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Original Article

A study on the influence of acotiamide hydrochloride hydrate on sex hormones, using a uterotrophic bioassay in rat

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ABSTRACT — Acotiamide hydrochloride hydrate (acotiamide-HH) is the first approved drug in the world for the treatment of patients with functional dyspepsia in Japan. A statistically significant increase in the incidence of endometrial adenocarcinoma was found in a 104-week carcinogenicity study in rats, in a non-dose-dependent manner, and it was considered that further evaluation was required to clarify this issue. Therefore, we performed a uterotrophic bioassay using immature female rats, which is mentioned in the Organization for Economic Co-operation and Development (OECD) Guideline 440, to evaluate the effect of acotiamide-HH on estrogen, which is one of the most important mechanisms causing increase in the incidence of endometrial adenocarcinoma. The positive control substance selected was 17α-ethinyl estradiol (EE). While EE caused a dose-dependent increase in uterine weight, no increase in uterine weight, histopathological changes, or endometrial proliferation activity were observed in the acotiamide-HH treatment groups at doses of up to 1000 mg/kg. Based on this result, we concluded that acotiamide-HH has no potential risk to cause imbalance of the sex hormone environment in female rats.

Key words: Acotiamide, Endometrial adenocarcinoma, Rat, Carcinogenicity, Sex hormone

INTRODUCTION

Acofide Tablet (generic name: acotiamide hydrochloride hydrate (acotiamide-HH)) is an acetylcholine esterase (AChE) inhibitor discovered by Zeria Pharmaceutical Inc., Ltd., which has been approved in Japan as the first drug in the world for functional dyspepsia (Acofide Tablets 100 mg: Review Report, 2013). Acotiamide-HH acts by increasing gastric antral motility and improving the reduced gastric motility by increasing the amount of ACh available at the cholinergic nerve terminal through its selective AChE inhibiting activity. Although theoretical concerns on carcinogenicity based on its pharmacological activity were not observed, an issue arose in the rat carcinogenicity study of acotiamide-HH, i.e., an increased incidence of non-dose-dependent endometrial adenocarcinoma in the middle-dose group.

The carcinogenicity study was conducted in the rat with acotiamide-HH at doses of 200, 600 and 2000 mg/kg/day. Among 50 animals in each dose group, the incidence of endometrial adenocarcinoma was 1, 5, 8 and 5 in the control, 200 mg/kg, 600 mg/kg and 2000 mg/kg groups, respectively (Shiga, 2002; Kuroda *et al.,* 2015). The results of no genotoxic findings in any of the genotoxicity studies and no treatment-related toxicological findings in reproductive and developmental studies suggested that the endometrial adenocarcinoma found in the rat carcinogenicity study possibly arose spontaneously at all doses (Review report, 2013). However, the incidence of endometrial adenocarcinoma in the carcinogenicity study in rat was a deviation from the background data of the conducting facility. Therefore, we considered that an additional study was necessary to clarify the causal relationship between the endometrial adenocarcinoma and acotiamide-HH (Kuroda *et al.,* 2015). For the additional study, we performed the uterotrophic bioassay using

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immature rat, mentioned in OECD Guideline 440, to evaluate if acotiamide-HH has an effect on the sex hormone environment, which is one of the most important factors in the causation of endometrial adenocarcinoma (OECD Guideline Test No. 440).

MATERIALS AND METHODS

This study was approved and conducted in accordance with the Ethics Committee of Zeria Pharmaceutical Co., Ltd., as well as Reliability Criteria of Application Document; Uterotrophic Study Guideline OECD 440, enforced on October 17, 2007; and Regulations and Guidelines on Scientific and Ethical Care and Use of Laboratory Animals, enforced on June 01, 2006.

Animals and environment of animal room

Female Sprague-Dawley pregnant rats were obtained from Charles River Laboratories, Inc. (Atsuqi, Japan) and bred under specific pathogen free (SPF) environment. CRF-1 pellet (Oriental Yeast Co. Ltd, Tokyo, Japan) and tap water were available to rats *ad libitum*. The juvenile rats were weaned from dams at 16 to 17 days after childbearing. The day after weaning, the animals were assigned to groups (both control and treatment) by randomized weight distribution, so that the mean body weight of each group was not statistically significant different from any other group. Each study group comprised of 6 intact juvenile female rats.

