Verification of a false positive in a two-year rat carcinogenicity study using dual control groups

Shuji Ogawa1, Hiroyuki Kuroda2, Toshiko Kinomoto1, Yoshihiro Kawabata1, Mayumi Kawabe3, Mayuko Suguro3 and Yuji Oishi4

1Central Research Laboratories, Zeria Pharmaceutical Co., Ltd., 2512-1 Oshikiri, Kumagaya-shi, Saitama 360-0111, Japan
2R&D PLANNING, Zeria Pharmaceutical Co., Ltd., 10-11 Nihonbashi Kobuna-cho, Chuo-ku, Tokyo 103-8351, Japan
3DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya-shi, Aichi 491-0113, Japan
4Department Molecular Pathology, Osaka City University, 1-4-3 Asahi-machi, Abeno-ku, Osaka-City, Osaka 545-8585, Japan

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ABSTRACT — There is sometimes controversy over whether or not statistically significant responses produced in carcinogenicity studies have biologically significance. Ambiguous results from our previous two-year oral carcinogenicity study on acotiamide hydrochloride hydrate (acotiamide-HH), a prokinetic drug for functional dyspepsia, in rats made it unclear whether the drug may exhibit uterine carcinogenicity. To check this finding, we performed a second long-term carcinogenicity study using two identical control groups to more accurately evaluate uterine carcinogenesis by considering the incidence of spontaneous neoplasms. Female Fischer 344 rats were divided into three groups: the two control groups (control 1 and 2) were administered vehicle (0.5% w/v methylcellulose) and the acotiamide-HH-treated group was administered 2,000 mg/kg/day of acotiamide-HH by oral gavage for two years. Among all groups, the incidence of endometrial adenocarcinoma (EmA) was highest in the control 2 group, followed by the acotiamide-HH-treated group and the control 1 group. Moreover, acotiamide-HH did not affect the incidence of precursor lesions of EmA. In cases where an ambiguous difference is observed, the use of two control groups allows for a more informed interpretation of the findings in the drug-treated groups. The outcomes in this study strongly support the hypothesis that the increase in EmA in rats treated with acotiamide-HH in our previous study is unrelated to administration of the drug.

Key words: Dual controls, Carcinogenicity, Endometrial adenocarcinoma, Rat, Acotiamide

INTRODUCTION

Carcinogenicity studies in animals are important for identifying the tumorigenic potential of compounds of interest and play a pivotal role in the risk assessment of new chemicals and drugs for human consumption. However, it is often controversial whether or not statistically significant results obtained in animal carcinogenicity studies have biological significance. In the development of acotiamide hydrochloride hydrate (acotiamide-HH), an acetylcholine esterase inhibitor developed by Zeria Pharmaceutical Co., Ltd., we previously observed a statistically significant increase in the incidence of endometrial adenocarcinoma (EmA) in only the middle-dose group (600 mg/kg) in a rat long-term carcinogenicity study. The EmA incidence in this group exceeded the range in historical background data from the study facility (Shiga, 2005). However, the low (200 mg/kg) and high (2,000 mg/kg) acotiamide-HH dosing groups showed no statistically significant increase in EmA incidence, although the incidence was slightly higher than that in the control group (Kuroda et al., 2015). We therefore considered this finding to be the result of biological variation rather than a biologically significant difference. With the aim to clarify that these previous results were indeed due to biological variation, we re-planned the two-year carcinogenicity study with a focus on EmA incidence.

Here, we conducted a carcinogenesis study with two control groups to more accurately evaluate uterine carcinogenesis by considering the incidence of spontaneous neoplasms. We also discuss the usefulness of dual control groups in long-term carcinogenicity studies.

