Pubertal exposure to saisentong: Effects on thyroid and hepatic enzyme activity in juvenile female rats

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Abstract

Saisentong, as a thiadiazole fungicide, is widely used in China. The structure of saisentong shows a closer relationship to N, N-methylene-bis (2-amino-1, 3, 4-thiadiazole) (Bis-A-TDA), which is a teratogen. A few studies have shown that some of the thiadiazole fungicides act as endocrine disruptors via disturbance in thyroid hormone homeostasis. Little is known about the effect of pubertal exposure to saisentong on the development in pubertal female rats. Based on the protocol of the 20-Day Pubertal Female Assay, we attempt to estimate the possible effect of exposure to saisentong on thyroid hormone and hepatic enzyme activity in female rats. Postnatal days (PND) 22 old SD rats were administered with saisentong daily by oral gavage at doses of 0, 5, 10 or 15 mg/kg/day for 20 days. After treatment, the rats were sacrificed for blood collection; the reproductive organs, liver, pituitary, adrenal and thyroid gland were harvested. The results indicated that saisentong administration increased thyroid weight and thyroid stimulating hormone (TSH) concentrations, and induced hepatic uridine diphosphate glucuronyl transferase (UDPGT) activities in the highest-dose group, although not statistically significant.

The high dose caused a decrease in weight at vaginal opening (VO), but the age at VO was unaffected by saisentong in all treatment groups. No histological changes were observed in uterus and ovaries. These data and changes demonstrate that saisentong is a potential thyroid disrupter in female rat following exposure during development, but does not affect the development of pubertal female rats.

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Keywords: Saisentong; Pubertal development; Thyroid hormone; Hepatic enzyme activity

Introduction

Finding novel green compounds with high activity and low toxicity is a development status and trend of future pesticide. Due to thiadiazole compound’s excellent bioactivities, especially to bacterial blight, which is one of the most destructive diseases of rice worldwide (Guo et al., 2005), and various chemical structures, it is becoming a new research hotspot for more and more scientists. Thiadiazole contains the five-membered diunsaturated ring structure composed of two nitrogen atoms and one sulfur atom (Fig. 1). With the introduction of thiadiazole, many compounds display broad-spectrum bioactivities. Thiadiazole and its
derivatives are used for biological activities such as antiviral, antibacterial, antifungal and antituberculous activities. Saisentong [N, N-methylene-bis (2-amino-5-sulfhydryl-1, 3, 4-thiadiazole) copper], which belongs to the sort of thiadiazole fungicide, is a Chinese-created systemic fungicide that has been used to control rice diseases Xanthomonas oryzae pv. Oryzae and Xanthomonas oryzae pvoryzicola, (Xing and He, 2007). Since saisentong has a broad spectrum of activity and low toxicity, it was used widely in southern China.

Indeed, the structure of saisentong shows a closer relationship to N, N-methylene-bis (2-amino-1, 3, 4-thiadiazole) (Bis-A-TDA), which is a teratogen (Xu and Gao, 1991; Gu and Qian, 1991). Meanwhile, in our previous study, we found bismerthiazol, another thiadiazole fungicide, to be likely a thyroid disrupter in female rat following exposure during development (Zhang et al., 2008). Nevertheless, little is known about saisentong and its possible effect on thyroid hormone disruption, hepatic enzyme activity and development in pubertal female rats.

Pubertal female rat assay, as one of the screening batteries for identifying thyroid toxicants, is used to quantify the effects of chemicals on pubertal development and thyroid function in the intact peripubertal female rat. Two essential endpoints that are routinely used for identifying compounds that alter thyroid function are thyroid hormone measurements and histopathology of the thyroid gland (O’Connor et al., 1999). In addition, since thyroid hormone concentrations can be considered as an indicator of thyroid toxicants and thyroid gland histopathology has been judged by Duke University Workshop to be the most sensitive parameter for the detection of compounds that adversely affect thyroid function, these two endpoints may be the most useful criteria for identifying thyroid toxicants. Furthermore, the pubertal female assay also examines the endpoints associated with the development of female sex organs and secondary sexual characteristics (Kim et al., 2002), and the vaginal opening (VO) can be used as an indicator of pubertal development.

In the present study, we used the pubertal female rat assay to detect the effect of saisentong on pubertal development, thyroid hormone disruption and hepatic enzyme activity in the intact juvenile female rat. We have examined the effect of saisentong on several thyroid endpoints (serum hormone concentrations, thyroid gland weight and thyroid gland histopathology), development endpoints (the age and weight of VO in the juvenile female rat and reproductive organs) and hepatic enzyme activity about 4-nitrophenol uridinephosphate-glucuronosyltransferase (UDPGT), which could help us understand whether pubertal exposure to saisentong acts as endocrine disrupters in female rats.