Study design

All test substances were administered orally at 5 mL/kg volume once a day for 3 days. In both the baseline positive study and the main study, general clinical observations, body weight and food consumption measurements were performed every day. At 24 hr after the last dosing, all rats were sacrificed by bleeding under isoflurane deep anesthesia. The necropsy was performed randomly across groups to avoid progression directly up or down dose groups that could subtly affect the data. Before the dissection, the vagina was examined for opening status. According to OECD Test Guideline 440 (OECD Guideline Test No. 440), the extraction of the uterus of each animal was performed carefully and appropriately. All uteri were weighed with and without the luminal fluid. The relative weight of each uterus was calculated based on the corresponding individual rat's body weight.

Baseline positive study

This preliminary study was performed with 6 animals of each group to evaluate the response of the uterus to EE administration. The doses of EE were set at 0.1, 0.3, 1 and 10 μg/kg, referring to a previous study (Kanno *et al*., 2001). Corn oil (CO Wako Corporate, Osaka, Japan) was set as the vehicle group. In addition, a group of acotiamide-HH with the dose of 1000 mg/kg, which is the highest administration dose in the Guideline, was also set in this study.

Main study

The animals were randomly assigned to comprise 6 animals in each group. In the control group, animals were dosed with 0.5 w/v% methylcellulose (MC, Wako Corporate, Osaka, Japan) solution, and 200, 600, and 1000 mg/kg of acotiamide-HH. The positive control group was set at 1.0 μg/kg of EE, which was found to have led to good estrogen response in the baseline positive study.

The uterus and vagina of all animals in the study were fixed in 10% neutral buffered formalin for histological examination after the uterine weighing. The histopathological assessment was performed on 6 transverse sections of the uterine horns and 1 section of the vagina after hematoxylin-eosin (HE) staining. To investigate endometrial cellular activity, immunohistochemical staining was performed with Proliferating Cell Nuclear Antigen (PCNA) (Dako Japan Co., Tokyo, Japan) using the Simple Stain MAX PO (MULTI) kit (Nichirei Biosciences Inc., Tokyo, Japan) on additional 6 sections from the same portion of the uterine horns. The numbers of all endometrial cells at the luminal surface and the number of cells reacting with PCNA at the luminal surface were counted in all sections. For each sample, 6 sites were examined to calculate the proportion of PCNA-positive cells.

Statistical analysis

Statistical analyses were performed by SAS non-clinical package (SAS System version 8.2, SAS Institute Inc., NC, USA). Steel test or Dunnett multiple comparison test was used for body weight; Aspin-Welch test was used for uterine weight; and Student t-test was used for cell proliferation activity by PCNA staining. Significance levels were set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULT

In the baseline positive study, there was no significant difference in body weight or in food consumption in any group compared with the control group (Table 1). The change in uterine weight is presented in Fig. 1 and Fig. 2. There was no significant difference in uterine weight between the control group and the acotiamide-HH group, while the uterine weight in the EE groups was

Table 1. Body weight and food consumption in the baseline positive control study.

Dosing Day	Corn oil	Ethinyl estradiol				Acotiamide-HH
		0.1 μg/kg	0.3 μg/kg	1.0 μg/kg	3.0 μg/kg	1000 mg/kg
Body weight (g) Mean ± S.D. of six rats						
1	33.2 ± 3.1	32.8 ± 2.9	32.1 ± 2.8	31.3 ± 1.8	33.2 ± 3.4	31.8 ± 2.5
2	36.3 ± 3.6	36.1 ± 3.0	35.9 ± 2.3	34.9 ± 3.3	37.3 ± 3.2	35.3 ± 2.4
3	41.4 ± 3.5	41.4 ± 3.2	41.2 ± 2.5	39.8 ± 3.0	43.1 ± 3.1	41.3 ± 2.3
final	46.5 ± 3.4	46.0 ± 3.2	46.3 ± 2.9	44.9 ± 3.0	48.0 ± 3.1	47.3 ± 2.1
Food consumption (g/day/animal)						
1-2	3.10	2.92	3.65	0.43	2.42	2.88
2-3	5.02	5.10	4.80	4.82	5.80	5.63
3-final	6.85	6.43	6.55	6.00	6.45	7.10