Correspondence: Shuji Ogawa (E-mail: syuujigawa@zeria.co.jp)
MATERIALS AND METHODS

This study was conducted in compliance with the Ministerial Ordinance on Good Laboratory Practice for Nonclinical Safety Studies of Drugs (Ordinance of the Ministry of Health and Welfare No. 21 of March 26, 1997, as last amended by the Ordinance of the Ministry of Health, Labour and Welfare No. 114 of June 13, 2008) and in accordance with the Guidelines on Carcinogenicity Tests of Drugs (Pharmaceutical Affairs Bureau Notification No. 1607) and the Guidance on Toxicokinetics (Evaluation of Systemic Exposure in Toxicity Studies, July 2, 1996, Evaluation and Licensing Division Notification No. 443).

Materials

Acotiamide-HH was synthesized by Zeria Pharmaceutical Co., Ltd. (Saitama, Japan). Heptyl 4-hydroxybenzoate (an internal standard for acotiamide-HH) and methylcellulose (MC) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan), respectively. All other chemicals were reagent grade.

Animals and housing conditions

Female specific pathogen-free Fischer 344 (F344) rats, F344/DuCrI/Crlj, were obtained from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were 4 weeks old on arrival and were quarantined/acclimated for 7 days at the test facility. At 5 weeks of age, rats with regular body weight gain without clinical abnormalities were selected and randomly assigned to three groups such that mean body weights were approximately equal among the groups. The groups comprised two control groups and one acotiamide-HH treatment group. Animals were housed individually in hanging stainless-steel wire mesh cages in an animal room under the following conditions: temperature, 22 ± 3°C; relative humidity, 55 ± 15%; air ventilation, more than 10 air changes per hour; and 12-hr light/dark cycle (lights on 07:00 to 19:00). CRF-1 pel- let or powder diet (15 kGy radiation-sterilized, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were available ad libitum. All cages and racks were rotated once a week to avoid environmental biases that could potentially affect the research outcomes. All animals were treated humanely according to institutional guidelines (DIM, 2015), and the experimental procedure was approved by the institutional ethics committee.

Carcinogenicity study

Rats in the acotiamide-HH group were administered acotiamide-HH at a dose of 2,000 mg/kg in 10 mL/kg 0.5% w/v MC by oral gavage every day for two years. The 2,000 mg/kg acotiamide-HH dose was selected based on the maximum tolerated dose determined in a previous rat carcinogenicity study (Kuroda et al., 2015). The control 1 and 2 groups were identically administered 0.5% w/v MC by oral gavage every day for two years. Each group consisted of 55 female rats. Animals were inspected twice daily for gross abnormalities or mortality and palpated weekly. Body weights were recorded once a week for the first 26 weeks and once every two weeks thereafter until the end of the study. Food consumption and food efficiency were recorded once a week for the first 26 weeks, once every two weeks thereafter until 56 weeks of administration, and then once a week until the end of the study. After 104 weeks of administration, all surviving animals were euthanized. All animals, including dead or moribund animals, were subjected to detailed necropsy, and all gross lesions were recorded. All major organs/tissues including any gross lesions were removed and preserved in 10% buffered formalin solution. According to our aim to clarify whether or not acotiamide-HH has carcinogenic potential in the uterus, only the uterus was evaluated by histopathological examination in this study. Uterine tissues were routinely processed for embedding in paraffin wax, and were sectioned horizontally and stained with hematoxylin and eosin for histopathological examination. Furthermore, a third party-authorized toxicological pathologist who was not directly associated with the study performed a pathological peer review to confirm our histopathological diagnosis and findings. In addition, plasma concentrations of acotiamide-HH were measured in separate groups of rats at 0.5 and 4 hr after administration of the initial drug treatment and at weeks 13 and 26. The free form concentration was calculated.

