrothorax in the thoracic cavity, attachment of red content in mucosa of the glandular stomach and recessed area, or red spots in the duodenum in either animal. In the histopathological examination, slight hemorrhage in the endometrium, and very slight edema, very slight foam cell accumulation in alveolus, and very slight capillary fibrinous thromboses in the lung were observed in the two females. The histopathological examination revealed no toxicological effects in other males and females.

Table 7 shows reproductive and developmental findings in rats given DPPD. One female at 8 mg/kg bw/day did not deliver pups by day 25 of gestation. An autopsy on day 26 of gestation revealed no implantations in this female. This female was excluded from the statistical evaluation of pregnant females. No changes attributable to the chemical were noted in the number of mated pairs, number of copulated pairs, copulation index, number of fertile males, fertility index, length of estrus cycle, pairing days until copulation, number of corpora lutea, number of implantations, implantation index, and number of pregnant females. Gestation lengths were significantly longer than the control group at 50 and 300 mg/kg bw/day.

Although no statistical significance was observed, the number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation dose dependently decreased. The number of live pups and viability index were also decreased on day 4 of lactation in treatment groups, especially at 300 mg/kg bw/day. No changes were observed in litter weights and body weights of pups on days 0 and 4 of the lactation period. No gross external or internal abnormalities were observed in pups.

4. Discussion

In the repeated dose study, no deaths were observed in any of the groups; there were no effects on the clinical observation, detailed

Table 6
Incidence of histopathological findings of rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)	Grade	Administration period				Recovery period	
		0	100	300	1000	0	1000
Male							
No. of animals		5	0	0	5	0	0
Lung							
Arterial mineralization	+	0	–	–	1	–	–
Foam cell accumulation	+	1	–	–	1	–	–
Heart							
Myocardial degeneration/fibrosis	+	0	–	–	1	–	–
Liver							
Microgramuloma	+	1	–	–	0	–	–
Extramedullary hematopoiesis	+	1	–	–	0	–	–
Kidney							
Hyaline droplet in the proximal tubular epithelium	+	4	–	–	5	–	–
Basophilic tubule	+	1	–	–	2	–	–
Thymus							
Hemorrhage	+	1	–	–	1	–	–
Spleen							
Extramedullary hematopoiesis	+	5	–	–	5	–	–
Deposition of a brown pigment	+	5	–	–	5	–	–
Prostate							
Interstitial lymphocytic infiltration	+	1	–	–	0	–	–
Female							
No. of animals		5	0	0	5	5	5
Lung							
Arterial mineralization	+	1	–	–	2	–	–
Osseous metaplasia	+	1	–	–	0	–	–
Liver							
Microgramuloma	+	2	–	–	1	–	–
Kidney							
Basophilic tubule	+	1	–	–	2	–	–
Solitary cyst	+	1	–	–	1	–	–
	++	1	–	–	0	–	–
Thymus							
Hemorrhage	+	0	–	–	1	–	1 (1)
Spleen							
Extramedullary hematopoiesis	+	5	–	–	5	–	–
Deposition of a brown pigment	+	5	–	–	5	–	–
Pituitary							
Remnant of Rahke's pouch	+	0	–	–	1	–	–
Utreus							
Dilatation of the lumen	+	0	–	1 (1)	0	–	–

Grade (+: slight change; ++: mild change; –: not applicable).
Parentheses indicate the number of rats examined.

Table 7
Reproductive and developmental findings in rats dosed with DPPD by gavage in the reproduction/developmental toxicity study.

Dose (mg/kg bw/day)	0	8	50	300
Number of mated pairs	13	13	12	13
Number of copulated pairs	13	13	12	13
Copulation index	100.0	100.0	100.0	100.0
Number of fertile males	13	12	12	13
Fertility index	100.0	92.3	100.0	100.0
Length of the estrous cycle in the pre-treatment period (days)	4.1 ± 0.3 (13)	4.2 ± 0.4 (13)	4.3 ± 0.5 (12)	4.1 ± 0.3 (13)
Length of the estrous cycle in the treatment period (days)	4.0 ± 0.0 (13)	4.1 ± 0.3 (13)	4.3 ± 0.5 (12)	4.2 ± 0.4 (12)
Pairing days until copulation	2.4 ± 1.3	2.7 ± 1.3	2.8 ± 1.5	2.7 ± 1.3
Number of corpora lutea	17.8 ± 2.2 (13)	18.4 ± 3.3 (12)	17.3 ± 1.3 (12)	16.9 ± 1.3 (11)
Number of implantations	15.9 ± 1.5 (13)	16.3 ± 2.7 (12)	16.2 ± 1.0 (12)	15.8 ± 1.9 (11)
Implantation index	90.7 ± 11.9 (13)	89.6 ± 16.3 (12)	94.0 ± 5.7 (12)	93.5 ± 8.1 (11)
Number of pregnant females	13	12	12	13
Number of pregnant females with live pups	13	12	12	11
Gestation length (days)	22.4 ± 0.5 (13)	22.8 ± 0.5 (12)	23.0 ± 0.0** (12)	23.0 ± 0.4** (11)
Day 0 of lactation				
Number of pups born	14.8 ± 2.1 (13)	14.8 ± 3.1 (12)	14.3 ± 1.5 (12)	13.7 ± 3.1 (11)
Delivery index	92.5 ± 7.5 (13)	90.7 ± 8.2 (12)	88.3 ± 8.7 (12)	86.7 ± 16.1 (11)
Number of live pups	14.7 ± 2.1 (13)	14.4 ± 2.7 (12)	13.8 ± 1.5 (12)	12.8 ± 4.1 (11)
Sex ratio	44.3 ± 18.3 (13)	39.4 ± 12.1 (12)	47.6 ± 14.1 (12)	48.1 ± 13.2 (11)
Birth index	92.1 ± 7.9 (13)	88.4 ± 7.1 (12)	85.8 ± 10.1 (12)	81.2 ± 24.7 (11)
Live birth index	99.5 ± 1.7 (13)	97.7 ± 5.4 (12)	97.2 ± 5.3 (12)	92.0 ± 20.7 (11)
Day 4 of lactation				
Number of live pups	14.5 ± 1.9 (13)	13.9 ± 2.6 (12)	13.8 ± 1.4 (12)	12.2 ± 5.0 (11)
Sex ratio	44.7 ± 18.2 (13)	39.4 ± 12.2 (12)	47.9 ± 14.3 (12)	48.0 ± 14.6 (10)
Viability index	99.1 ± 2.2 (13)	97.0 ± 8.5 (12)	99.5 ± 1.8 (12)	87.5 ± 30.0 (11)

