Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.9, No.2, 31-36, 2022

The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Absence of *in vivo* mutagenicity of 4,4'-oxybis(benzenesulfonohydrazide) in liver and glandular stomach of Muta™ Mouse

Takako Iso[1], Masakatsu Natsume[2], Yasumasa Murata[1], Yoshiyuki Shigeta[1], Nozomu Hirose[1], Takaaki Umano[1], Katsuyoshi Horibata[3], Kenichi Masumura[3], Kei-ichi Sugiyama[3], Mariko Matsumoto[1] and Akihiko Hirose[1]

[1]Division of Risk Assessment, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan
[2]Genotoxicology Laboratory, BioSafety Research Center Inc., 582-2 Shioshinden, Iwata, Shizuoka 437-1213, Japan
[3]Division of Genetics and Mutagenesis, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan

(Received March 12, 2022; Accepted March 19, 2022)

ABSTRACT — 4,4'-Oxybis(benzenesulfonohydrazide) (OBSH) is a blowing agent widely used in the manufacture of porous plastics and rubber. OBSH was notified as an additive in the Japanese positive list system for food utensils, containers and packaging. The *in vitro* mutagenicity of OBSH was shown extensively in bacterial reverse mutation assays, a DNA repair test, and a chromosomal aberration test. Few studies exist on *in vivo* genotoxic evaluation on OBSH apart from an *in vivo* micronuclei test. To clarify *in vivo* mutagenicity, we conducted a transgenic rodent gene mutation (TGR) assay (OECD TG 488). We dosed male Muta™ Mouse with OBSH by oral gavage at 0 (negative control), 25, 50, and 100 mg/kg/day for 28 consecutive days, and evaluated mutant frequencies (MFs) of *lacZ* in the liver and glandular stomach (5 mice/group). We observed two deaths and a reduction in body weight at 100 mg/kg/day. Although we exposed Muta™ Mouse to OBSH orally for 28 days up to the maximum tolerated dose, we did not detect *in vivo* mutagenicity in the liver and glandular stomach. In contrast, in the positive control we detected significantly increased MFs. The results of this study suggest that OBSH is not mutagenic *in vivo*.

Key words: 4,4'-Oxybis(benzenesulfonohydrazide), *In vivo* mutagenicity, Transgenic rodent gene mutation assay, Risk assessment

INTRODUCTION

4,4'-Oxybis(benzenesulfonohydrazide) (OBSH) (Fig. 1) has been used as a blowing agent for plastic and rubber in various industrial products. OBSH was notified as an additive in the Japanese positive list (PL) system for food utensils, containers and packaging (UCP). Both rule-based and statistically based quantitative structure–activity relationship (QSAR) models predict that OBSH bears a structural alert to mutagenicity in the Ames test. We used Derek Nexus (ver.6.0.1, Lhasa limited, UK) as rule-based and CASE Ultra (ver.1.6.2.2, MultiCASE Inc., USA) as statistically based QSAR models, and both models classified OBSH as able to release hydrazine (Fig. 1), a known *in vitro* mutagen (De Flora, 1981; McMahon *et al.*, 1979; Parodi *et al.*, 1981). In fact, an Ames test using

Correspondence: Takako Iso (E-mail: takako.iso@nihs.go.jp)

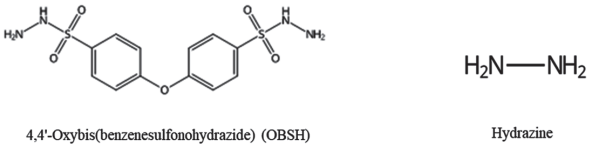

Fig. 1. Chemical structure of 4,4‘-oxybis (benzenesulfonohydrazide) (OBSH) (CASRN 80-51-3) and hydrazine (CASRN 302-01-2).

Salmonella typhimurium TA1535, TA1537, TA98, TA100, and *Escherichia coli* WP2 at a maximum concentration of 3,000 μg/plate with or without S9 mix showed positive results for OBSH (NIER, 1999 cited by OECD, 2006). Subsequently, another Ames test conducted using the same strains of *S. typhimurium* and *E. coli* at a maximum dose of 5,000 μg/plate with or without S9 also reported positive results with TA100 and TA1535 (MHLW, 2003a). OBSH treatment also induced unscheduled DNA synthesis in rat hepatocytes (Mori *et al.*, 1988). A chromosomal aberration test using Chinese hamster lung (CHL/IU) cells at a maximum dose of 1,700 μg/mL with or without S9 showed weak induction of structural abnormalities (MHLW, 2003b). An *in vivo* micronucleus test demonstrated that OBSH did not induce micronuclei in mouse bone marrow after two doses ranging from 375 to 1,500 mg/kg/day, although the detailed dosing condition was not clear (NIER, 2000 cited by OECD, 2006).

