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OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals

Kanji Yamasaki [a,*], Ryo Ohta [b], Hirokazu Okuda [c]

[a] *Chemicals Evaluation and Research Institute, 3-822 Ishii, Hita, Oita 877-0061, Japan*
[b] *Food Drug Safety Center, Kanagawa, Japan*
[c] *Japan Bioassay Research Center, Kanagawa, Japan*

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Abstract

The Organization for Economic Co-operation and Development (OECD) has initiated the development of new guidelines for the screening and testing of potential endocrine disrupters. The Hershberger assay is one of the assays selected for validation based on the need for in vivo screening to detect androgen agonists or antagonists by measuring the response of five sex accessory organs and tissues of castrated juvenile male rats: the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis. The Phase 1 feasibility demonstration stage of the Hershberger validation program has been successfully completed with a single androgen agonist and a single antagonist as reference substances. The Phase 2 validation study was performed, employing a range of additional androgen agonists and antagonists. Recently, the Phase 3 validation study was conducted and performed in several International laboratories. Three Japanese laboratories have contributed to the blind study using coded materials of Phase 3 validation. Four coded test substances in the agonistic version and seven substances in the antagonistic version were orally administered by gavage for 10 consecutive days, respectively. In the antagonist version of the assay, 0.2 mg/kg/day of testosterone propionate (TP) was coadministered by subcutaneous injection. All five accessory sex reproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window in both versions. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Keywords: Blind study; Endocrine; Hershberger assay; OECD validation

1. Introduction

Certain reproductive and developmental toxicants may have the potential to interfere with normal sexual differentiation and development in animals and humans by modulating or interfering with the endocrine system (McLachlan, 1993; McLachlan and Korach, 1995). The Organization for Economic Co-operation and Development (OECD) has initiated an activity to revise existing guidelines and develop new screening and testing guidelines to aid in the identification and assessment of such toxicants (OECD, 1998, 2000, 2002).

One proposed assay, referred to as the Hershberger assay, uses the androgen sensitivity of several accessory sex organs and tissues of the male reproductive tract. The assay was originally developed in the 1930s by Korenchevsky and coworkers, and a number of accessory sex organs and tissues were shown to be use-

* Corresponding author. Tel.: +81 973 24 7211; fax: +81 973 23 9800.
E-mail address: yamasaki-kanji@ceri.jp (K. Yamasaki).

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ful by these and other investigators including the ventral prostate (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the seminal vesicles and coagulating glands (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the preputial glands (Bülbring and Burn, 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), Cowper's glands (Wainman and Shipounoff, 1941), and the glans penis (Bülbring and Burn, 1935; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b). In the 1940s, it was discovered that the levator ani and bulbocavernosus muscles also responded to androgens, but in a differential way from the other tissues (Wainman and Shipounoff, 1941; Eisenberg et al., 1949; Eisenberg and Gordan, 1950). The basis for this differential sensitivity is the presence of 5α-reductase in most accessory tissues of the male reproductive tract, but its absence in the muscle complex (Di Salle et al., 1994). The capabilities of the assay were demonstrated in 1953 by Hershberger et al. when they analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones (Hershberger et al., 1953).

In the 1970s and 1980s, with the discovery of the androgen receptor and the first compounds such as cyprotone acetate that were antagonists of the receptor, the assay was modified to address antagonistic activity. Briefly, a set dose of a reference agonist was coadministered to several groups of animals to whom a set of doses of the purported antagonist was also administered. This modified system was successfully used by several investigators for assaying androgen antagonists (Peets et al., 1973; Raynaud et al., 1980, 1984; Wakeling et al., 1981).

Therefore, based upon the recommendation of scientific workshops, both the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) (USEPA, 1998) and the OECD Endocrine Disrupter Testing and Assessment Group (EDTA) of the OECD (OECD, 2000) have proposed this assay as a Tier-1 screen to identify possible reproductive and developmental toxicants acting through androgen agonist and antagonist mechanisms.

The OECD Phase 1 validation program for the Hershberger assay was completed in 2001. In this phase, a standardized protocol using the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis was successfully tested against a reference androgen compound, testosterone propionate (TP), and a reference antagonist, flutamide (OECD, 2002). The OECD proposed a Phase 2 validation program using additional androgen agonistic and antagonists as the next step to validate the assay, but the final results of Phase 2 studies were not opened by the OECD.