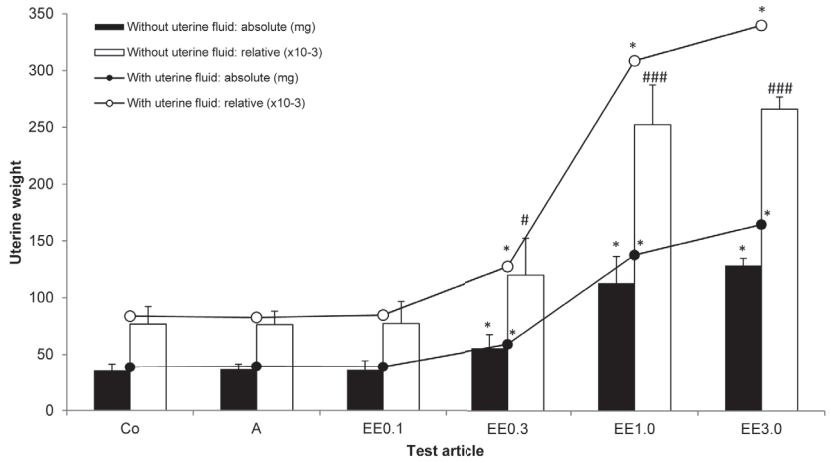

Fig. 1. Uterine weight in the baseline positive control study. Each point, column and vertical bar represents the mean ± S.D. of six rats. CO: corn oil, A: acotiamide-HH, EE: ethinyl estradiol. *p < 0.05 vs. CO (vehicle group) Steel two-sided test. #p < 0.05, ###p < 0.001 vs. Corn oil (vehicle group) Dunnett multiple comparison two-sided test. *** p < 0.001 vs. 0.5 w/v% MC Aspin-Welch test.

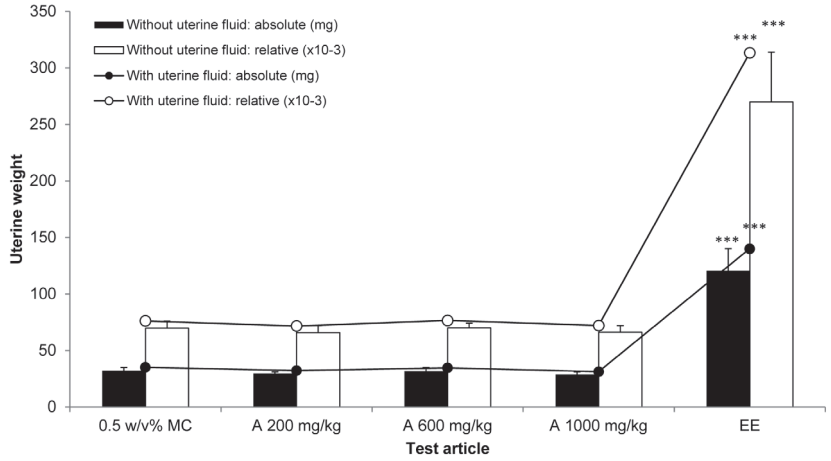

Fig. 2. Uterine weight in the main study. Each point, column and vertical bar represents the mean ± S.D. of six rats. Co: corn oil, A: acotiamide-HH, EE: ethinyl estradiol. *** p < 0.001 vs. 0.5 w/v% MC Aspin-Welch test.

markedly increased, with a dose-dependent increase at doses more than 0.3 µg/kg, and a tendency of the uterine weight gain to plateau at the 3.0 µg/kg dose (Fig. 1). Based on these results, the reliability of the uterotrophic bioassay using immature female rats was confirmed. EE in a dose of 1.0 µg/kg, which elicits clear uterine response, was selected for the positive control and for the main study.

In the main study, the body weight and food consumption showed no change in the EE and acotiamide-HH groups, except for inhibition of body weight gain in the 1000 mg/kg acotiamide-HH group. Although inhibition of body weight gain was observed in the 1000 mg/kg group 1 to 3 days after initial treatment, compared with the 0.5 w/v% MC administration group, the body weight had recovered on the final day of the study (Table 2).