Animal studies with mice, rats and hamsters have shown the carcinogenicity of hydrazine or compounds with a hydrazine structure by oral exposure (Biancifiori, 1970; Bosan *et al.*, 1987; Matsumoto *et al.*, 2016). Tumor development has been also reported in the rat uterus and the mouse lung (Steinhoff and Mohr, 1988; Toth, 1972). The study conducted by Douglas *et al.* (1995) showed *in vivo* non-mutagenicity of hydrazine in the liver, lung, and bone marrow of *lacZ* transgenic mice. However, since the study design allowed for a single oral dose up to toxic concentrations, whether the compound was adequately exposed to the target tissues remains questionable, and no conclusions were reached regarding *in vivo* mutagenicity. The carcinogenicity of hydrazine or compounds with hydrazine structures raises health concerns due to long-term exposure to OBSH, but no data was available on *in vivo* mutagenicity or carcinogenicity, other than the result of OBSH not inducing micronuclei in mouse bone marrow. Here we aimed to evaluate the *in vivo* mutagenicity of OBSH using Muta™ Mouse. We selected the liver and glandular stomach as target organs because the liver is a major organ in chemical metabolism and is sensitive in the assay, and the glandular stomach is first exposed to unchaged OBSH after oral administration.

MATERIALS AND METHODS

Testing institute

This study was conducted at the BioSafety Research Center (BSRC; Shizuoka, Japan) in accordance with the Organization for Economic Cooperation and Development (OECD) guideline 488 (OECD, 2013). Animals were treated in accordance with the Act on Welfare and Management of Animals (MOE, 2014), the Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals (MOE, 2006), and the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (METI, 2003).

Test chemicals

We purchased the test chemical OBSH [lot no. 19HBL62J, Chemical Abstracts Service Registry Number (CASRN) 80-51-3] from AK Scientific, Inc. (San Francisco, CA, USA) with 94% purity, and a positive control, *N*-ethyl-*N*-nitrosourea (ENU; CASRN 759-73-9), from Toronto Research Chemicals Inc. (Toronto, ON, Canada). We purchased corn oil from NACALAI TESQUE, INC. (Kyoto, Japan). OBSH was suspended in corn oil and dosing formulations were stored at room temperature until use and used for treatment within 8 or 10 days after preparation. Stability and uniformity during storage were verified. The positive control ENU was dissolved in phosphate buffer (pH: 5) prior to administration.

Animals and treatment

We purchased both male and female CD2F1, and male Muta™ Mouse (CD2-LacZ80/HazfBR) aged 8 weeks from Japan SLC, Inc. (Shizuoka, Japan) and Trans Genic Inc. (Fukuoka, Japan), respectively, and acclimatized them for 8 days before treatments. Food (CRF-1, Oriental Yeast, Japan) and water were provided *ad libitum*. Animals were maintained at a room temperature of 20°C–26°C, a relative humidity of 35%–70%, a 12 hr light/dark cycle, and 12 air changes per hour. After an acclimatization period, we randomly assigned CD2F1 and Muta™ Mouse to groups including 3 and 6 or 8 mice per group, respectively. We also assigned 6 Muta™ Mouse to each of the positive and negative control groups.

We set the highest dose based on a range-finding 14-day study in both male and female CD2F1 mice testing dose levels of 0, 30, 100, 300, and 1,000 mg/kg/day by oral gavage. In the range-finding study, we observed excessive toxicity—that is, mortality and moribundity—within

8 days in male and female mice at a dose of 300 mg/kg/day and above. Based on the results of the study, we considered doses of 25, 50, and 100 mg/kg/day to be appropriate for 28-day repeated administrations. Animals were monitored daily for general condition and regularly for body weight until the dissection day. We administered the dosing formulations of OBSH (25, 50, and 100 mg/kg/day) or corn oil (negative control) by oral gavage at a dosing volume of 10 mL/kg once daily for 28 consecutive days. We treated the positive control animals with ENU at 100 mg/kg/day by intraperitoneal injection once daily for 2 days. Animal experiments were conducted in accordance with the regulations of the Animal Care and Use Committees of the National Institute of Health Sciences and the BioSafety Research Center Inc..

