Lack of hepatocarcinogenicity of 2,2’-[1,2-ethanediylbis(oxymethylene)]bis-oxirane, 3-hydroxy-2-naphthoic acid, and acetoacetanilide in a medium-term rat liver bioassay

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Abstract: The carcinogenicity of 2,2’-[1,2-ethanediylbis(oxymethylene)]bis-oxirane (ethylene glycol diglycidyl ether; EGDE), 3-hydroxy-2-naphthoic acid (HNA), and acetoacetanilide (AAA) was investigated using a medium-term rat liver bioassay for an occupational safety assessment. F344 male rats were administered a single intraperitoneal injection of diethylnitrosamine (200 mg/kg body weight (bw)/day) and then starting 2 weeks later, they received EGDE at 6, 20, and 60 mg/kg bw/day, HNA at 20, 60, and 200 mg/kg bw/day, or AAA at 60, 200, and 600 mg/kg bw/day by oral gavage for 6 weeks. The animals in the positive control group received phenobarbital sodium solution (PB, 25 mg/kg bw/day) by oral gavage and those in the negative control group received a vehicle (water/corn oil) during the administration period of test substances in this model. All animals were subjected to two-thirds partial hepatectomy at week 3 and euthanized at week 8. Neither the number nor the area of hepatocellular foci positive for glutathione S-transferase placental form (GST-P) increased in any of the EGDE, HNA, or AAA treated groups. However, the number and area of GST-P-positive foci significantly increased in the positive control group treated with PB. The results indicate that EGDE, HNA, and AAA lack hepatocarcinogenicity in rats. (DOI: 10.1293/tox.2022-0010; J Toxicol Pathol 2022; 35: 313–320)

Key words: 2,2’-[1,2-ethanediylbis(oxymethylene)]bis-oxirane (EGDE), 3-hydroxy-2-naphthoic acid (HNA); acetoacetanilide (AAA), medium-term liver bioassay, tumor promotion, F344 rats
EGDE, HNA, and AAA Do Not Have a Hepatocarcinogenicity

Materials and Methods

Animals and chemicals

Five-week-old specific pathogen-free male Fischer 344 rats were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). The animals were housed in a solid-floor plastic cage with bedding in a barrier-sustained animal room maintained at 23 °C ± 3 °C, with a relative humidity of 50% ± 20%, air ventilation at 10 to 15 vol/h and a 12-hour light/dark cycle. The animals were allowed free access to pelleted radiation-sterilized diet CR-LPF (Oriental Yeast, Tokyo, Japan) and tap water. The present animal studies were approved by the Institutional Animal Care and Use Committee, and the test facility, Gotemba laboratory of BoZo Research Center Inc. (Shizuoka, Japan) is fully accredited by the AAALAC International. Diethylnitrosamine (DEN; purity >99%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and dissolved in physiological saline (Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at a concentration of 40 mg/mL. Phenobarbital sodium (PB), 2,2’-[1,2-ethanediylbis(oxy)methylene]bis-oxirane (commonly known as ethylene glycol diglycidyl ether; EGDE), 3-hydroxy-2-naphthoic acid (HNA), and acetoacetanilide (AAA) were also purchased from Tokyo Chemical Industry Co., Ltd. The chemical characteristics, stability, and concentration and homogeneity in the dose formulations of PB (vehicle: water for injection), Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), EGDE (vehicle: water for injection), HNA (vehicle: corn oil, for biochemistry, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and AAA (vehicle: corn oil) were ensured by chemical analyses at the test facility under GLP.

Experimental design

Five test groups, including three dose groups of the test substance, a negative (vehicle) control group, and a positive control group, were set for each study (Table 1). Initially, each group consisted of 25 male rats; however, the number of effective animals for evaluation was eventually reduced for some reasons including euthanasia for spontaneous hepatodiaphragmatic nodule (impossibility of surgery) and deaths due to the test substance toxicity. All rats received an intraperitoneal injection of 200 mg/kg body weight (bw) of DEN as an initiation treatment. From 2 weeks after the DEN treatment, the test substances were administered to rats at 6, 20, and 60 mg/kg bw at a dose volume of 5 mL/kg bw for