**Materials and methods**

**Animals**

Sprague–Dawley rats were purchased from SINO-BRITISH SIPPR/BK Laboratory Animal Ltd. in Shanghai under specific pathogen-free (SPF) condition, bred in the animal room of the National Shenyang Center for Drug Safety and Evaluation Research (GLP). Juvenile female rats were derived from individually housed pregnant females that were bred in-house. All dams were pregnant for the first time and timed to deliver on the same day. Dams delivered their pups naturally. Any litters with fewer than 8 total litter (including both males and females) and any litters not delivered by gestation day (GD) 23 were excluded from the study. To maximize uniformity in growth rates, the litters were standardized to 8 pups per litter in postnatal days (PND) 4. Body weights are monitored weekly and any unthrifty litters or runty pups were excluded from the study. Before being placed on study, dams with litters of 21-day-old female rats were housed together in clear polycarbonate cages. All animals were maintained in a well-ventilated room at a temperature of 22±2°C and a relative humidity of 55±10%, with a 12 h light/12 h dark cycle. Food and tap water were provided ad libitum. The sterilized feed was purchased from Qianming Animal Feed Factory, at Yuhong district in Shenyang. All animals, including the control group used in this experiment, were handled in an accredited Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) facility. On PND 22, the animals were allocated to different treatment groups in accordance with their body weight (b.w.).
Experimental protocol

The study design consisted of 10 animals per group. In the present study, the highest dose level was chosen as a dose to reduce the terminal body weight not more than 10% of the mean for the controls, and no clinical signs of toxicity associated with the dose level are observed throughout the study in the preliminary pilot study. To evaluate the dose-dependent relationship, common division by 2 from the highest level was selected. Saisentong (CB No. CB61145982; purity 95%) was obtained from Zhejiang Dongfeng Chemicals Co., Ltd. (Wen Zhou, Zhejiang Province, China). It was suspended in the soybean oil; the dosed group was administered daily from PND 22 for 20 days at dosages of 5, 10 and 15 mg/kg by oral gavages and the control group was administered with vehicle alone. The test compound was prepared daily before treatment. The dose volume was 4 ml/kg body weight and the concentration and homogeneity of saisentong in the suspension for each dose were confirmed within the limits before administration. The animals were administered the dose and examined daily between 9:00 and 10:00. Their body weights were measured daily prior to treatment, and recorded. Clinical observations are also recorded daily. Twenty-four hours after the last treatment, rats were anesthetized with CO2. Blood samples were collected from the abdominal vein and their volumes recorded according to the schedule for examination of serum hormone levels. The livers were homogenized in ice-cold 0.05 M Tris–KCl buffer (pH 7.4) and the crude homogenate was centrifuged at 10,000 g for 20 min at 4 °C and the supernatant (S9) was preserved at −70 °C until hepatic enzyme activity analysis.

Vaginal opening

Each animal was examined daily for VO beginning on PND 22. On the day the VO was first detected, the age and body weights were recorded.

Hepatic enzyme activity assay

UDPGT activity in the liver S9 suspension was measured using 4-nitrophenol as substrate. UDPGT activities toward 4-nitrophenol were determined by a modification of the method of Bock et al. (1983) as previously described (O’Connor et al., 1999). Each sample was measured in duplicate. Protein content was determined by the Bradford method (1976) using bovine serum albumin as a standard.

Hormone measurement

Serum total thyroxin (T4) and thyroid stimulating hormone (TSH) were determined by enzyme-linked immunosorbent assay kits (Adlitteram Diagnostic Laboratories, Inc., USA), which were adapted for use with rat, according to the manufacturer's instructions. Briefly, samples, including standards of known T4/TSH concentrations and unknowns, are pipetted into wells. The T4/TSH antigen and a biotinylated monoclonal antibody (goat anti-rat T4/TSH) are simultaneously incubated. After washing, the enzyme (streptavidin peroxidase) is added. After incubation and washing to remove the entire unbound enzyme, a substrate solution that is acting on the bound enzyme is added to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of T4/TSH present in the samples. For measurement of hormone concentrations, serum prepared from the collected blood was stored at −70 °C until its assay and measured in duplicate with the same assay. All data are expressed as nmol/L serum T4 and mU/L serum TSH.