Detection of gene mutations

All treated mice except the positive control were sacrificed by carbon dioxide asphyxiation 3 days after the last treatment. The positive control mice were sacrificed 10 days after the last treatment in the same manner. For each dose group, we subjected five mice to mutation analysis. Liver and stomach tissues were collected, stomach tissues were divided into forestomach and glandular stomach, and genomic DNA was extracted from liver and glandular stomach samples according to previously reported methods (Matsumoto *et al.*, 2014).

DNA packaging was performed according to the instruction manual of Transpack (Agilent Technologies, Santa Clara, CA, USA). In brief, DNA solutions (100–600 μg/mL) were gently mixed with the Transpack packaging extract and were incubated 2 times at 30°C, for 1.5 hr; SM buffer containing NaCl, $MgSO4 \cdot 7H_2O$, Tris–HCl (pH: 7.5), and gelatin was then added. Packaged phages were absorbed into *E. coli* cells at room temperature for 20–30 min. An appropriately diluted *E. coli* cell suspension was mixed with LB top agar for titer plates, and selection plates were produced by mixing the remaining cell suspension with LB top agar containing P-gal (phenyl-β-D-galactoside). Selection plates were then incubated overnight at 37°C, and packaging was repeated to reach a total number of 300,000 plaques. MFs were calculated as follows: MFs = total numbers of plaques on selection plates/total numbers of plaques on titer plates (x dilution factor).

Statistical analysis

Homogeneity of variance of MFs in treated groups were analyzed with Bartlett's test. For homogeneity of variance, significant differences between the control and treated groups were determined using Dunnett's multiple comparison test and in case of homogeneity of variance is not met, non-parametric Steel test was used. MFs between the negative and positive controls were compared by the Student's t-test or Aspin–Welch's t-test after F test. Five percent levels of probability were used as the criterion for significance.

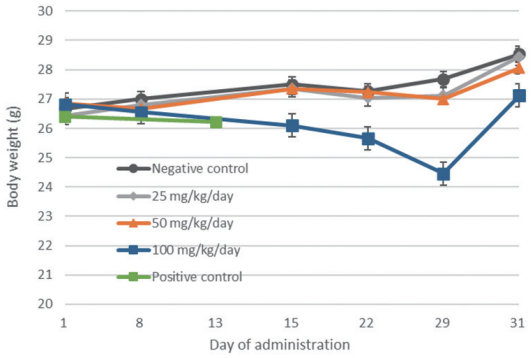

Fig. 2. Body weights of Muta™ Mouse through the 28-day administration period of 4,4'-oxybis(benzenesulfonohydrazide) (OBSH) to the date of organ collection. Values are Mean ± SD (n = 5). Positive control: treated with ENU at 100 mg/kg/day by intraperitoneal injection once daily for 2 days.

RESULTS

Body weights and general condition examination

One death was observed on day 26 during treatment, and another death was occurred 3 days after the last treatment at a dose of 100 mg/kg/day. We analyzed MFs on five mice alive at the organ collection 3 days after the final administration. In Fig. 2, we show the changes in body weight of the animals subjected to mutation analysis. We recorded body weight loss on day 22 and day 29 at a dose of 100 mg/kg/day, with no clinical signs of toxicity observed in all treatment groups. No gross pathological abnormalities in the liver or glandular stomach at necropsy were found (data not shown).

Analysis of MFs

MFs of *lacZ* in the liver and glandular stomach of animals treated with OBSH were not significantly higher than those in the respective negative controls (Tables 1–2). In contrast, the positive control ENU significantly increased MFs in the liver and glandular stomach ($p < 0.05$).

Table 1. Mutant frequencies in liver of Muta™ Mouse treated with 4,4'-oxybis(benzenesulfonohydrazide) (OBSH).

Substance	Dose (mg/kg/day, p.o.)	Animal ID	No. of total plaques	No. of mutants	Mutant frequency ($\times 10^{-6}$)	Mean ± S.D. ($\times 10^{-6}$)
Corn oil	0	3001	597,600	18	30.1	
		3002	425,700	11	25.8	
		3003	681,300	20	29.4	31.2 ± 11.7
		3004	806,400	16	19.8	
		3005	531,900	27	50.8	
OBSH	25	3101	650,700	13	20.0	
		3102	451,800	19	42.1	
		3103	735,300	14	19.0	30.1 ± 10.3
		3104	779,400	25	32.1	
		3105	855,000	32	37.4	
	50	3201	742,500	19	25.6	
		3202	504,900	16	31.7	
		3203	516,600	15	29.0	32.1 ± 7.2
		3204	585,000	26	44.4	
		3205	767,700	23	30.0	
	100	3301	716,400	16	22.3	
		3302	1,041,300	32	30.7	
		3304	697,500	20	28.7	30.8 ± 5.8
		3305	591,300	22	37.2	
		3306	486,900	17	34.9	
ENU	100	3401	527,400	48	91.0	
		3402	436,500	45	103.1	
		3403	350,100	33	94.3	98.7 ± 9.6*
		3404	642,600	73	113.6	
		3405	513,000	47	91.6	