Parentheses indicate the number of dams.
Copulation index = (number of copulated pairs/number of mated pairs) × 100%.
Fertility index = (number of fertile males/number of copulated pairs) × 100%.
Delivery index = (number of pups born/number of implantations) × 100%.
Birth index = (number of live pups on day 0/number of implantations) × 100%.
Live birth index = (number of live pups on day 0/number of pups born) × 100%.
Sex ratio = (number of male live pups/number of live pups) × 100%.
Viability index on day 4 of lactation = (number of live pups on day 4/number of live pups on day 0) × 100%.
* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett type test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett type test).

clinical observation, sensory function, motor activity, body weight, urinary examination, hematological findings, organ weights, or histopathological findings. In the blood chemistry examination, total bilirubin levels significantly increased in all treatment groups at the end of the treatment period in males; however, bilirubin and urobilinogen levels in urine did not increase. In addition, no related effects such as histopathological changes in the liver were observed. Because both bilirubin and DPPD contain –NH substitutes, the interference of DPPD with bilirubin measurements was anticipated. When DPPD was added to rat serum, bilirubin levels measured by the diazo method increased in a concentration-related manner with or without the rat S9 mix. Therefore, increased bilirubin levels in the present study were considered to be due to interference by DPPD. However, it is of interest that these effects were not observed in females.

Information on absorption, distribution, metabolism, and excretion (ADME) of DPPD is available in male rats (Umeniwa et al., 1985). DPPD dosed by an intraduodenal route was rapidly metabolized to DPPD glucuronide and was also suggested to be metabolized to hydroxylated DPPD. After a 6-day oral dosing, the total fecal excretion of DPPD was 55.4% (unchanged) and total urinary excretion of unchanged DPPD and glucuronide DPPD was 0.04%; unchanged DPPD was temporary detected in fat tissues. There is no information available on sex differences for the ADME of DPPD, and it is difficult to predict whether sex differences do indeed exist. Results of the present study may suggest that detectable DPPD or DPPD metabolites by the diazo method were very low in the serum of female rats for some reason.

In the reproduction/developmental toxicity study presented here, no effects were observed in male fertility function. The number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation dose dependently decreased, but they were not significant. No changes were observed in litter weights and body weights of pups on days 0 and 4 of the lactation period. We confirmed that gavage doses of DPPD significantly prolonged the gestation period in rats.

Marois (1998) investigated a possible mechanism of the prolonged gestation period caused by DPPD. When prostaglandin $F_{2\alpha}$, a regulator of uterus contraction, was injected into rats given 40 mg DPPD from the 14th day of pregnancy, observed adverse effects decreased. Prostaglandin E production was markedly inhibited by DPPD in rabbit kidney medulla slices (Fujimoto et al., 1984; Fujita et al., 1982). Prostaglanidins E_2 and $F_{2\alpha}$ induce uterus contraction (Parkington et al., 1999), and the prolonged gestation period was considered to be due to low prostaglandins levels caused by DPPD administration, similar to non-steroidal anti-inflammatory drugs (NSAIDs). If DPPD acts like NSAIDs, adverse effects such as gastrointestinal disturbances, antiplatelet activity, and kidney failure, known side effects of NSAIDs (Ejaz et al., 2004), can be caused by repeated doses of DPPD.

Prostaglandins are synthesized from arachidonic acid by cyclooxygenase (COX). Prostaglandins play an important role in modulating mucosal integrity and various functions of the gastrointestinal tract, and NSAIDs are known to damage the gastrointestinal tract by reducing these functions (Al-Saeed, 2012; Takeuchi et al., 2010). In the reproduction/developmental toxicity study, hemorrhage in the stomach and duodenum were observed in dead or sacrificed dams at 300 mg/kg bw/day, but no toxicologically significant effects were observed in food consumption. In the repeated dose study, food consumption significantly decreased at 300 and 1000 mg/kg bw/day in males; there is a possibility that DPPD affected the gastrointestinal tract in males. However, these changes

were not considered to be toxicologically significant because of high food consumption in the control group, no differences in body weights, and no gross- or histo-pathological effects in the gastrointestinal tract in the repeated dose study. This result was consistent with a 2-year feeding study in which no histopathological effects were found in the gastrointestinal tract (Hasegawa et al., 1989).

Prostaglandins also regulate platelet aggregation, and NSAIDs are known to inhibit platelet aggregation (Fabre et al., 2001). In the reproduction/developmental toxicity study, pale skin and hemorrhage in the uterus, stomach, and duodenum were observed in dead or sacrificed dams at 300 mg/kg bw/day. It is questionable if these observations may suggest inhibitory effects of platelet aggregation. In the repeated dose study, slight hydrometra in the uterus was observed in one female at 300 mg/kg bw/day at the end of administration period, but it was not dose dependent. In addition, heamorrhage in the thymus in one female was observed at 1000 mg/kg bw/day at the end of recovery period in the repeated dose study, but it was not observed at the end of administration period. Therefore, hydrometra in the uterus and heamorrhage in the thymus observed in the repeated dose study were considered to be incidental.