Quantification of acotiamide-HH by high performance liquid chromatography (HPLC)

The plasma samples (100 μL) were added to pure water (1 mL) and transferred to an OASIS HLB 30 μm solid-phase extraction plate (Waters, Milford, MA, USA), and the analytes were eluted with 3 mL methanol. A 2% propylene glycol solution (100 μL) was added to each eluate. Each eluate was evaporated to dryness under nitrogen gas in a water bath at 50°C and dissolved in 300 μL of mobile phase, and 20 μL aliquots were then subjected to HPLC. HPLC analysis was performed using a LC-VP series (Shimazu, Kyoto, Japan) with a reversed phase column (Inertsil ODS-3; GL Sciences Inc., Tokyo, Japan).
Ultraviolet (UV) absorbance was detected at 266 nm. The mobile phase consisted of 0.6% potassium dihydrogenphosphate solution (pH 3.0) and methanol (1:4) and sodium dodecyl sulfate (10 mM), and was pumped at a flow rate of 1.0 mL/min.

Statistical analysis

Statistical analysis of survival rate, body weight, food consumption, food efficiency and histopathological data was performed between the acotiamide-HH group and each control group, as well as between the control 1 and 2 groups. Survival rate was compared using a log-rank test. Body weight, food consumption and food efficiency were analyzed using an F-test for equality of variances. When the equality of variances was not significant, a two-sided Student’s t-test was conducted. When the equality of variances was significant, a two-sided Welch test was conducted. Graded histopathological changes in non-neoplastic lesions were analyzed using the Wilcoxon signed-rank test, while non-graded histopathological changes in neoplastic lesions were analyzed using a two-sided Fisher’s exact test. A two-sided p value of less than 0.05 was considered statistically significant.

RESULTS

Survival rate, general conditions, body weight, food consumption and food efficiency

A summary of the survival rate is shown in Table 1. One rat in the control 1 group died following an accidental fall at 17 weeks of administration and was subsequently excluded from the study. The number of surviving animals at terminal sacrifice was 37 out of 54 (69%), 34 out of 55 (62%) and 34 out of 55 (62%) in the control 1, control 2 and acotiamide-HH groups, respectively. The survival rate in the acotiamide-HH group was within the range observed for both control groups and was not significantly different from either of the control groups. Unexpected sudden deaths that revealed only retention of food in the oropharyngeal cavity or the esophagus without other necropsy findings sporadically occurred during a period from 44 to 57 weeks of administration in 3, 4 and 2 rats in the control 1, control 2 and acotiamide-HH groups, respectively. To reduce sudden deaths, the feed was changed from pellet to powder diet at 58 weeks of administration because a previous report showed that changing the feed is effective in reducing sudden deaths in F344 rats (Ohishi et al., 2008; Shirai et al., 2005). No sudden deaths of this type occurred after changing the feed. Although the number of sudden deaths in the acotiamide-HH group was lower than that in the control groups, survival to terminal sacrifice was not significantly different between the acotiamide-HH group and either of the control groups even after excluding the animals that experienced sudden death in each group. The other deaths or moribund sacrifices occurred from 52 weeks of administration. Hematopoietic or pituitary tumor was considered to be the main cause of those deaths or moribund sacrifices because enlarged spleen or pituitary was frequently observed in those animals.

Major general conditions are shown in Table 2. Nodules or masses localized to the skin or subcutis, crust formation on the footpads, emaciation, decreased locomotor activity and anemia were observed at high frequency. Among these, emaciation, decreased locomotor activity and anemia were mainly observed under deteriorating conditions in tumor-bearing animals. The incidence of each major general condition was similar among the groups.

Body weight change and food consumption are shown in Figs. 1 and 2, respectively. The body weight and food consumption of the acotiamide-HH group were close to the ranges observed in the two control groups throughout the administration period. There were no toxicologically

Table 1. Survival rate.