Recently, the OECD conducted a Phase 3 validation program as a final blind study using coded agonistic and antagonistic chemicals (OECD, 2003). In Phase 3, the coded test substances were to be used to investigate the reliability of the assay, including a demonstration of the protocol's transferability among laboratories and the reproducibility of the protocol's results. Three Japanese laboratories participated in the Phase 3 validation study using four coded agonistic test substances and seven antagonistic substances. The participation of the laboratories in the OECD Phase 3 validation study was performed as part of a national validation program in Japan.

2. Materials and methods

2.1. Laboratories

The three participating Japanese laboratories were: the Chemicals Evaluation and Research Institute (CERI); the Food Drug Safety Center; and the Japan Bioassay Research Center. Each laboratory performed the study in compliance with the principles of Good Laboratory Practice guidelines.

2.2. Test substance

All coded test substances except for TP were sent to each laboratory from a centralized chemical repository at TNO, Zeist, the Netherlands. TP and corn oil as vehicles were prepared in each laboratory. The coded substances A, B, L and E were used in the agonistic version, and F, G, I, C, K, D and H were used in the antagonistic version. We did not receive any information regarding the coded substances before all tests were started.

2.3. Animals

Laboratory details regarding rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage are summarized in Table 1. Two laboratories used Crj:CD (SD) rats castrated at 6-weeks old, and the test substances were administered 1 week after castration. One laboratory used Brl Han: WIST Jcl (GALAS) rats castrated at 6-weeks old, and the test substances were administered 2 weeks after castration. In all the laboratories, the rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups after they had recovered from their operation. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water and

Table 1
Laboratory detail for rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage

Lab	Rat strain	Age of castration	Age at start of dosing	Day of autopsy	Diet	Number of rats per cage
1	Brl Han: WIST Jcl (GALAS)[a]	6-weeks old	8-weeks old	10-weeks old	MF[b]	3
2	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CE-2[d]	1
3	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CRF-1[d]	1

[a] Clear Japan Inc., Tokyo, Japan.
[b] Oriental Yeast Co., Ltd., Tokyo, Japan.
[c] Charles River Japan, Kanagawa, Japan.
[d] Clear Japan Inc.

a commercial diet ad libitum. The animals were kept under SPF conditions. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by The Japanese Association for Laboratory Animal Science.

2.4. Administration

We performed each test according to the protocol proposed by the OECD (OECD, 2000, 2002, 2003). Each test substance was orally administered via a stomach tube for 10 consecutive days at approximately the same time each day. A vehicle control group receiving only corn oil was used in both versions. For the antagonistic version, 0.2 mg/kg/day of TP was coadministered each day by subcutaneous injection in the dorsal region after the oral administration of each chemical. The volume of the corn oil solution containing the TP was 0.5 ml/kg. In the agonistic version, a positive control group of animals received TP injections alone. The group size in all cases was six rats. The volume of the corn oil solutions containing each of the test chemicals was 5 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 h after receiving their final dosage. The five mandatory tissues, the ventral prostate and fluid, seminal vesicle and fluid, LABC, glans penis, and Cowper's gland, were carefully dissected free of adhering fat and weighed to the nearest 0.1 mg. We also weighed the liver in three laboratories, and paired kidney and adrenal weights were measured in one laboratory.

2.5. Statistical analysis

We received the information from the coordinator of this Phase 3 validation after all tests were finished that the participating laboratories received pairs of the test chemicals (i.e. L and E, F and G, I and C, or K and D), so we analyzed the data using the following analytical methods between the vehicle control group and the same chemical groups in the agonistic version, and the TP group and the same chemical groups in the antagonistic version. In addition, coded A and F were nonylphenol, B and G were dinitrophenol, E and L were trenbolone, C and I were *p,p'*-DDE, and D and K were linurone. Body weight and organ weight data were analyzed by Bartlett's test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed with Dunnett's test. If the variance was not homogeneous, the Kruskal–Wallis test was used. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed by the non-parametric Dunnett's test. On the other hand, differences in body weight and organ weight between the control group and the TP group, coded A or B in the agonistic version and between TP group and the group using coded H, G or F in the antagonistic version were assessed for statistical significance by the two-tailed Student's *t*-test. For graphical presentation, the sex accessory organ data were normalized to visually compare the shapes of the responses produced by each laboratory. For this normalization, the control value was set to 100% in the agonistic study, and 100% in the TP without coded compound in the antagonistic study. Analyses of variance were performed on the data from each laboratory and for the pooled laboratory data; these normalized values were not analyzed statistically.