Gavage doses of DPPD showed weaker effects than a previously reported feeding dose study. In a feeding study by Oser and Oser (1956), the mean gestation period was significantly longer [22.9 days (22–24 days), 24.1 days (22–25 days), 25.2 days (23–29 days), and 24.7 (22–27 days) at 0.025, 0.10, 0.40, and 1.60% (7, 28, 113, and 450 mg/kg bw/day: conversion data from RTECS)] than that of the control group [22.1 days (21–23 days)] (Oser and Oser, 1956). An increased gestation length was associated with higher birth weights due to the longer growth period in the uterus and resulted in dystocia and stillbirths in the feeding study. It was considered that feeding doses of DPPD continuously inhibited prostaglandin synthesis, while gavage doses allowed prostaglandin synthesis intermittently.

In the 28-day repeated dose study, neither deaths nor dose-related adverse effects were observed up to 1000 mg/kg bw/day (the highest dose tested) in both sexes. Therefore, the NOAEL of repeated dose toxicity was considered to be 1000 mg/kg bw/day in rats. In the reproduction/developmental toxicity study, no adverse effects were found in male reproduction up to 300 mg/kg bw/day (the highest dose tested). However, significant longer gestation length was observed at 50 and 300 mg/kg bw/day in dams, and the NOAEL of reproduction/developmental toxicity was considered to be 8 mg/kg bw/day in rats.

Although a reproductive toxicity study is important for risk assessment, sometimes it is not conducted by predicting the effects from available repeated dose studies. When low reproductive toxicity is expected from repeated dose studies, only a prenatal developmental toxicity study can be conducted to observe the developmental effects of chemicals, but effects on fertility and parturition are not observed in this study. In case of DPPD, the results of a long term feeding study in rats (Hasegawa et al., 1989), and the current repeated dose study indicated very low toxicity; it could be expected that DPPD is unlikely to cause reproductive effects. However, our reproductive/developmental toxicity study showed a huge discrepancy in NOAEL with these repeated dose studies. Our experience suggests that conducting a reproduction/developmental study, which includes mating and parturition, is important for the risk assessment of reproductive toxicity.

In conclusion, the results of the current study sufficiently provide initial toxicity data for repeated dose and reproduction/developmental toxicities of DPPD. The NOAEL of repeated dose toxicity was considered to be 1000 mg/kg bw/day based on no adverse effects. The NOAEL of reproduction/developmental toxicity was considered to be 8 mg/kg bw/day based on a longer gestation length at 50 and 300 mg/kg bw/day.

Conflict of Interest

None of the authors have any conflicts of interest associated with this study.

Acknowledgement

This study was supported by the Ministry of Health, Labour and Welfare, Japan.

References

Al-Saeed, A., 2012. Gastrointestinal and cardiovascular risk of nonsteroidal anti-inflammatory drugs. Oman Med. J. 26, 385–391.
Chemicalland21, 2012. N,N'-Diphenyl-p-Phenylenediamine. <http://www.chemicalland21.com/>.
Ejaz, P., Bhojani, K., Joshi, V.R., 2004. NSAIDs and kidney. J. Assoc. Phys. India 52, 632–640.
Fabre, J.E., Nguyen, M., Athirakul, K., Coggins, K., McNeish, J.D., Austin, S., Parise, L.K., FitzGerald, G.A., Coffman, T.M., Koller, B.H., 2001. Activation of the murine EP3 receptor for PGE2 inhibits cAMP production and promotes platelet aggregation. J. Clin. Invest. 107, 603–610.
Fujimoto, Y., Tanioka, H., Toibana, E., Yamamoto, T., Fujita, T., 1984. Inhibition of prostaglandin synthesis in rabbit kidney medulla slices by antioxidants. J. Pharm. Pharmacol. 36, 195–197.
Fujita, T., Fujimoto, Y., Tanioka, H., 1982. Antioxidant effects on prostaglandin synthesis in rabbit kidney medulla slices. Experientia 38, 1472.
HSDB, 2012. P-Phenylenediamine, N,N'-Diphenyl (CASRN: 74-31-7). <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>.
Hasegawa, A., Suzuki, S., 2005. Analysis of chemical substances in the leachate of landfill by LC/MS. Kanagawaken Kankyo Kagaku Senta Gyomu Hokoku 28, 45–51.
Hasegawa, R., Fukushima, S., Hagiwara, A., Masui, T., Masuda, A., Ito, N., 1989. Long-term feeding study of N, N'-diphenyl-p-phenylenediamine in F344 rats. Toxicology 54, 69–78.
Labor Hygiene and Occupational Diseases, 1966. Gigiena Truda i Professional'nye Zabolevaniya (V/O Mezhdunarodnaya Kniga, 113095 Moscow, USSR) 10, 49 (cited in RTECS).
MOE, 2005. Chemicals in the Environment. <http://www.env.go.jp/chemi/kurohon/2005/http2005/pdfs/1-hyoka.pdf>.
Marhold, J., 1986. Prehled Prumyslove Toxikologie; Organicke Latky, Prague, Czechoslovakia, Avicenum (cited in RTECS).
Marois, M., 1998. Antioxidants, prostaglandins and prematurity. Bull. Acad. Natl. Med. 182, 581–595, discussion 595–587.
OECD, 2012. Manual for the Assessment of Chemicals. <http://www.oecd.org/document/7/0,2340,en_2649_34379_1947463_1_1_1_1,00.html>.
Oser, B., Oser, M., 1956. Feed antioxidants, inhibitory effect of feed grade diphenyl-p-phenylenediamine (DPPD) of parturition in rats. Agric. Food Chem. 4, 796–797.
Parkington, H.C., Tonta, M.A., Davies, N.K., Brennecke, S.P., Coleman, H.A., 1999. Hyperpolarization and slowing of the rate of contraction in human uterus in pregnancy by prostaglandins E2 and f2alpha: involvement of the Na+ pump. J. Physiol. 514 (Pt 1), 229–243.
Takeuchi, K., Kato, S., Amagase, K., 2010. Prostaglandin EP receptors involved in modulating gastrointestinal mucosal integrity. J. Pharmacol. Sci. 114, 248–261.
Umeniwa, K., Matsuda, T., Maeda, M., Miyazaki, K., Arita, T., 1985. Biological fate of N, N'-diphenyl-p-phenylenediamine. Yakugaku Zasshi 105, 65–76.