Corn oil: Negative control (10 mL/kg).
ENU: Positive control (*N* -ethyl-*N* -nitrosourea, 10 mL/kg, i.p., dose once a day, for 2 days, expression period; 10 days).
*: $p < 0.05$, Significant difference from negative control by Student's *t* test.

DISCUSSION

OBSH was notified as an additive in the Japanese PL system for UCP. OBSH is added as a foaming agent in the manufacturing process to create bubbles in plastic and provide lightness, thermal insulation, and buffering. OBSH decomposes to nitrogen gas, water vapor and 4,4'-oxydibenzenesulfinic acid by heat, and the generated gas expands plastics to obtain foamed plastics (Kondo, 2001). Consumers are unlikely to be exposed to OBSH in plastics (OECD, 2006), but the residual OBSH in UCP currently remains unknown. No data are available on absorption and distribution of OBSH, but due to the physico-chemical properties of OBSH—the molecular weight is 358.4 and the estimated log Kow is 0.08 (OECD, 2006)—OBSH is considered orally absorbable, and the duration of exposure for consumers is expected to be lifelong. Since OBSH is mutagenic *in vitro* and lacks long-term exposure toxicity information, it is necessary to clarify *in vivo* mutagenicity from the aspect of risk management of OBSH. In the present study, we conducted the TGR assay in the liver and glandular stomach to systematically assess *in vivo* mutagenicity; the result was negative in both organs following 28-day gavage treatment. In the TGR assay, body weight loss was observed at a maximum dose of 100 mg/kg/day, but no other effect was found. We set the administration dosages from the range-finding study and observed excessive toxicity, such as emaciation or moribundity, above 300 mg/kg/day, indicating a maximum tolerated dose of 300 mg/kg/day. Hence, the liver and glandular stomach were adequately exposed to OBSH under test conditions. Since the results of the present TGR assay conducted in the liver and glandular stomach were negative, we concluded that the mutagenicity exhibited in *in vitro* studies did not occur in the living body. Although the health effects of long-term exposure to OBSH currently remain unclear, the results of this study suggest that OBSH is not mutagenic *in vivo*.

Table 2. Mutant frequencies in glandular stomach of Muta™ Mouse treated with 4,4'-oxybis(benzenesulfonohydrazide) (OBSH)

Substance	Dose (mg/kg/day, p.o.)	Animal ID	No. of total plaques	No. of mutants	Mutant frequency ($\times 10^{-6}$)	Mean ± S.D. ($\times 10^{-6}$)
Corn oil	0	3001	635,400	18	28.3	
		3002	470,700	15	31.9	
		3003	660,600	24	36.3	34.8 ± 7.8
		3004	566,100	27	47.7	
		3005	569,700	17	29.8	
OBSH	25	3101	326,700	9	27.5	
		3102	654,300	28	42.8	
		3103	748,800	19	25.4	35.0 ± 10.7
		3104	803,700	40	49.8	
		3105	675,000	20	29.6	
	50	3201	606,600	19	31.3	
		3202	903,600	15	16.6	
		3203	1,000,800	22	22.0	29.6 ± 11.3
		3204	842,400	39	46.3	
		3205	664,200	21	31.6	
	100	3301	513,000	21	40.9	
		3302	432,900	11	25.4	
		3304	514,800	16	31.1	29.9 ± 6.8
		3305	525,600	15	28.5	
		3306	847,800	20	23.6	
ENU	100	3401	612,000	238	388.9	
		3402	499,500	237	474.5	
		3403	539,100	208	385.8	394.2 ± 62.2*
		3404	855,000	358	418.7	
		3405	623,700	189	303.0	

Corn oil: Negative control (10 mL/kg).
ENU: Positive control (*N* -ethyl-*N* -nitrosourea, 10 mL/kg, i.p., dose once a day, for 2 days, expression period; 10 days).
*: $p < 0.05$, Significant difference from negative control by Aspin-Welch's *t* test.