Concerning uterine weight, there was no significant difference in the absolute and relative uterine weights, with or without intra-uterine fluid, in all the acotiamide-HH dose groups, including the 1000 mg/kg group, compared with the control group. On the other hand, a statistically significant increase in uterine weight, with or without intra-uterine fluid, was observed in the positive control group, with values of uterine weight 3 to 4 times those seen in the control group (Fig. 2). No abnormality in general clinical observations was observed in any of the animals and no animal showed vaginal opening in any of the groups. On histopathological examination with HE staining, no abnormal findings in the uterus or vagina were observed in any of the acotiamide-HH groups. In the positive control group, endometrial hypertrophy, i.e., high columnar luminal epithelial cell with enlarged cytoplasm, mitosis and apoptosis were observed in the uterus, while squamous epithelial keratinization was observed in the vaginal mucosa (Table 3, Fig. 4, and Fig. 5). No significant difference in the averages of PCNA-positivity of luminal epithelial cells was observed between any of the acotiamide-HH groups and the 0.5 w/v% MC group. The averages of PCNA-positivity of luminal epithelial cells (mean ± S.D.) were 6.4 ± 0.9, 4.5 ± 1.4, 4.9 ± 2.2 and 3.9 ± 1.2 in the 0.5 w/v% MC, 200 mg/kg, 600 mg/kg and 1000 mg/kg of acotiamide-HH groups, respectively (Fig. 3, Fig. 6). On the other hand, the average of PCNA-positivity of luminal epithelial cells at the luminal surface in the 1.0 µg/kg EE group (mean ± S.D.) was 46.6 ± 2.5, which was 7 times higher than that in the 0.5 w/v% MC group ($p < 0.001$) (Fig. 3, Fig. 6).

DISCUSSION

In the rat, the carcinogenicity study of acotiamide-HH showed unclear uterine tumorigenesis with a statistically significant increase in the incidence of endometrial adenocarcinoma observed on histopathological examination in the 600 mg/kg group, which was the middle dose of aco-

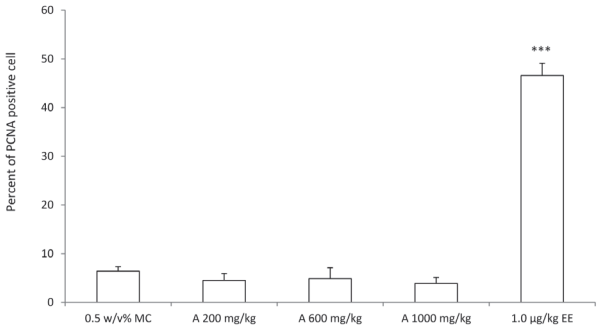

Fig. 3. Cell proliferation activity by PCNA staining. Each point, column and vertical bar represents the mean ± S.D. of six rats. ***p < 0.001vs 0.5 w/v% MC Student-t test.

Table 2. Body weight and food consumption in the main study.

Dosing Day	0.5 w/v% MC	Acotiamide-HH			Ethinyl estradiol
		200 mg/kg	600 mg/kg	1000 mg/kg	1.0 µg/kg
Body weight (g) Mean ± S.D. of six rats					
1	32.3 ± 0.5	31.0 ± 1.6	31.9 ± 1.9	30.5 ± 0.5*	31.5 ± 1.6
2	35.2 ± 1.3	34.2 ± 2.0	34.1 ± 2.5	31.8 ± 0.9##	35.1 ± 1.7
3	41.1 ± 1.1	40.0 ± 2.2	39.3 ± 2.3	37.2 ± 0.8##	40.5 ± 2.4
final	46.2 ± 1.3	45.1 ± 2.8	45.3 ± 2.5	43.2 ± 1.1	44.7 ± 2.6
Food consumption (g/day/animal)					
1-2	3.52	2.20	3.07	1.63	3.30
2-3	5.93	5.55	5.60	4.77	5.43
3-final	7.15	6.87	6.77	6.62	5.97

*$p < 0.05$ vs.0.5 w/v% MC, Steel's test two-sided.

##$p < 0.01$ vs. 0.5 w/v% MC, Dunnett multiple comparison test two-sided.

Table 3. Histopathological examination in uterus and vagina.