〈内分泌撹乱物質スクリーニング〉

第18章　BG1Luc細胞を用いるエストロゲン受容体転写活性化試験法

(OECD TG 457)

小野 敦
(Atsushi Ono)

1　はじめに

OECD（経済協力開発機構）では，1998年に重点活動項目の1つとして，内分泌かく乱作用を有する可能性のある化学物質のスクリーニングおよび詳細試験のテストガイドラインの整備を開始し，スクリーニングから確定試験までに有用と考えられる試験法を5レベルからなる“内分泌かく乱作用を有する可能性のある化学物質の試験および評価に関するOECD概念枠組み”として整理し，各レベルにおける試験法のガイドラインの整備を進めている[1]。

本試験法は，エストロゲン受容体（ER）を介する下流遺伝子転写活性への化学物質の影響を評価する転写活性化試験法（Transactivation（TA）試験）であり，OECD概念枠組みレベル2の「機構に関する情報をもたらす *in vitro* 試験」に相当する。

ERは，遺伝子転写因子であり，エストロゲンとの相互作用により下流遺伝子転写を介して細胞増殖，胎児の発達，恒常性維持などにおける細胞機能を制御している。正常なエストロゲン作用のかく乱は，様々な健康影響を引き起こす可能性がある。TA試験は，レポータージーン試験とも呼ばれ，化学物質のホルモン受容体への結合を介した転写活性化能を測定する方法であり，化学物質を暴露してレポータータンパク質の発現量から転写活性化率を評価する[2]。

本試験では，ヒト卵巣腺癌細胞由来BG-1細胞をホスト細胞として，4回繰り返しエストロゲン応答配列下流にマウス乳腺腫瘍ウイルスプロモーター（MMTV）を繋いだホタルルシフェラーゼ遺伝子を組み込んだレポータープラスミドを安定形質転換したBG1Luc4E2細胞をレポーター細胞として用い，内因性ERによるレポーター遺伝子の発現を検出する。BG1Luc4E2細胞は，エストロゲン以外のステロイドや非ステロイドホルモンとの交差反応性はほとんど無いことが示されている[3]。

脊椎動物では，少なくともERαとβの2種の主要なサブタイプが知られている。ERαとβは異なる遺伝子にコードされており，組織分布や生物学的機能やリガンド特異性に差がある[4~7]。古典的なエストロゲンの作用は，主にERαを介していることからER TA試験の多くは，ERαを介した作用のみを検出するよう設計されている[8]。BG1細胞は，ERαとともに発現量は少な

いもののER βを内因性に発現しており[9]．本試験系ではER α，ER βいずれを介した作用であっても検出可能である。

BG1Luc4E2細胞を用いたERアゴニストおよびアンタゴニスト検出のためのプロトコルは，ICCVAM（The Interagency Coordinating Committee on the Validation of Alternative Methods）が中心となり，ECVAM（European Centre for the Validation of Alternative Methods）および日本動物実験代替望評価センター（JaCVAM：Japanese Center for the Validation of Alternative Methods）の協力により，日米欧3試験施設が参加したバリデーション試験の結果をもとに，第3者公開評価会議による評価を受けて最終化された[10]。第3者評価結果および最終プロトコルを基にICCVAMよりOECDガイドラインが提案され，2012年にTG 457として成立した[11]。第3者公開評価会議における評価資料や評価レポートおよび最終プロトコルなどはICCVAMウェブサイトから入手可能である[12]。

2 材料および試薬

2.1 生物材料

(1) BG1Luc4E2細胞

測定に用いるBG1Luc4E2細胞は，カリフォルニア大学（Davis, California, USA）[*1]もしくはXenobiotic Detection Systems（Durham, North Carolina, USA）[*2]から入手する。細胞の入手には，技術ライセンス契約が必要である。

(2) 培養試薬

- RPMI 1640培地，L-グルタミン含有（RPMI 1640）
- Dulbecco's Modification of Eagle's培地（DMEM），4.5 g/Lグルコース，ピルビン酸ナトリウム含有，フェノールレッド，L-グルタミン不含
- ウシ胎児血清（FBS）
- ウシ胎児血清，charcoal/dextran処理，triple 0.1 μm sterile filtered（charcoal/dextran処理FBS）[Hyclone, Cat. No. SH30068.03]
- 10xトリプシン，2.5% in Hank's balanced salt solution（HBSS），Ca, Mg, フェノールレッド不含
- ペニシリン／ストレプトマイシン5000LU溶液（Pen-Strep）。ペニシリン，5000 μg/mLストレプトマイシン