<table>
<thead>
<tr>
<th>Period (week)</th>
<th>Control 1</th>
<th>Control 2</th>
<th>Acotiamide-HH</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>55/55 (100%)</td>
<td>55/55 (100%)</td>
<td>55/55 (100%)</td>
</tr>
<tr>
<td>26</td>
<td>54/54 (100%)</td>
<td>55/55 (100%)</td>
<td>55/55 (100%)</td>
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<tr>
<td>52</td>
<td>50/54 (93%)</td>
<td>51/55 (93%)</td>
<td>54/55 (98%)</td>
</tr>
<tr>
<td>78</td>
<td>48/54 (89%)</td>
<td>49/55 (89%)</td>
<td>52/55 (95%)</td>
</tr>
<tr>
<td>105b</td>
<td>37/54 (69%)</td>
<td>34/55 (62%)</td>
<td>34/55 (62%)</td>
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</tbody>
</table>

*One rat was excluded from the study due to accidental death at 17 weeks of administration. bTerminal sacrifice. Values indicate n (%). Sudden deaths that revealed only retention of food in the oropharyngeal cavity or the esophagus without other necropsy findings occurred from 44 to 57 weeks of administration in 3, 4 and 2 rats in the control 1, control 2 and acotiamide-HH groups, respectively. The other deaths or moribund sacrifices occurred from 52 weeks of administration. Hematopoietic or pituitary tumor was considered to be the main cause of those deaths or moribund sacrifices.
meaningful changes in food efficiency among the groups (data not shown).

**Plasma drug concentration**

Plasma concentrations of acotiamide-HH are shown in Table 3. Plasma concentrations of the free form of acotiamide-HH were similar to those reported in a previous report (Kuroda et al., 2015), indicating good reproducibility.

**Pathological examination**

Major findings of the gross pathology examination are shown in Table 4. Various findings including enlarged horn and discolored lesions or nodules were observed in the uterus. The nodules, which suggest uterine tumors, was observed in 11 out of 54, 24 out of 55, and 14 out of 55 rats in the control 1, control 2 and acotiamide-HH groups, respectively. The number of nodules observed in the acotiamide-HH group was within the range observed in both control groups. Although enlarged spleen, atrophic thymus, enlarged or discolored pituitary, enlarged liver, and nodules or masses localized to the skin or subcutis, which occurred spontaneously, were observed in all groups, the incidence of each finding in the acotiamide-HH group was similar or below the range observed in the control groups.

As seen from Table 5, EmA was histopathologically observed in 5 out of 54 (9%), 7 out of 55 (13%), and 6 out of 55 (11%) rats in the control 1, control 2 and
acotiamide-HH groups, respectively. The incidence of EmA in the acotiamide-HH group was within the range observed in the control groups and was not significantly different from either of the control groups. Furthermore, the incidence of endometrial adenoma or epithelial/glandular endometrial hyperplasia, which are considered to be precursor lesions of EmA, in the acotiamide-HH group was likewise not significantly different from either of the control groups.

Meanwhile, the incidence of epithelial/glandular endometrial hyperplasia in the present study was higher than that reported in our previous study (Kuroda et al., 2015). We hypothesize that this is due to the performance of a more detailed histopathological examination from the tip of the uterine horn to the portio vaginalis uteri in the present study.

**Comparison between dual control groups**

The body weights of rats in the control 2 group were significantly increased compared to those in the control 1 group from 14 weeks to 92 weeks of administration. Significant increases in food consumption were also observed sporadically in the control 2 group compared to the control 1 group. Furthermore, the control 2 group tended to have many incidences of uterine nodules, enlarged pituitary, and nodules or masses localized to the skin or subcutis.