	0.5w/v% MC				Acotiamide-HH 200 mg/kg				Acotiamide-HH 600 mg/kg				Acotiamide-HH 1000 mg/kg				EE 1.0 μg/kg			
Uterus	n = 6				n = 6				n = 6				n = 6				n = 6			
Score	-	+	‡	‡‡	-	+	‡	‡‡	-	+	‡	‡‡	-	+	‡	‡‡	-	+	‡	‡‡
Endometrial hypertropy	6	0	0	0	6	0	0	0	6	0	0	0	6	0	0	0	0	4	2	0
Miosis	6	0	0	0	6	0	0	0	6	0	0	0	6	0	0	0	0	3	3	0
Apoptosis	6	0	0	0	6	0	0	0	6	0	0	0	6	0	0	0	0	6	0	0
Vagina	n = 6				n = 6				n = 6				n = 5*				n = 6			
Score	-	+	‡	‡‡	-	+	‡	‡‡	-	+	‡	‡‡	-	+	‡	‡‡	-	+	‡	‡‡
Squamous cell hyperkeratosis	6	0	0	0	6	0	0	0	6	0	0	0	5	0	0	0	6	0	0	0

Score: - ; normal, +; slight, ++; moderate, +++; severe
*: One vagina was lost in necropsy.

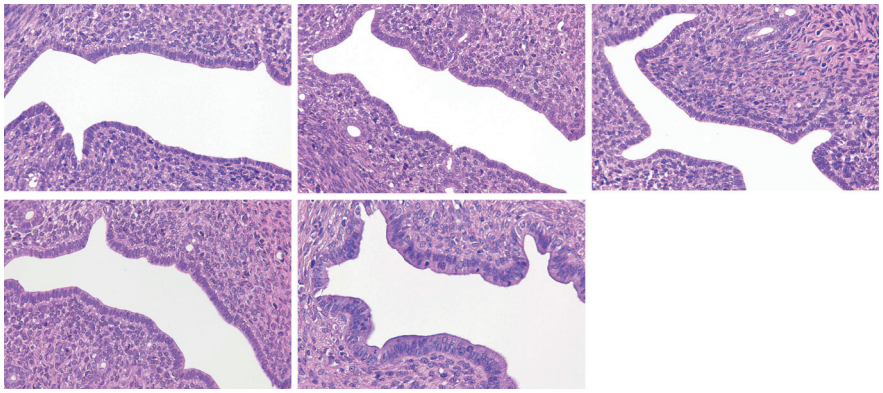

Fig. 4. Hematoxylin-eosin staining of uterus. (x 200). No histopathological findings were observed in 200 mg/kg group (upper left), 600 mg/kg group (upper middle) and 1000 mg/kg group (upper right) of actiamide-HH. And also no histopathological findings were observed in 0.5 w/v% MC group (lower left). Hypertrophy of endometrium was observed in 1.0 μg/kg EE group (lower right).

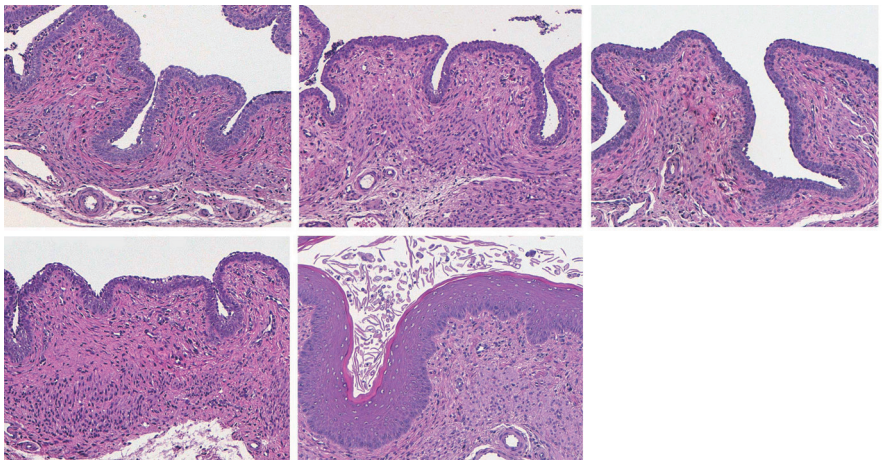

Fig. 5. Hematoxylin-eosin staining of vagina. (x 200). No histopathological findings were observed in 200 mg/kg group (upper left), 600 mg/kg group (upper middle), and 1000 mg/kg group (upper right) of acotiamide-HH. And also no histopathological findings were observed in 0.5 w/v% MC group (lower left). Hyperkeratosis of Vaginal epithelial cell was observed in 1.0 μg/kg EE group (lower right).