[*1] UC Davis, Office of Research Technology Transfer Services 1850 Research Park Drive, Suite 100 Davis, CA 95618. (530) 754-8649. E-mail：msdenison@ucdavis.edu

[*2] Xenobiotic Detection Systems, Inc. 1601 E Geer St # S Durham, NC 27704. (919) 688-4804. E-mail：info@dioxius.com

・リン酸緩衝液（PBS, 1x）Ca, Mg 不含
・Gentamycin Sulfate（G418）, 50 mg/mL
・L-グルタミン, 29.2 mg/mL

2.2　試薬[*3]

(1) ルシフェラーゼ測定用試薬
・5x Lysis 溶液［Promega, Cat. No. E1531］
・Luciferase Assay System（10-Pack）［Promega Cat. No. E1501］

(2) 溶媒
・Dimethyl sulfoxide（DMSO, CAS No. 67-68-5）
※被験物質を溶解可能かつ細胞培養液と混和可能な他の溶媒も使用可能である。DMSO, 水およびエタノール（95％以上）等が推奨される。被験物質とコントロールは, 同一の100％溶媒に溶解した後, 適切な濃度まで培養液で希釈して用いる。溶媒の培地中最終濃度は細胞毒性を示さず, 測定結果に影響を与えない事が確認済みの濃度以下とする。DMSO を用いる場合は, 最終濃度 1.0％以下とする。本稿では, 溶媒として DMSO を用い, 培地中最終濃度 1.0％で添加する場合について記載した。

(3) 陽性コントロール化合物
・アゴニスト・アンタゴニストアッセイ共通：17β-estradiol（E2, CAS No. 50-28-2）
・アゴニストアッセイ：p, p'-methoxychlor（Meth, CAS No. 72-43-5）
・アンタゴニストアッセイ：Raloxifene（Ral, CAS No. 84449-90-1）, Tamoxifen（Tam, CAS No. 10540-29-1）

2.3　器具・機器
・マイクロプレートルミノメーター：Berthold Orion L, II［Titertek-Berthold, AL, USA］など
※細胞培養およびルシフェラーゼ化学発光測定に用いる一般的な器具および理化学機器を用いる。具体的な例について表 S1（本書添付 CD に収載）に示す。

3　試験方法

本試験法は, 化学物質による ER 活性化を検出するアゴニストアッセイと E2 による ER 活性化の阻害を検出するアンタゴニストアッセイから構成される。各アッセイは独立したプロトコ

[*3] （ ）内には, 本文中で用いた略称および CAS ナンバーを示す。また, 一部の試薬については, ［ ］内に代表的なメーカー・カタログ番号を示したが, 同等品であれば使用可能である。

ル[13,14]であるが，多くの操作は共通であることから，本稿では異なる操作が必要となる部分についてのみ個別の項目を設けて記載した。

3.1 細胞の準備

3.1.1 培地および関連試薬の調整

・RPMI 細胞増殖用培地（PRMI）

RPMI 1640 に 0.9 % Pen-Strep と 8.0 % FBS を添加して RPMI 増殖培地を調整する。RPMI1640 500 mL ボトルで調整する場合，室温に戻した FBS 44 mL，Pen-Strep 5 mL を RPMI1640 500 mL ボトルに添加して混和する。

・エストロゲン除去 DMEM 測定用培地（EFM）

DMEM に 4.5% charcoal/dextran 処理 FBS，1.9% L-グルタミン，0.9% Pen-Strep を添加して調整する。DMEM 500 mL ボトルで調整する場合，charcoal/dextran 処理 FBS 24 mL，L-グルタミン 10 mL および Pen-Strep 5 mL を DMEM 500 mL ボトルに添加して混和する。

・1x トリプシン溶液

10x トリプシン溶液は 10 mL ずつ分注して－20℃で保存する。10x トリプシン溶液 1 mL と 1x PBS 9 mL を 15 mL 滅菌済み遠心チューブで混和する（10 本調整する）。1x トリプシン溶液は，－20℃で保管する。

・1x 細胞溶解液

15 mL 遠心チューブに 5x Lysis 溶液 2 mL と脱イオン蒸留水 8 mL を混和して調整する。5x 溶液，1x 溶液ともに－20℃で保管し，1 年以内に使用する。

・ルシフェラーゼ試薬

ルシフェラーゼ試薬は，ルシフェラーゼバッファーと凍結乾燥ルシフェラーゼ基質から構成される。未開封のルシフェラーゼ試薬は，－70℃で保管し 1 年以内に使用する。ルシフェラーゼバッファー10 mL を凍結乾燥ルシフェラーゼ基質容器に加え，ボルテックスミキサーを用いて軽く撹拌して基質を溶解させる。溶解したら 15 mL 遠心チューブに分注して－20℃で保存し，一ヶ月以内に使用する。

3.1.2 細胞の増殖

細胞は，細胞培養フラスコを用いて CO_2 インキュベーター（温度：37℃ ±1℃，湿度：90% ±5% および CO_2 濃度：5.0% ±1%）で単層培養する。培養中の細胞は，倒立位相差顕微鏡を用いて細胞形態や接着の程度を毎日観察記録する。細胞培養の手順は，Good Cell Culture Practice ガイドラインに準拠して行う[15,16]。

① 凍結保存された BG1 を解凍して，マイクロピペットを用いて 2 mL クライオバイアルから 50 mL コニカル遠心チューブに移す。クライオバイアルを 1x PBS で 2 回リンスして，リ

ンス液も細胞と同じコニカル遠心チューブに移す。RPMI 20 mLをコニカル遠心チューブに添加し，1000 xg，8分間遠心してペレットにする。(ペレットにならない場合は，さらに5分遠心する。)