3. Results

3.1. Agonistic version

3.1.1. Body weights, clinical observations, and optional organ weights

The body weights and the optional organ weight changes are shown in Table 2. Terminal body weights in rats given L were significantly lower than in rats given vehicle alone in Labs 2 and 3, and tendency towards lowering of the terminal body weights was observed in Lab 1. No abnormal clinical signs were observed in any of the rats that were treated with each substance. The paired kidney weights in rats given substance A and TP were significantly higher than in rats given only the vehicle in Lab 3, and the liver weights in rats given A and TP were also higher than in rats given the vehicle only in Lab 2.

Table 2
Optional organ weights including the liver, adrenal, and kidney in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Starting body wt. (g)	214.8 ± 10.6	214.4 ± 11.0	213.1 ± 10.1	219.1 ± 10.5	215.0 ± 8.0	214.9 ± 9.3
	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Liver (g)	10.0 ± 1.1	10.2 ± 1.3	9.6 ± 0.5	11.1 ± 0.7	9.7 ± 0.7	10.1 ± 0.8
2	Starting body wt. (g)	231.0 ± 5.3	227.5 ± 5.0	229.6 ± 6.9	229.1 ± 3.9	226.8 ± 7.9	230.9 ± 6.9
	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Liver (g)	11.1 ± 0.6	12.1 ± 0.8*	10.7 ± 1.1	11.5 ± 0.6	11.5 ± 0.9	12.8 ± 1.2*
3	Starting body wt. (g)	257.1 ± 8.9	256.7 ± 8.9	256.0 ± 8.3	257.4 ± 7.9	256.8 ± 10.1	255.0 ± 12.2
	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Liver (g)	12.9 ± 0.9	14.0 ± 1.9	13.0 ± 1.3	12.2 ± 1.6	13.0 ± 1.3	13.6 ± 2.0
	Adrenals (mg)	58.8 ± 9.3	57.0 ± 9.2	61.5 ± 10.6	49.9 ± 7.8	50.2 ± 5.1	51.0 ± 11.7
	Kidneys (mg)	2110 ± 72	2344 ± 138*	2290 ± 162	2229 ± 226	2189 ± 192	2435 ± 244*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

3.1.2. Accessory sex organ weights

Five accessory sex organ and total five organ weight changes are shown in Table 3, and normalized organ weight changes are shown in Fig. 1. The accessory sex organ weights of rats given TP only in all laboratories were higher than these of rats given the vehicle alone, confirming the reliability of this study. Almost all accessory sex organ weights and total five organs in rats given L were higher than in rats given the vehicle in all laboratories. The LABC weights in rats given E was significantly higher than in rats given the vehicle in Lab 2, but the normalized change in this organ was