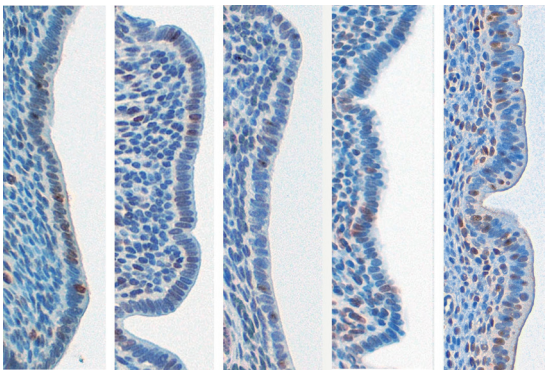

Fig. 6. Immunostaining using PCNA antibody of uterus. (x 200). No number of PCNA positive cell were increased in 200 mg/kg group (left), 600 mg/kg group (middle left) and 1000 mg/kg group (center) of acotiamide-HH groups compared with 0.5 w/v% MC group (middle right). Also, no histopathological abnormalities were found in these groups. Hypertrophy of endometrium with increasing the number of PCNA positive cell were observed in 1.0 μg/kg EE group (right).

tiamide-HH (Shiga, 2002). On this issue, we have previously reported that the increased incidence of endometrial adenocarcinoma was not a dose-dependent change, with no difference in the incidence of pre-neoplastic lesions, and no histopathological findings in the genital tract and endocrine systems compared with the vehicle control group. Further, there was no difference between the acotiamide-HH group and the vehicle control group in histopathological findings in non-neoplastic areas (Kuroda *et al.,* 2015). Although these data suggested that acotiamide-HH did not have carcinogenic activity, we conducted this additional examination to clarify the causal correlation between acotiamide-HH administration and endometrial adenocarcinoma.

In general, endometrial adenocarcinoma is one of the most common malignant tumors in women and it has been reported that sex hormone imbalances are associated with tumor causation (Takahashi *et al*., 1995). Indeed, it has been reported that the incidence of endometrial adenocarcinoma in women who had taken estrogen treatment is 4.5 times higher than women who had not taken it, and that estrogen plays an important role in the progression of endometrial adenocarcinoma (Takahashi *et al.*, 2001). Therefore, we performed a uterotrophic bioassay using immature rats to evaluate the carcinogenic potential of acotiamide-HH. This test method requires an intact hypothalamic-pituitary-gonadal (HPG) axis, and shows response to substances that interact with the HPG axis rather than just the estrogen receptor. This test method is a better and high priority method compared with ovariectomized rats in terms of animal protection and management because it does not require any surgery such as ovariectomy. Furthermore, it has been validated and has shown reliability and reproducibility between intra- and inter-test facilities (Kanno *et al.,* 2001*,* 2003a and 2003b). In the uterotrophic bioassay using immature rats, we evaluated general observation of the animal, vaginal opening, uterine weight, histopathological examination in HE staining, and proliferation activity of luminal epithelial cell by PCNA immunological staining of the uterus and vagina.

A statistically significant increase in uterine weight was observed, along with hypertrophy of luminal epithelial cells of the uterus, and squamous epithelial change with keratinization in the vagina on histopathological examination, in the 1.0 μg/kg EE group that was set as the positive control group. A statistically significant increase of PCNA-positive cell count in luminal epithelial cells was observed in the 1.0 μg/kg EE group. No increase in uterine weight, histopathological findings in uterus and vagina, or PCNA-positive cell count of endometrial membrane were observed in the acotiamide-HH groups up to the 1000 mg/kg dose, which is approximately 200 times higher than the recommended clinical dose (300 mg/person/day).

Based on the results stated above, acotiamide-HH does not have any direct proliferative activity on endometrium and no potential for induction of abnormal endocrine secretion from the HPG axis. We also demonstrated that acotiamide-HH does not have any effect on the sex hormone environment. However, in view of the statistically significant increase in the incidence of endometrial adenocarcinoma with the 600 mg/kg acotiamide-HH group in the carcinogenicity study that was an aberration from the background data of the facility that conducted the rat carcinogenicity study at that time, an alternative mechanism for development of endometrial adenocarcinoma without causal association of sex hormone imbalance should be considered. To exclude the possibility of hormone-independent carcinogenicity of acotiamide-HH, it is recommended to use an alternative test model in future research on acotiamide-HH.

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

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