② 培地を吸引除去した後，RPMI 5 mLを添加し，1.0 mLセロロジカル・ピペットを用いて細胞塊が残らないように再懸濁する。細胞懸濁液を25 cm^2細胞培養フラスコ（T25）に移して，80～90%コンフルエントに達するまで約48～72時間培養する。

③ T25が，80～90%コンフルエントに達したら，PRMIを吸引除去し，1xPBS 5 mLを加え細胞を洗浄し，PBSを吸引除去した後，1xトリプシン溶液2 mLを加え，CO_2インキュベーターで5～10分間消化して細胞を剥離する。(細胞の剥離が不十分時は，さらに2分間インキュベートする。)細胞が剥離したら，1x PBS 5 mLを加えて細胞を懸濁し，50 mLコニカルチューブに移す。T25は，1x PBS 5 mLでさらに1回リンスして，リンス液も50 mLコニカルチューブに移す。50 mLコニカルチューブに移した細胞懸濁液には，トリプシン活性を阻害するため，直ちに20 mL RPMIを加え，遠心してペレットにしてPRMIを吸引除去した後，RPMI 10 mLで再懸濁する。細胞懸濁液を75 cm^2細胞培養フラスコ（T75）に移して，80～90%コンフルエントに達するまで約48～72時間培養する。

④ T75が，80～90%コンフルエントに達したら，上述と同様に1xトリプシン2 mLで細胞を剥離した後，20 mL RPMIに再懸濁して150 cm^2細胞培養フラスコ（T150）に移してさらに約48～72時間培養する。

⑤ T150が，80～90%コンフルエントに達したら，同様に3 mL 1xトリプシンで細胞を剥離した後，40 mL RPMIに再懸濁してT1502本に各20 mLずつ移してさらに約48～72時間培養する。

3.1.3　アッセイ用細胞の前培養

試験に用いる細胞は，エストロゲン非存在下で前培養を行う。

T150 2本の細胞を4本のT150に分割し，うち2本を測定用としてEFMで培養し，うち2本は継代用としてRPMIで培養を行う。EFMで培養する測定用細胞については，RPMIのコンタミに十分注意する。

(1) 細胞懸濁液の調整

80～90%コンフルエントに達したT150 2本それぞれについて，PRMIを吸引除去し，1xPBS 5 mLを加え細胞を洗浄した後，1xPBSを吸引除去して1xトリプシン3 mLを添加して前述同様に細胞を剥離する。細胞が剥離したら，片方のT150に1xPBS 5 mLを加えて細胞を懸濁し，もう一方のT150に移す。T150 2本分の細胞懸濁液を50 mLコニカルチューブに移した後，1xPBS 5 mLで同様の操作により両方のフラスコをリンスして，リンス液を50 mLコニカルチューブに移す。50 mLコニカルチューブに移した細胞懸濁液には，トリプシン活性を阻害するため，直ちにEFM 20 mLを加える。一度，遠心してペレットにし，培地を吸引除去した後，

EFM 4 mL に再懸濁する。

(2) 継代用細胞

T150 2本に RPMI 20 mL を分注し，220 μL G418 を添加し，上記で準備した細胞懸濁液 1 mL を加えて培養する。G418 添加 24 時間後に培養液を交換して，レポータープラスミドを発現していない死細胞を除去する。交換後の培養液に，G418 を添加する必要はない。80～90％コンフルエントに達するまで約 48～72 時間培養を行い，前述に従い継代する。

(3) 測定用細胞

① T150 2本に EFM 20 mL を分注し，150 μL G418 を添加し，上記で準備した細胞懸濁液 1 mL を加えて培養する。G418 添加 24 時間後に培養液を交換して，レポータープラスミドを発現していない死細胞を除去する。交換後の培養液に，G418 を添加する必要はない。80～90％コンフルエントに達するまで約 48～72 時間培養する。

② 測定用細胞が，80％～90％コンフルエントに達したら，上述と同様に 1x トリプシン 3 mL で細胞を剥離した後，50 mL コニカル遠心チューブを用いてエストロゲン除去 DMEM 20 mL に再懸濁し，細胞懸濁液 15 μL を分取して，血球計算板を用いて細胞密度を計測し，結果をもとに 200,000 cells/mL になるように EFM を加えて希釈する。

③ 200,000 cells/mL に調整した細胞懸濁液を 96 ウェルプレートに 200 μL/ ウェル（40,000 cells/ ウェル）播種して，19～24 時間前培養する。前培養は最大 48 時間以内とする。測定に用いないウェルには，EFM 200 μL のみを加える。

④ 通常，80～90％コンフルエントの T150 2本分の細胞で，96 ウェルプレート 3～4 枚分の測定が実施可能である。

3. 1. 4 細胞の安定性

安定な測定結果を得るため，凍結ストックから起眠した細胞は，RPMI 細胞増殖用培地にて数回継代を行う。また，30 代以上継代した細胞は測定には用いない。BG1Luc4E2 細胞は，約 3 カ月で 30 代となる。

3. 1. 5 細胞生存率の目視判定

培養後の 96 ウェルプレートをブロッティングペーパーに転倒して培養液を除去する。各ウェルを 1x PBS 50 μL で洗浄し，すぐに除去する。倒立顕微鏡を用いて全てのウェルの細胞を観察し，表 1 に示す基準に従いスコアを付ける。

各スコアに相当する細胞の観察例は，ICCVAM ウェブサイトに掲載されている "Visual Observation Cell Viability Manual"[17] を参考にすると良い。また，バリデーション試験における検討から，目視判定スコアが 1 となる場合，一般的に用いられる定量的評価法により細胞生存率が 80％以上，目視判定スコアが 2 以上の場合，細胞生存率が 80％以下となることが確認されている[18]。