Histopathological examination

At the time of necropsy, the thyroid gland, liver, kidney, pituitary, adrenals, uterus and ovaries were dissected and weighed from rats immediately, then thyroid gland, kidney, uterus and ovaries were fixed in 10% buffered formalin for at least 5 days. Each was processed in an automaticissue processor and embedded in paraffin. Thin sections were cut at 4–5 μm thickness, and stained with hematoxylin and eosin for pathological evaluation under a microscope.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) (n = 10 animals). All statistical analyses were performed on SPSS 15.0 for windows. Data for mean initial or necropsy body weights, organ weights and hormone levels were analyzed statistically for homogeneity of variance using Levene’s test. If homogenous, the data were analyzed by a one-way analysis of variance (ANOVA). When samples were proved to be heterogeneous, nonparametric analysis of variance was applied. When a significant treatment effect was present, Dunnett’s test was used to compare treatment groups. Data for the incidences of histopathological findings were analyzed with Fisher’s exact probability test. The level of statistical significance was set a priori at α = 0.05.
Results

General observation and body weights

During the study period, there were no clinical signs of toxicity in any treatment group with the exception of dose-related decreases in body weight occurring at the time of VO and necropsy in the high-dose group, and the compound had no significant effect on mean body weight gains (Fig. 2).

Organ weights

As summarized in Table 1, a significant increase in thyroid weight and pituitary were found at medium- and high-treated group of saisentong, compared to those of the control group. No significant changes in the weights of the other organs (liver, kidney, adrenals, uterus and ovaries) were observed for any dose group.

Vaginal opening

The high dose caused a significant decrease in weight at the age of VO between the control and saisentong-treated groups, but the age at VO was unaffected by saisentong in all treatment groups (Table 2).

Effects on hepatic enzyme activity

There was a significant change in S9 4-nitrophenol UDPGT activity in the treatment groups. Interestingly, we note a statistically significant reduction in S9 4-nitrophenol UDPGT activity in the group treated with the low dose and middle dose of saisentong, compared to controls (Fig. 3).

Hormone measurement

No significant changes were observed in total T4 and TSH in any of the treatment groups (Table 2).

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Table 1. Absolute organ weights in SD rats treated with saisentong in the Female Pubertal Assay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage (mg/kg)</th>
<th>Initial BW</th>
<th>Final BW</th>
<th>Ovarian</th>
<th>Uterine</th>
<th>Adrenals</th>
<th>Thyroid</th>
<th>Liver</th>
<th>Kidney</th>
<th>Pituitary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>46.60 ± 2.58</td>
<td>149.40 ± 9.50</td>
<td>62.80 ± 16.21</td>
<td>0.33 ± 0.15</td>
<td>38.90 ± 9.22</td>
<td>18.60 ± 5.28</td>
<td>5.39 ± 0.66</td>
<td>1.43 ± 0.14</td>
<td>6.50 ± 2.55</td>
</tr>
<tr>
<td>Saisentong</td>
<td>5</td>
<td>47.56 ± 3.91</td>
<td>145.78 ± 13.54</td>
<td>59.00 ± 21.17</td>
<td>0.33 ± 0.14</td>
<td>34.33 ± 8.63</td>
<td>23.11 ± 7.94</td>
<td>5.59 ± 0.96</td>
<td>1.40 ± 0.19</td>
<td>10.11 ± 3.55</td>
</tr>
<tr>
<td>10</td>
<td>45.88 ± 2.42</td>
<td>143.00 ± 7.78</td>
<td>54.75 ± 18.45</td>
<td>0.38 ± 0.11</td>
<td>40.13 ± 5.96</td>
<td>35.50 ± 19.94</td>
<td>5.48 ± 0.92</td>
<td>1.39 ± 0.13</td>
<td>16.25 ± 8.60*</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>46.63 ± 2.62</td>
<td>138.38 ± 10.88*</td>
<td>54.63 ± 16.28</td>
<td>0.36 ± 0.12</td>
<td>37.13 ± 7.43</td>
<td>33.25 ± 15.76*</td>
<td>4.84 ± 0.74</td>
<td>1.28 ± 0.17</td>
<td>12.00 ± 6.76*</td>
<td></td>
</tr>
</tbody>
</table>

Note: Values are mean ± SD (n = 10 animals per treatment group). BW, body weight; Initial BW, body weight on the first day of treatment (22 days of age). Necropsy BW, body weight at necropsy (42 days of age). Body weights, liver, kidney and uterus weights given in g; pituitary, adrenal, thyroid and ovary weights given in mg.