Table 3
Mean body weights and mean organ weights in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Ventral prostate (mg)	16.8 ± 1.0	17.0 ± 3.2	15.6 ± 3.4	34.1 ± 8.0	14.9 ± 1.2	93.6 ± 11.0*
	Seminal vesicles (mg)	27.9 ± 5.6	26.5 ± 1.9	25.7 ± 4.9	61.2 ± 9.6*	29.0 ± 3.5	190.4 ± 19.1*
	LABC (mg)	136.8 ± 22.2	128.2 ± 19.2	128.3 ± 11.0	298.5 ± 28.1*	141.5 ± 15.2	312.3 ± 26.9*
	Glans penis (mg)	29.5 ± 5.6	29.9 ± 2.8	28.3 ± 7.2	49.8 ± 6.5*	32.6 ± 5.1	64.4 ± 6.0*
	Cowper's glands (mg)	4.1 ± 1.3	4.4 ± 1.4	3.9 ± 1.5	10.3 ± 2.5*	4.5 ± 1.1	20.4 ± 3.5*
	Total of five organs (mg)	215.0 ± 21.3	206.0 ± 23.5	201.9 ± 21.3	453.8 ± 48.3*	222.4 ± 19.0	681.0 ± 42.3*
2	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Ventral prostate (mg)	16.0 ± 5.2	19.8 ± 5.1	15.8 ± 6.5	33.4 ± 6.3*	18.4 ± 3.2	121.8 ± 25.6*
	Seminal vesicles (mg)	42.0 ± 14.5	40.9 ± 11.2	38.8 ± 12.2	178.7 ± 60.4*	41.6 ± 11.5	420.4 ± 32.1*
	LABC (mg)	163.6 ± 38.3	178.6 ± 23.5	189.9 ± 30.4	426.8 ± 46.2*	216.3 ± 17.3*	527.5 ± 23.5*
	Glans penis (mg)	44.1 ± 4.3	42.9 ± 2.2	41.8 ± 2.3	58.5 ± 3.7*	45.5 ± 2.5	73.9 ± 3.9*
	Cowper's glands (mg)	5.8 ± 1.3	5.6 ± 1.1	4.4 ± 1.7	10.1 ± 2.6*	5.8 ± 1.8	34.4 ± 8.1*
	Total of five organs (mg)	271.6 ± 51.3	287.8 ± 29.7	290.7 ± 35.3	707.6 ± 104.7*	327.7 ± 14.6	1177.9 ± 35.1*
3	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Ventral prostate (mg)	22.0 ± 3.1	20.6 ± 1.4	24.0 ± 1.7	43.7 ± 11.5*	26.2 ± 3.8	186.5 ± 48.4*
	Seminal vesicles (mg)	61.2 ± 5.9	58.1 ± 7.0	58.4 ± 8.2	165.5 ± 37.1*	61.2 ± 10.9	431.3 ± 55.1*
	LABC (mg)	191.3 ± 16.0	178.6 ± 25.2	190.7 ± 6.6	452.3 ± 34.5*	221.1 ± 35.8	543.5 ± 83.5*
	Glans penis (mg)	53.0 ± 8.0	54.6 ± 5.4	54.8 ± 5.6	72.9 ± 3.2*	52.0 ± 2.6	95.1 ± 8.0*
	Cowper's glands (mg)	8.5 ± 2.2	7.4 ± 1.8	8.0 ± 1.3	18.2 ± 5.2*	8.8 ± 2.4	37.1 ± 6.6*
	Total of five organs (mg)	336.0 ± 19.9	319.3 ± 29.6	336.0 ± 13.7	752.5 ± 66.4*	369.4 ± 45.8	1293.6 ± 112.7*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