表 1　細胞生存率の目視判定スコア

細胞生存率スコア	細胞の状況
1	細胞密度，形態とも正常である。
2	細胞形態に変化が認められる。もしくは，細胞間に若干，隙間がある。
3	細胞形態に変化が認められる。もしくは，細胞間に大きな隙間がある。
4	細胞が，僅かしか（もしくは，全く）認められない。
P	被験物質の析出が認められる。

3. 2　コントロールの準備

下記では，◆は，アゴニストアッセイ，◇は，アンタゴニストアッセイで用いる試薬を示す。

3. 2. 1　ストック溶液の準備

◆ 1.0×10^{-2} μg/mL E2 ストック溶液

① E2 10 mg/mL 100% DMSO 溶液を調整する。

② ①で調整した E2 溶液 10 μL に 990 μL の DMSO を加えてボルテックスで撹拌する。

③ ②をさらに 2 回繰り返して 1.0×10^{-2} μg/mL 溶液を調整する。

◇ 5.0×10^{-3} μg/mL E2 ストック溶液

1.0×10^{-2} μg/mL E2 ストック溶液を 100% DMSO で 2 倍希釈する。

◆ 313 μg/mL Meth ストック溶液

① Meth 10 mg/mL 100% DMSO 溶液を調整する

② ①で調整した Meth 溶液 94 μL に DMSO 2906 μL を加えてボルテックスで撹拌する。

◇ 2.5 μg/mL Ral ストック溶液

① Ral 10 mg/mL 100% DMSO 溶液を調整する。

② ①で調整した Ral 10 mg/mL 溶液 10 μL に DMSO 990 μL を加え 100 μg/mL 溶液を調整する。

③ ②で調整した Ral 100 μg/mL 溶液 150 μL に DMSO 2.850 μL 加え 5 μg/mL 溶液を調整する。

④ ③で調整した Ral 5 μg/mL 溶液 1.5 mL に DMSO 1.5 mL を加え 2.5 μg/mL 溶液を調整する。

※各物質の DMSO ストック溶液は，室温で約 3 以内もしくは，各試薬の分析証明により定められた有効期限内に使用する。

3. 2. 2　用量設定試験で用いる標準液の調整

◆◇ DMSO 標準液：13 mm ガラス試験管を用いて，DMSO 10 μL を EFM 1000 μL と混合する。

◆アゴニストアッセイ用 E2 標準液

① 4 mL コニカルチューブを用いて，#1 から #4 の希釈系列を調整する。

#1　1.0×10^{-2} μg/mL E2 ストック 6 μL＋100%DMSO 6 μL

#2　1.0×10^{-2} μg/mL E2 ストック 6 μL＋100%DMSO 18 μL

#3　#2 E2 溶液 6 μL＋100%DMSO 18 μL

#4　#3 E2 溶液 6 μL＋100%DMSO 18 μL

② 13 mm ガラス試験管を 4 本用意して，それぞれに 600 μL の EFM を準備し，①で調整した #1～#4 の E2 希釈系列をそれぞれ 6 μL 加えて，アッセイ用標準液を調整する。

※E2 標準液の最終濃度は，それぞれ 1.84×10^{-10}，4.59×10^{-11}，1.15×10^{-11}，2.87×10^{-12}M となる。

◇アンタゴニストアッセイ用 Ral/E2 標準液

① 4 mL コニカルチューブを用いて，#1～#4 の Ral 希釈系列を調整し，#2～#4 を用いる。

#1　6.25×10^{-1} μg/mL 溶液：2.5 μg/mL Ral ストック溶液 250 μL＋DMSO 750 μL

#2　3.13×10^{-1} μg/mL 溶液：6.25×10^{-1} μg/mL Ral 溶液 500 μL＋DMSO 500 μL

#3　7.81×10^{-2} μg/mL 溶液：3.13×10^{-1} μg/mL Ral 溶液 250 μL＋DMSO 750 μL

#4　1.95×10^{-2} μg/mL 溶液：7.81×10^{-2} μg/mL Ral 溶液 125 μL＋DMSO 375 μL

② #2～#4 の Ral 溶液各 500 μL と 5×10^{-3} μg/mL E2 ストック溶液 500 μL を混合する

③ 13 mm ガラス試験管を 3 本用意して，それぞれに 600 μL の EFM を準備し，②で調整した #2～#4 の Ral/E2 溶液をそれぞれ 6 μL 加えて，アッセイ用標準液を調整する。

※Ral/E2 標準液の最終濃度は，それぞれ Ral 3.06×10^{-9}，7.67×10^{-10}，1.92×10^{-10} M（E2 9.18×10^{-11} M 共存）となる。

3. 2. 3　本試験で用いる標準液の調整

◆ DMSO 標準液：DMSO 10 μL を EFM 1000 μL と混合する。

◆ E2 標準液

① 4 mL コニカルチューブを用いて，11 段階の 2 倍希釈系列を調整する。

#1　1.0×10^{-2} μg/mL E2 ストック 6 μL

#2　1.0×10^{-2} μg/mL E2 ストック 6 μL＋DMSO 6 μL

#3　#2 で調整した E2 溶液 6 μL＋100% DMSO 6 μL

#4～#11　#3 と同様に 2 倍希釈系列を調整

② 13 mm ガラス試験管を 11 本用意して，それぞれに 600 μL の EFM および①で調整した 11 段階（#1～#11）の E2 希釈系列をそれぞれ 6 μL 加えて，試験用標準液とする。