| Table 1. Treatment Groups |
|---------------------------|
| Study 1                   | Study 2                   | Study 3                   |
| Vehicle control (water)   | Vehicle control (corn oil)| Vehicle control (corn oil)|
| EGDE 6 mg/kg/day          | HNA 20 mg/kg/day          | AAA 60 mg/kg/day          |
| EGDE 20 mg/kg/day         | HNA 60 mg/kg/day          | AAA 200 mg/kg/day         |
| EGDE 60 mg/kg/day         | HNA 200 mg/kg/day         | AAA 600 mg/kg/day         |
| Positive control PB 25 mg/kg/day | Positive control PB 25 mg/kg/day | Positive control PB 25 mg/kg/day |
EGDE, 20, 60, and 200 mg/kg bw at a dose volume of 10 mL/kg bw for HNA, and 60, 200, and 600 mg/kg bw at a dose volume of 10 mL/kg bw for AAA by oral gavage once daily for 6 weeks. Animals in the positive control group received 25 mg/kg bw PB at a dose volume of 5 mL/kg bw and those in the vehicle control group received a vehicle in the same manner.

The high dose level of each test substance was selected as the maximum-tolerated-dose based on the results of a preliminary 1-week toxicity range-finding study in normal rats and the 2-week study in rats that received partial hepatectomy (PH). In the range-finding study of EGDE, one rat died at 600 mg/kg bw and a severe decrease in the body weight gain (~11% from the vehicle control group) was observed at 200 mg/kg bw. In addition to the above, in a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test in SD rats, a decrease in the body weights (~7% from the vehicle control group) were observed in males at 50 mg/kg bw. In the range-finding study of HNA, death occurred at 400 and 300 mg/kg bw, but no apparent toxicity was observed at 130 mg/kg bw or below. In the range-finding study of AAA, death occurred at 2,000 and 1,000 mg/kg bw and a mild decrease in the body weight gain (~6% from the vehicle control group) was observed at 600 mg/kg bw. All rats were subjected to two-thirds partial hepatectomy (PH) under anesthesia by isoflurane inhalation (concentration 2.0%) on the last day in week 3 after the DEN treatment. Carprofen (Rimadyl Injectable Solution: Zoetis Japan Co., Ltd., Tokyo, Japan) was administered to all rats at 5 mg/kg bw as an analgesic treatment. Carprofen (Rimadyl Injectable Solution: Zoetis Japan Co., Ltd., Tokyo, Japan) was administered to all rats at 5 mg/kg bw by subcutaneous injection as an analgesic before PH and on the day after PH. The administration period of the test substances was 6 weeks. The body weights and food consumption were recorded at least once a week during the study. On the day after the end of the administration period, all surviving animals were necropsied and the liver weights were recorded. Histopathological and immunohistochemical examinations of the liver were performed, and thus, the number and area of the GST-P-positive foci, which are regarded as preneoplastic lesions in the liver, were determined. The present studies were conducted in compliance with GLP (Ministry of Health, Labour, and Welfare Ordinance No. 76, 1988).

Immunohistochemical analysis
The livers were weighed, excised, and fixed in phosphate buffered 10% formalin. One slice each was made from the right lateral lobe, caudate process of the caudate lobe, and papillary process of the caudate lobe and processed for paraffin-embedding. These slices were cut for immunohistochemical staining of sections for GST-P as well as staining with hematoxylin and eosin for histopathological examination. Briefly, deparaffinized sections were subjected to blockage of endogenous peroxidase activity by treatment with 3% H\textsubscript{2}O\textsubscript{2} for 10 min. Then, inhibition of non-specific binding with a blocking reagent (Abcam plc, Cambridge, UK) was performed for 5 min. Sections were exposed to rabbit anti-rat GST-P antibodies (1:4,000; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) in 1% bovine serum albumin/0.01M phosphate-buffered saline overnight at 4 °C and then biotinylated secondary antibody (anti-rabbit IgG), and StreptAB Complex/horseradish peroxidase treatment was performed using the LSAB2 kit (Dako Japan, Tokyo, Japan) for 10 min. The sites of peroxidase binding were demonstrated with 3,3′-diaminobenzidine/H\textsubscript{2}O\textsubscript{2} as the chromogen. Sections were counterstained with hematoxylin and cover-slipped for microscopic examination. The numbers and areas of GST-P-positive foci larger than 0.2 mm in diameter and total areas of the liver sections were measured with an image analyzer (automatic image processing analyzer LUZEX AP, Nireco Co., Ltd., Tokyo, Japan), a slide scanner (Aperio ScanScope XT, Leica Microsystems, Inc., Tokyo, Japan) and image processing software (AperioPathology image analysis solution, Leica Microsystems, Inc., Tokyo, Japan).