ACKNOWLEDGMENTS

This study was supported by MHLW, Japan.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Biancifiori, C. (1970): Hepatomas in CBA/Cb/Se mice and liver lesions in golden hamsters induced by hydrazine sulfate. J. Natl. Cancer Inst., **44**, 943-953.

Bosan, W.S., Shank, R.C., MacEwen, J.D., Gaworski, C.L. and Newberne, P.M. (1987): Methylation of DNA guanine during the course of induction of liver cancer in hamsters by hydrazine or dimethylnitrosamine. Carcinogenesis, **8**, 439-444.

De Flora, S. (1981): Study of 106 organic and inorganic compounds in the *Salmonella*/microsome test. Carcinogenesis, **2**, 283-298.

Douglas, G.R., Gingerich, J.D. and Soper, L.M. (1995): Evidence for *in vivo* non-mutagenicity of the carcinogen hydrazine sulfate in target tissues of *lacZ* transgenic mice. Carcinogenesis, **16**, 801-804.

Kondo, H. (2001): The Characteristic of Foaming Agents. NIPPON GOMU KYOKAISHI, **74**, 406-411.

Matsumoto, M., Masumori, S., Hirata-Koizumi, M., Ono, A., Honma, M., Yokoyama, K. and Hirose, A. (2014): Evaluation of *in vivo* mutagenicity of hydro-quinone in Muta mice. Mutat. Res. Genet. Toxicol. Environ. Mutagen., **775-776**, 94-98.

Matsumoto, M., Kano, H., Suzuki, M., Katagiri, T., Umeda, Y. and Fukushima, S. (2016): Carcinogenicity and chronic oxicity of hydrazine monohydrate in rats and mice by two-year drinking water treatment. Regul. Toxicol. Pharmacol., **76**, 63-73.

McMahon, R.E., Cline, J.C. and Thompson, C.Z. (1979): Assay of 855 test chemicals in ten tester strains using a new modification of the Ames test for bacterial mutagens. Cancer Res., **39**, 682-693.

METI (2003): Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Act No. 97 of 2003, as amended in Act No.18 of 2017).

MHLW. (2003a): Japan Existing Chemical Database (JECDB), Bacterial Reverse Mutation Test (in Japanese) (https://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF80-51-3e.pdf).

MHLW. (2003b): Japan Existing Chemical Database (JECDB), In

Vitro Mammalian Chromosome Aberration Test (in Japanese) (https://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF80-51-3f.pdf).

MOE (2006): Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment No. 88 of 2006).

MOE (2014): Act on Welfare and Management of Animals (Act No. 105 of 1973, as amended in Act No.46 of 2014).

Mori, H., Sugie, S., Yoshim, N. and Iwata, H. (1988): Genotoxicity of a variety of hydrazine derivatives in the hepatocyte primary culture/DNA repair test using rat and mouse hepatocytes. Jpn. J. Cancer Res., **79**, 204-211.

NIER. (1999): National Institute of Environmental Research. Bacterial reverse mutation test of 4,4′-oxybis (benzenesulfonyl hydrazide) (Study No. S137), Tested by LG Life Science/Toxicology Center. Korea (unpublished data) cited by OECD, 2006, SIDS Initial Assessment Report For SIAM 22.

NIER. (2000): National Institute of Environmental Research. *In vivo* micronucleus test of 4,4'-oxybis(benzenesulfonyl hydrazide) in male ICR mice (Report No. G00114), tested by KRICT, Korea (unpublished data) cited by OECD, 2006, SIDS Initial Assessment Report For SIAM 22.

OECD (2006): SIDS Initial Assessment Report For SIAM 22

OECD (2013): TG488, OECD Guideline for the Testing of Chemicals 488 (26 June: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays).

Parodi, S., De Flora, S. and Cavanna, M. (1981): DNA-damaging activity *in vivo* and bacterial mutagenicity of sixteen hydrazine derivatives as related quantitatively to their carcinogenicity. Cancer Res., **41**, 1469-1482.

Steinhoff, D. and Mohr, U. (1988): The question of carcinogenic effects of hydrazine. Exp. Pathol., **33**, 133-143.

Toth B. (1972): Hydrazine, methylhydrazine and methylhydrazine sulfate carcinogenesis in swiss mice. failure of ammonium hydroxide to interfere in the development of tumors. Int.J. concer, **9**, 109-118.