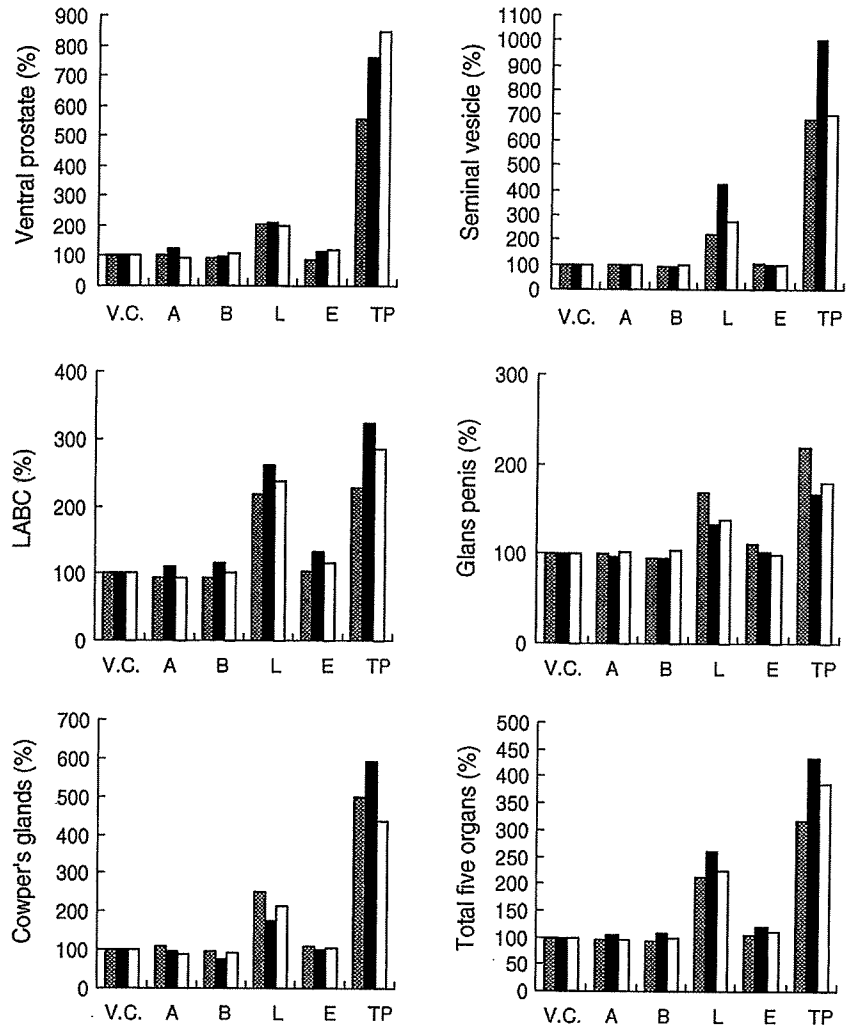

Fig. 1. Organ weights of accessory sex organs in agonistic version. Values from each laboratory were normalized to the control value set equal to 100%. LABC: levator ani and bulbocavernosus muscle; V.C.: vehicle control; A, B, L, and E: coded chemicals; TP: testosterone propionate. $n=6$ rats/group/Lab (▩, Lab 1; ■, Lab 2; □, Lab 3).

not apparent. Normalized weight changes of the glans penis in rats given coded L showed the weakest response among five organs (Fig. 1).

3.2. Antagonistic version

3.2.1. Body weights, clinical general observations, and optional organ weights

The body weight changes and the optional organ weight changes are shown in Table 4. Two rats given I plus TP died with toxic signs such as decreasing body weight, soft feces, reddish urine, and weakness at 7–10 days after the administration in Labs 2 and 3, respectively. The terminal body weights in rats given I plus TP or K plus TP were significantly lower than in rats given TP only in two laboratories. The paired adrenals in rats given K plus TP were significantly higher than in rats given TP in Lab 3. The liver weights in rats given I plus TP were higher than in rats given TP in all laboratories, and increased liver weights were also observed in rats given C in Lab 1.

3.2.2. Accessory sex organ weights

Five accessory sex organ and total organ weight changes are shown in Table 5, and normalized organ weight changes are shown in Fig. 2. All accessory sex organ weights of rats given H, which is a positive compound, flutamide, plus TP were lower than those of rats given TP, confirming the reliability of this version. Almost all the accessory sex organ weights in rats given I plus TP and K plus TP were significantly lower than in rats given TP in all laboratories. Some accessory sex organ weights in rats given C plus TP and D plus TP were also lower than in the rats given TP. Although the

Table 4
Optional organ weights including the liver, adrenal, and kidney in antagonistic version

Lab	Body weights/organ weights	Substances								
		V.C.	TP	F	G	I	C	K	D	H
1	Starting body wt. (g)	216.2 ± 10.9	216.1 ± 8.8	214.9 ± 10.6	216.6 ± 10.8	218.1 ± 12.5	216.5 ± 8.1	216.0 ± 8.2	219.4 ± 11.3	215.2 ± 9.7
	Terminal body wt. (g)	260.0 ± 17.4	267.4 ± 16.5	263.3 ± 17.8	272.2 ± 9.5	248.7 ± 17.1	266.3 ± 11.7	245.7 ± 11.1*	263.6 ± 11.8	261.5 ± 12.5
	Liver (g)	10.0 ± 1.0	10.4 ± 0.9	11.3 ± 1.3	10.9 ± 0.9	17.3 ± 1.2*	12.5 ± 0.8*	9.6 ± 0.8	10.4 ± 0.5	10.0 ± 0.8
2	Starting body wt. (g)	256.8 ± 11.0	258.9 ± 9.2	259.3 ± 11.6	257.6 ± 10.9	256.8 ± 9.6	258.9 ± 10.8	258.0 ± 10.6	258.9 ± 10.7	255.4 ± 12.7
	Terminal body wt. (g)	313.2 ± 14.7*	331.9 ± 14.3	326.1 ± 16.4	332.2 ± 20.7	270.8 ± 44.6*	329.8 ± 18.7	309.5 ± 13.9*	335.3 ± 15.6	327.4 ± 23.1
	Liver (g)	13.8 ± 0.7	15.6 ± 2.1	15.8 ± 2.3	15.1 ± 0.9	22.8 ± 3.6*	17.7 ± 1.7	14.1 ± 0.8	15.6 ± 1.4	15.1 ± 2.2
3	Starting body wt. (g)	254.1 ± 13.8	254.4 ± 13.6	255.1 ± 12.3	255.1 ± 12.4	255.6 ± 13.1	255.3 ± 14.7	255.2 ± 14.9	254.3 ± 15.9	254.6 ± 16.6
	Terminal body wt. (g)	303.3 ± 16.4	314.7 ± 20.9	307.0 ± 24.0	317.5 ± 10.9	248.1 ± 62.4*	322.7 ± 23.5	292.7 ± 18.1	310.6 ± 23.1	313.2 ± 24.2
	Liver (g)	12.9 ± 1.2	13.7 ± 1.7	14.2 ± 1.7	13.7 ± 0.5	18.6 ± 2.5*	16.0 ± 1.3	13.0 ± 0.9	12.8 ± 1.4	13.5 ± 2.5
	Adrenals (mg)	57.0 ± 10.8	49.5 ± 8.3	54.3 ± 12.5	58.0 ± 5.9	55.5 ± 3.6	56.6 ± 3.5	63.2 ± 7.0*	56.5 ± 7.8	55.5 ± 7.7
	Kidneys (mg)	2106 ± 212	2207 ± 228	2316 ± 273	2342 ± 34	2152 ± 216	2329 ± 197	2236 ± 132	2184 ± 198	2195 ± 201