Statistical analysis
Numerical data were tested by Bartlett’s test for homogeneity of variance (level of significance: 0.01). When the variances were homogeneous, Dunnett’s test was applied to compare the mean value in the vehicle control group with that in each treated group (levels of significance: 0.05 and 0.01, two-tailed). When the variances were heterogeneous, Steel’s test was applied to compare the mean rank in the vehicle control group with that in each treated group (levels of significance: 0.05 and 0.01, two-tailed). In addition, the data were tested by F test for homogeneity of variance (level of significance: 0.05). When the variances were homogeneous, Student’s t test was applied to compare the mean value between the vehicle control group and the positive control group (levels of significance: 0.05 and 0.01, two-tailed). When the variances were heterogeneous, Aspin & Welch t test was applied to compare the mean value between the vehicle control group and the positive control group (levels of significance: 0.05 and 0.01, two-tailed).

Results

EGDE
There were no EGDE-related changes in the mortality or clinical signs. At 60 mg/kg bw, a statistically significant decrease in food consumption was (sporadically) observed in the early stage of the dosing period, and a statistically significant increase in food consumption was (sporadically) observed in the later stage of the dosing period, although this was not accompanied by any change of body weight (Fig. 1). Although relative liver weights increased at 60 mg/kg bw (Table 2), no EGDE-related histological change was observed in the liver. There were no differences in the number or area of GST-P-positive foci in the liver as compared to the vehicle control group (Table 3).

Animals in the positive control group showed increases in the body weight, food consumption (Fig. 1), and absolute and relative liver weights (Table 2) as well as hypertrophy of the centrlobular hepatocytes in all rats evaluated (data not
shown). In addition, statistically significant higher values in both the number and area of GST-P positive foci were observed in the positive control group (Table 3).

**HNA**

There were no HNA-related changes in the mortality or clinical signs. Suppression of body weight gain (~8% from the vehicle control group) and decreased food consumption were observed at 200 mg/kg bw (Fig. 2). Although a statistically significant decrease in the absolute liver weight was noted at 200 mg/kg bw, it was considered to be attributable to the decreased body weights at necropsy (Table 4). There were neither HNA-related histopathological changes nor significant differences in the number or area of GST-P positive foci in the liver of the test substance-treated groups as compared to the vehicle control group (Table 5).

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**Table 2.** Body and Liver Weights in the Medium-term Rat Liver Bioassay of EGDE

| Dose (mg/kg/day) | Effective No. of animals | Body weight before necropsy (g) | Absolute liver weights (g) | Relative liver weights (g/100 g) |
|------------------|--------------------------|--------------------------------|-----------------------------|---------------------------------|
| 0               | 20                       | 260 ± 11 a                     | 7.91 ± 0.39                 | 3.04 ± 0.09                     |
| 6               | 20                       | 261 ± 9                        | 7.96 ± 0.50                 | 3.05 ± 0.12                     |
| 20              | 23                       | 259 ± 11                       | 8.05 ± 0.53                 | 3.10 ± 0.11                     |
| 60              | 22                       | 258 ± 11                       | 8.26 ± 0.57                 | 3.20 ± 0.16**                   |
| PB 25           | 20                       | 273 ± 11**                     | 10.24 ± 0.48**              | 3.75 ± 0.12**                   |

PB: phenobarbital.

*Mean ± SD.*

**Significantly different from the vehicle control group (p≤0.01).**

**Table 3.** GST-P-positive Foci in the Medium-term Rat Liver Bioassay of EGDE

| Dose (mg/kg/day) | Effective No. of animals | GST-P-positive foci |
|------------------|--------------------------|---------------------|
|                  |                          | Number (No./cm²)    | Area (mm²/cm²)        |
| 0                | 20                       | 5.369 ± 1.744 a     | 0.470 ± 0.197         |
| 6                | 20                       | 5.562 ± 1.764       | 0.533 ± 0.237         |
| 20               | 23                       | 6.161 ± 1.890       | 0.625 ± 0.242         |
| 60               | 22                       | 5.645 ± 1.420       | 0.558 ± 0.220         |
| PB 25            | 20                       | 9.852 ± 2.076**     | 1.067 ± 0.340**       |

PB: phenobarbital.