※E2 の最終濃度範囲は，3.67×10^{-10} から 3.59×10^{-13} M となる。

◆ Meth 標準液：13 mm ガラス試験管を用いて，313 μg/mL Meth ストック 10 μL を EFM 1000 μL と混合する。

※Meth 最終濃度は，9.06×10^{-6} M となる。

◇ DMSO 標準液：DMSO 8 μL を EFM 800 μL と混合する。

◇E2 標準液：5×10^{-3} μg/mL E2 ストック溶液 4 μL および DMSO 4 μL を EFM 800 μL と混合する。

※E2 最終濃度は，9.18×10^{-11} M となる。

◇Ral/E2 溶液

① 4 mL コニカルチューブを用いて，下記 9 段階の 2 倍希釈系列を各 500 μL 調整する。

#1　2.5 μg/mL Ral ストック溶液 500 μL

#2　2.5 μg/mL Ral ストック溶液 500 μL＋DMSO 500 μL

#3　#2 で調整した Ral 溶液 500 μL＋DMSO 500 μL

#4～#9　#3 と同様に 2 倍希釈系列を調整

※#9 は，調整後 500 μL を除去して 500 μL にする。

② #1～#9 の Ral 溶液各 500 μL と 5×10^{-3} μg/mL E2 ストック溶液 500 μL を混合する。

◇Tam/E2 溶液：2.5 mg/mL Tam 溶液を調整し，5×10^{-3} μg/mL E2 ストック溶液と 1 対 1 の割合で混合する。

◇Ral/E2 標準液，Tam/E2 標準液：13 mm ガラス試験管を用意して，それぞれに 600 μL の EFM を準備し，上記で調整した溶液をそれぞれ 6 μL 加えて，試験用標準液とする。

※Ral/E2 標準液の最終濃度は，Ral 濃度範囲 3.67×10^{-10} から 3.59×10^{-13} M（E2 9.18×10^{-11} M 共存）となる。Tam/E2 標準液の最終濃度は，Tam 3.36×10^{-6} M（E2 9.18×10^{-11} M 共存）となる。

3. 3　被験物質の準備

被験物質溶液は，媒体中での安定性が確認されていない場合には，調整後 24 時間以内に使用する。

3. 3. 1　アゴニスト試験

(1) 用量設定試験

① まず始めに 100 mg/mL DMSO 溶液を調整する。ボルテックスにより撹拌して，被験物質が溶解しない場合は，1/10 濃度（10 mg/mL）の溶液を再調整し，溶解するまで 1/10 ずつ濃度を下げて最大溶解濃度溶液を調整する。

② 最大溶解濃度を 1 段階目として 7 段階の 10 倍希釈系列 4 mL コニカルチューブを用いて調整する。

③ 13 mm ガラス試験管を 7 本用意して，それぞれに 600 μL の EFM を準備し，被験物質の最大溶解濃度溶液および①で調整した 7 段階の被験物質希釈系列を各 6 μL 加える。

(2) 本試験

① 用量設定試験結果をもとに決定した最大試験濃度および希釈倍率に従って，11 段階の DMSO 希釈系列を 4 mL コニカルチューブを用いて調整する。

② 13 mm ガラス試験管を 11 本用意して，それぞれに 800 μL の EFM を準備し，①で調整した被験物質希釈系列を各 8 μL 加える。

3.3.2 アンタゴニスト試験

(1) 用量設定試験

① アゴニスト試験と同様に最大溶解濃度溶液を 1 段階目として 7 段階の 10 倍希釈系列を調整する。ただし，最大溶解濃度が，10 mg/mL 以上の場合は，10 mg/mL を 1 段階目として 7 段階の 10 倍希釈系列を調整する。

② 13 mm ガラス試験管を 11 本用意して，それぞれに 800 μL の EFM を準備し，①で調整した被験物質希釈系列を各 4 μL と 5×10^{-3} μg/mL E2 ストック溶液 4 μL を加える。

(2) 本試験

① 用量設定試験結果をもとに決定した最大濃度および希釈倍率に従って，11 段階の DMSO 希釈系列を 4 mL コニカルチューブを用いて調整する

② 13 mm ガラス試験管を 11 本用意して，それぞれに 800 μL の EFM を準備し，①で調整した被験物質希釈系列を各 4 μL と 5×10^{-3} μg/mL E2 ストック溶液 4 μL を加える。

3.4 各試験施設における背景データベースの構築

試験結果の採用基準を決定するためアゴニストアッセイおよびアンタゴニストアッセイそれぞれの標準物質とコントロールについて，本試験プレートデザインに従い少なくとも 10 回以上の実施日の異なる測定を行い背景データベースを構築する。4 項で示す基準項目が背景データの標準偏差の 2.5 倍以内であれば試験結果を採用する。背景データベースには，以降の測定において基準を満たした試験結果を追加する。なお，測定機器等を変更した場合には，新たに背景データベースを構築する。

3.5 技術実証試験

試験施設の試験実施技術レベルを実証し，測定結果の信頼性を保証するため，背景データを整備した後，表 2 および 3 に示すアゴニストおよびアンタゴニストの実証物質の測定を実施し，各表に示した陽性陰性の結果が得られることを確認しなければならない。表に示す EC50 もしくは IC50 値は，参考情報であり同程度の値が得られれば良い。

それぞれの実証物質について，本試験プレートデザインに従い少なくとも実施日の異なる 2 回の測定を実施する。本試験における測定開始濃度は濃度設定試験結果をもとに決定する。2 回の試験結果の判定が一致しない，もしくは 1 回の試験結果が判定不能であった場合には追加測定を実施する。それぞれの実証物質について表に示す判定により技術レベルの実証を行う。実証試験は，新たに試験を実施する全ての試験者が必ず実施する。