V.C., vehicle control; TP, testosterone propionate. Each substance was coadministered with 0.2 mg/kg TP. $n = 6$ rats/group/Lab.
* Significantly different from TP group at $P < 0.05$.

LABC weight in rats given G plus TP was significantly lower than that in the TP group in Lab 2, the normalized change of this organ was not so apparent. The total of the five accessory sex organ weights in rats given I plus TP and K plus TP was lower than in rats given TP in all laboratories. The seminal vesicle weight changes in rats given I plus TP and K plus TP were most sensitive among the five organs (Fig. 2).

4. Discussion

Japanese laboratories performed the validation studies of Phase 2 using methyltestosterone, vinclozolin, and *p,p'*-DDE as a part of the national validation program with the result that the Hershberger assay proposed by the OECD was suggested to be a good screening assay to detect androgen agonistic and antagonistic effects (Yamasaki et al., 2003a).

We also performed the Hershberger assay using coded chemicals as part of a national validation Phase 3 as the next step for the OECD guideline process of this assay. The weights of all the accessory sex organs from the experimental animals in all the laboratories exhibited significantly the same changes in the agonistic version; almost all organ weights increased in the rats given coded substance L, and no organ showed any response in rats given coded substances A and B. We received the information from the coordinator of this validation study after all tests were finished that a group of L and E was the same compound and a dose of L was higher than that of E, and that A and B were reported to have no agonistic properties and L and E were a weak agonistic compound. In addition, the normalized weights of all the tissues treated with coded substances in each assay fell within narrow ranges. Therefore, we think that the Hershberger assay is a good screening assay for detecting the androgen agonistic effects of chemicals. The findings that the terminal body weights in rats given coded L were depressed in all laboratories and no body weight changes were detected in rats given coded substance E in all laboratories means that a dose of L was a toxic level and a dose of E had no observed effect. The androgen agonistic effects were detected by the administration of toxic level in this study, but weak agonistic and antagonistic properties of some weak chemicals were detected when non-toxic level doses were administered (Yamasaki et al., 2003a,b).

In the antagonistic version, almost all the sex accessory organs decreased in rats given coded substances I plus TP and K plus TP in all laboratories compared with each organ weight in the rats given TP only, and some organ weights also decreased in the coded substance C