*Mean ± SD.*

**Significantly different from the vehicle control group (p≤0.01).**
Animals in the positive control group showed increases in the body weights, food consumption (Fig. 2), and absolute and relative liver weights (Table 4), in addition to hyper trophy of the centrilobular hepatocytes in all rats evaluated and hepatocellular adenoma in 1 of 24 rats (data not shown). Furthermore, statistically significant higher values in both the number and area of GST-P positive foci were observed in the positive control group (Table 5).

**AAA In the clinical signs, pale skin was observed in all rats at 600 mg/kg bw from day 14 of AAA administration. One rat died at day 19 due to AAA-related effects and suppression of body weight gain (~10% from the vehicle control group) was observed at 600 mg/kg bw (Fig. 3). At necropsy, an enlarged spleen was observed at 200 mg/kg bw and above. Histopathological examination revealed brown
pigmentation of Kupffer cells and extramedullary hematopoiesis in the liver of almost all rats at 600 mg/kg bw (data not shown). In addition, hypertrophy of the centrilobular hepatocytes was observed in 12 of 23 rats at 200 mg/kg bw and in all rats at 600 mg/kg bw (data not shown), and was accompanied by increased absolute liver weights at ≥60 mg/kg bw and relative liver weights at 600 mg/kg bw (Table 6). However, there were no statistically significant differences in the number or area of GST-P positive foci in the liver of AAA-treated groups as compared to the vehicle control group (Table 7).

In the positive control group, there were increases in the body weights, food consumption (Fig. 3) and absolute and relative liver weights (Table 6), and hypertrophy of the centrilobular hepatocytes in all rats evaluated (data not shown). In addition, statistically significant higher values in both the number and area of GST-P positive foci were observed in the positive control group (Table 7).

Table 6. Body and Liver Weights in the Medium-term Rat Liver Bioassay of AAA

| Dose (mg/kg/day) | Effective No. of animals | Body weight before necropsy (g) | Absolute liver weights (g) | Relative liver weights (g/100 g) |
|-----------------|--------------------------|--------------------------------|---------------------------|---------------------------------|
| 0               | 22                       | 249 ± 11*                       | 7.82 ± 0.48               | 3.14 ± 0.11                     |
| 60              | 23                       | 247 ± 15                        | 7.97 ± 0.63               | 3.22 ± 0.13*                    |
| 200             | 23                       | 242 ± 14                        | 8.18 ± 0.59               | 3.37 ± 0.13**                   |
| 600             | 23                       | 221 ± 12**                      | 8.41 ± 0.52**             | 3.81 ± 0.11**                   |
| PB 25           | 23                       | 270 ± 13**                      | 10.20 ± 0.70**            | 3.78 ± 0.14**                   |

PB: phenobarbital.
a Mean ± SD.
* Significantly different from the vehicle control group (p≤0.05).
** Significantly different from the vehicle control group (p≤0.01).

Table 7. GST-P-positive Foci in the Medium-term Rat Liver Bioassay of AAA

| Dose (mg/kg/day) | Effective No. of animals | GST-P-positive foci |
|-----------------|--------------------------|---------------------|
|                |                          | Number (No./cm²)    | Area (mm²/cm²) |
| 0               | 22                       | 2.896 ± 1.022*      | 0.356 ± 0.182 |
| 60              | 23                       | 3.189 ± 1.189       | 0.391 ± 0.189 |
| 200             | 23                       | 2.652 ± 1.113       | 0.415 ± 0.406 |
| 600             | 23                       | 2.727 ± 0.979       | 0.264 ± 0.107 |
| PB 25           | 23                       | 8.431 ± 1.946**     | 1.192 ± 0.324** |

PB: phenobarbital.
a Mean ± SD.
** Significantly different from the vehicle control group (p≤0.01).
Discussion

Long-term administration studies of chemical substances using rats and mice have been the standard for evaluating the carcinogenic potential of these chemicals. This standard has been used worldwide, but 2-year carcinogenicity studies are very expensive and time consuming to clarify the carcinogenic potential; additionally, there is a demand to decrease the number of animals used for carcinogenicity studies because of animal welfare considerations. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) proposed a revised guideline that recommends reducing long-term studies using only one rodent species and replacing the second long-term rodent study with an alternative bioassay using neonatal rodents, transgenic mice, or two-stage carcinogenesis models, based on the results of the short-term studies\(^3\). Since it has been shown that the medium-term rat liver bioassay is a rapid, reliable, and practical tool for the prediction of the carcinogenic potential of chemicals\(^2\), this test system is now internationally well recognized and recommended as an alternative carcinogenicity test\(^6\).