*Denotes value significantly different from controls at *p* < 0.05.

Table 2. Effects of saisentong on VO and serum hormone concentration in the Female Pubertal Assay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage (mg/kg)</th>
<th>VO (day)</th>
<th>BW at VO day</th>
<th>T4 (nmol/L)</th>
<th>TSH (mU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>32.76 ± 0.59</td>
<td>119.21 ± 3.53</td>
<td>109.64 ± 6.47</td>
<td>6.09 ± 0.38</td>
</tr>
<tr>
<td>Saisentong</td>
<td>5</td>
<td>32.00 ± 1.05</td>
<td>114.00 ± 5.31</td>
<td>111.68 ± 7.83</td>
<td>5.95 ± 0.37</td>
</tr>
<tr>
<td>10</td>
<td>33.75 ± 1.11</td>
<td>119.00 ± 2.87</td>
<td>114.72 ± 6.80</td>
<td>5.98 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>33.57 ± 0.86</td>
<td>108.71 ± 2.73*</td>
<td>110.34 ± 10.11</td>
<td>6.22 ± 0.45</td>
<td></td>
</tr>
</tbody>
</table>

Note: Values are mean ± SD (n = 10 animals per treatment group). BW, body weight; VO, vaginal opening. Body weights at VO day given in g.

*Denotes value significantly different from controls at *p* < 0.05.
Histopathological findings

Histology of the thyroid glands showed no notable change in the all treated groups, compared to the controls (Fig. 4).

Discussion

There are few studies on the effect of pubertal exposure of saisentong on thyroid function. Since the structure of saisentong shows a close relationship to Bis-A-TDA, which is toxic to thyroid (Zhu et al., 1992; Liu et al., 1992), we have a hypothesis that saisentong can lead to thyroid dysfunction in pubertal female rats. Thus, we have examined several thyroid endpoints using pubertal female rat assay to test the hypothesis.

Thyroid weight provides a measure of its stimulation by TSH over time; thus, if thyroid hormone levels are altered slightly for some duration, thyroid weight may reflect this change before technical assays can detect changes in serum hormone levels (Shin et al., 2006). In our study, absolute thyroid weight increased significantly in all treatment groups compared to the control group. From the result, we could find that the saisentong affects thyroid weight in pubertal female rats. These proved that thyroid weights are an appropriate endpoint for detecting compound-related effects on thyroid weight. Furthermore, we found that the saisentong also affect pituitary weight. We have known that thyroid hormones T₄ are secreted from the thyroid gland under the control of thyroid-stimulating hormone (TSH) from the pituitary, which, in turn, is controlled in part by thyrotropin releasing hormone (TRH) from the hypothalamus. From the result, it is indicated that the saisentong may affect the hypothalamic-pituitary-thyroid axis (HPT axis); the mechanisms for disruption of the HPT axis need to be studied further.

Many of the endocrine-disrupting chemicals appear to have dose-responsive U-shaped and/or inverted U-shaped curves (Putz et al., 2001; Almstrup et al., 2002; Ahn et al., 2005). Interestingly, we did observe an inverted U-shaped trend of serum total T₄ and a U-shaped trend for both 4-nitrophenol UDPGT activity and TSH. Though this U-shaped or inverted U-shaped observation is not statistically significant for T₄ or TSH, this finding may be biologically meaningful.

In rodents, UDPGT is an important hepatic enzyme that metabolizes T₄, helping characterize possible biochemical mechanisms for thyroid hormone. From Table 2, a reduction in T₄ was observed in doses of 15 mg/kg/day; at the same time, in Fig. 3, 15 mg/kg/day treatment groups showed significant induction of UDPGT activity, compared to the doses 10 mg/kg/day. Whether this phenomenon hints at a relationship between serum T₄ depletion and induction of the UDPGT activity needs to be studied further.

In conclusion, the result of this study finds pubertal exposure to saisentong is able to increase thyroid weight and thyroid serum TSH concentrations, as well as induce hepatic UDPGT activity in female rat in the highest-dose group, though it is not statistically significant. Furthermore, the saisentong may affect the HPT axis since it increases both thyroid and pituitary
weight significantly. Although there were only three doses in the dose-response relationship, the preliminary results of this study provide some important information on one of the most commonly used and environmentally most relevant thiadiazole fungicide for human and ecological risk assessment. Further efforts are required for using environmentally relevant doses of saisentong, and exploring its metabolite in vivo will be necessary.

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