**DISCUSSION**

In this additional carcinogenicity study, acotiamide-HH administration did not affect any observation items including viability, general conditions, body weight, and food intake. Further, detailed histopathological examination of the uterus showed that acotiamide-HH had no
effect on the uterus including tumorigenesis, indicating that our previous ambiguous result of a statistically significant increase in the incidence of EmA in the middle-dose group (600 mg/kg) was a false positive that resulted from the incidence spontaneous tumors in F344 rats (Kuroda et al., 2013). Various endometrial proliferative lesions including adenocarcinoma, adenoma, epithelial/glandular hyperplasia and cystic endometrial hyperplasia were observed in all groups. Cystic endometrial hyperplasia, however, is thought to be attributed to prolonged estrogen stimulation and is not believed to be preneoplastic (Leininger and Jokinen, 1990). The number of animals with epithelial/glandular endometrial hyperplasia, adenoma and adenocarcinoma, which are regarded as a series of proliferative lesions, was 28 out of 54 (52%), 30 out of 55 (55%), and 30 out of 55 (55%) in the control 1, control 2 and acotiamide-HH groups, respectively. Even when considering endometrial proliferative lesions including precursor lesions of adenocarcinoma, acotiamide-HH-treated animals did not exhibit increased incidence of lesions. Numerous studies have reported the incidence of spontaneous neoplasms in F344 rats (Ando et al., 2008; Dinse et al., 2010; Haseman et al., 1998; Iwata et al., 1991; Kuroiwa et al., 2013; Maekawa et al., 1983; Maita et al., 1987; Takanobu et al., 2015). According to these studies, the incidence of EmA ranges from 0% to 22%. Furthermore, Nyska et al. (1994) reported an unusually high incidence of spontaneous EmA (24%) in a life-span oncogenicity study. Therefore, the variability in EmA incidence among studies is relatively large. Taking into account the present study results, the increased incidence of EmA (16%) in the middle-dose group in our previous study (Kuroda et al., 2015), which falls within the published range, can be considered to be the result of spontaneous tumor bias.

We observed various gross pathological findings such as uterine nodules, enlarged spleen, enlarged pituitary, and nodules or masses localized to the skin or subcutis in all groups. It is well known that the most common spontaneous tumors in female F344 rats include pituitary adenomas, uterine endometrial stromal polyps, mammary fibroadenomas and mononuclear cell leukemias (Dinse et al., 2010; Haseman et al., 1998; Iwata et al., 1991; Maekawa et al., 1983; Takanobu et al., 2015). Our gross pathological findings reflect these reports.

We observed some degree of difference even between our concurrent control groups under the same environmental condition. The body weight and food consumption of the control 2 group tended to be higher than those of the control 1 group throughout the administration period. Additionally, the control 2 group tended to have more gross pathological lesions in tissues such as the pituitary gland and skin than those of the control 1 group. Roe (1987) reported that overfeeding predisposes rats to increased incidence of tumors, particularly endocrine and mammary tumors. Therefore, the tendency towards higher body weight or food consumption in the control 2 group might be associated with the higher incidence of pituitary and skin lesions compared to the control 1 group.

The results from two identical control groups can be used to identify the extent of control variability, help evaluate the biological significance of increases in tumor incidence in the treated groups (i.e., true increases versus noise) (CDER, 2001), and enable easier interpretation of findings in drug-treated groups (Baldrick, 2005; Baldrick and Reeve, 2007). In the present study, histopathological examination revealed that the incidence of EmA in the acotiamide-HH group (11%) was within the range observed in the two control groups (9-13%), suggesting that acotiamide-HH has no potential for increasing the incidence of EmA. Our study therefore demonstrated that the use of two identical control groups allowed for a more informed interpretation of the findings in the acotiamide-HH group, although the range from the two control groups was not as large as expected. Furthermore, gross pathological examination showed that the incidence of most kinds of lesions in the acotiamide-HH group was also within the range observed in the two control groups, suggesting that the use of two identical control groups may also be useful in carcinogenicity studies in which organs other than the uterus are examined in detail.

In conclusion, the use of two control groups was useful in the carcinogenic assessment of acotiamide-HH in rats and we showed that acotiamide-HH did not increase the incidence of EmA compared to control. This outcome strongly supports the suggestion that the increased incidence of EmA observed in our previous study was a false positive that resulted from the incidence of spontaneous tumors in F344 rats. Dual controls are therefore useful for identifying the extent of variability in control animals and allow for a more informed interpretation of the results related to the test article-treated group.

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

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DIMS (2015): Standards for Care and Use of Laboratory Animals at DIMS Institute of Medical Science, Inc.