In a combined repeated-dose toxicity and reproductive toxicity study in rats orally administered EGDE (0, 12.5, 50, and 200 mg/kg bw), squamous cell hyperplasia in the forestomach and/or chronic ulcer in the glandular stomach were observed in both sexes at 200 and 50 mg/kg bw\(^4\). However, there were neither toxic lesions in the other tissues/organs nor proliferative lesions suggestive of preneoplastic changes in the organs/tissues of the treated animals. No carcinogenicity study was conducted on this substance. 1,4-Butanediol diglycidyl ether (CAS No. 2425-79-8), a chemical resembling EGDE, was tested for carcinogenicity in mice dosed for 2 years, but the result was negative\(^7\). In a 28-day repeated oral dose toxicity study of HNA in rats (0, 12, 60, and 300 mg/kg bw), necrosis of the adrenal cortex and increased liver weights in females were found at 60 mg/kg bw and more than 300 mg/kg bw, respectively\(^8\). There was no proliferative lesion suggestive of preneoplastic changes in the organs/tissues of the treated animals. No carcinogenicity bioassay has been conducted in this substance and its analogues. In a 28-day repeated oral dose toxicity study of AAA in rats (0, 12, 100, and 850 mg/kg bw), males at 850 mg/kg bw showed decreased body weights and food consumption, and dose-dependent changes in hematology and serum chemistry indicative of hemolytic anemia and met-hemoglobinemia were observed at 100 and 850 mg/kg bw. Spleen weights increased at 100 and 850 mg/kg bw. Microscopic examination revealed extramedullary hematopoiesis in the liver with hemosiderosis and hemosiderin deposition in the spleen and kidney in these groups\(^9\). No proliferative lesions suggestive of preneoplastic changes were detected in the organs/tissues of the treated animals. No carcinogenicity bioassay has been conducted in this substance and its analogues. Taken together, the results of these repeated dose toxicity studies on EGDE, HNA, and AAA in rodent species indicated that no proliferative lesions suggestive of preneoplastic changes were induced by these substances. In the present studies, clear increases in the number and area of GST-P positive foci in the liver of the positive control group (PB-treated group) were observed, confirming that this system could appropriately detect hepatocellular carcinogens or promoters. In contrast, no apparent differences in the number and area of GST-P positive foci were observed in the livers of rats administered EGDE, HNA, and AAA, indicating that these chemical substances do not induce hepatocarcinogenicity.

As mentioned in the Introduction section, EGDE, HNA, and AAA have been shown to exert in vitro genotoxic activity. It is known that such a genotoxicity does not always correlate with carcinogenicity, but the medium-term rat liver bioassay has been demonstrated to be excellent for the detection of liver carcinogens because this liver assay is susceptible to non-genotoxic hepatocarcinogens as well as genotoxic hepatocarcinogens\(^10\). In contrast, carcinogens targeting organs/tissues other than the liver cannot always be detected by this liver assay. Therefore, the results of the present studies suggest the possibility that EGDE, HNA, and AAA are carcinogens targeting the organs/tissues other than the liver. In addition, the mutagenic response of EGDE, HNA, and AAA may be unique to bacteria. For example, the mutagenic response may be specific to the bacteria or bacterial-specific metabolism (nitroreductase reaction), exceeding a detoxification threshold or the induction of oxidative damage to which bacteria may be more sensitive than mammalian cells or tissues\(^10\). Therefore, future investigation on the mutagenicity tests using mammalian cells would provide useful information for the mutagenicity/carcinogenicity assessment of these chemicals.

In conclusion, the results of the present studies indicate that EGDE, HNA, and AAA do not have a hepatocarcinogenic activity in the present experimental condition, but the possibility that they are carcinogens targeting organs/tissues other than the liver cannot be ruled out.

Disclosure of Potential Conflicts of Interest: All authors disclose here that there are no conflicts of interest that could inappropriately influence the outcome of the present study. This work was a public contract of the Ministry of Health, Labour and Welfare of Japan.

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