Table 5
Mean body weights and mean organ weights in antagonistic version

Lab	Body weights/organ weights	Chemicals								
		V.C.	TP	F	G	I	C	K	D	H
1	Terminal body wt. (g)	260.0 ± 17.4	267.4 ± 16.5	263.3 ± 17.8	272.2 ± 9.5	248.7 ± 17.1	266.3 ± 11.7	245.7 ± 11.1*	263.6 ± 11.8	261.5 ± 12.5
	Ventral prostate (mg)	14.4 ± 1.5*	102.4 ± 17.6	82.0 ± 7.7	85.9 ± 19.3	28.3 ± 5.9*	78.9 ± 14.6*	41.4 ± 5.9*	80.6 ± 10.7*	24.9 ± 3.6*
	Seminal vesicles (mg)	23.8 ± 3.1*	194.8 ± 38.9	191.4 ± 40.0	185.7 ± 33.5	35.5 ± 6.2*	144.5 ± 29.1	60.6 ± 8.2*	154.7 ± 32.5	28.2 ± 3.8*
	LABC (mg)	119.0 ± 7.0*	306.2 ± 39.1	307.8 ± 40.0	302.3 ± 29.4	125.7 ± 13.4*	280.7 ± 29.7	155.8 ± 21.8*	302.9 ± 40.4	148.3 ± 18.6*
	Glans penis (mg)	29.4 ± 2.9*	60.9 ± 5.3	61.9 ± 5.5	61.2 ± 2.1	32.0 ± 3.9*	53.6 ± 3.3*	41.7 ± 6.4*	64.5 ± 6.1	30.3 ± 4.2*
	Cowper's glands (mg)	3.2 ± 1.3*	18.4 ± 4.6	20.1 ± 1.7	16.2 ± 3.5	4.9 ± 0.8*	16.4 ± 1.9	8.3 ± 1.8*	18.1 ± 3.3	4.7 ± 1.3*
	Total of five organs (mg)	189.9 ± 10.6*	682.7 ± 85.8	663.2 ± 81.3	651.2 ± 65.6	226.5 ± 20.6*	573.9 ± 62.2	307.7 ± 25.4*	620.8 ± 74.6	236.3 ± 24.2*
2	Terminal body wt. (g)	313.2 ± 14.7*	331.9 ± 14.3	326.1 ± 16.4	332.2 ± 20.7	270.8 ± 44.6*	329.8 ± 18.7	309.5 ± 13.9*	335.3 ± 15.6	327.4 ± 23.1
	Ventral prostate (mg)	17.7 ± 2.7*	144.9 ± 19.4	135.0 ± 12.9	129.0 ± 31.5	28.0 ± 8.9*	126.5 ± 40.0	61.6 ± 25.7*	115.5 ± 26.3	28.2 ± 8.9*
	Seminal vesicles (mg)	47.5 ± 8.4*	463.2 ± 70.1	405.9 ± 69.8	439.7 ± 68.7	77.8 ± 26.4*	352.5 ± 80.3*	177.7 ± 43.8*	357.1 ± 40.3*	65.9 ± 11.2*
	LABC (mg)	217.7 ± 25.2*	575.3 ± 31.8	539.6 ± 53.0	529.9 ± 32.2*	218.1 ± 39.6*	496.4 ± 71.6*	336.0 ± 46.3*	532.4 ± 56.5	235.9 ± 29.0*
	Glans penis (mg)	49.9 ± 2.9*	73.0 ± 3.7	75.6 ± 5.9	75.2 ± 2.1	49.8 ± 1.7*	71.7 ± 2.5	63.9 ± 6.2*	73.0 ± 3.3	49.7 ± 2.0*
	Cowper's glands (mg)	5.7 ± 0.9*	33.0 ± 5.4	28.5 ± 5.6	31.0 ± 2.6	10.5 ± 3.8*	30.9 ± 6.5	22.9 ± 9.1	25.7 ± 6.2	10.4 ± 3.4*
	Total of five organs (mg)	338.4 ± 29.0*	1290.2 ± 111.4	1184.6 ± 97.1	1204.6 ± 115.7	384.2 ± 71.6*	1078.0 ± 174.3*	662.0 ± 111.1*	1103.7 ± 89.4*	390.0 ± 29.6*
3	Terminal body wt. (g)	303.3 ± 16.4	314.7 ± 20.9	307.0 ± 24.0	317.5 ± 10.9	248.1 ± 62.4	322.7 ± 23.5	292.7 ± 18.1	310.6 ± 23.1	313.2 ± 24.2
	Ventral prostate (mg)	20.7 ± 3.5*	159.1 ± 31.2	142.0 ± 34.5	132.8 ± 20.1	36.2 ± 9.4*	107.2 ± 17.8*	67.8 ± 24.5*	133.6 ± 24.2	39.8 ± 5.2*
	Seminal vesicles (mg)	53.9 ± 5.2*	459.7 ± 97.2	437.1 ± 120.5	411.6 ± 59.7	73.9 ± 7.9*	323.7 ± 59.0	159.6 ± 45.0*	317.1 ± 62.3*	76.3 ± 10.8*
	LABC (mg)	193.1 ± 11.3*	518.1 ± 74.2	460.2 ± 84.7	494.4 ± 50.1	165.4 ± 35.5*	396.4 ± 50.9*	262.6 ± 47.8*	452.4 ± 64.2	221.8 ± 51.5*
	Glans penis (mg)	50.9 ± 3.8*	93.5 ± 4.6	86.3 ± 7.9	89.6 ± 7.6	60.2 ± 7.3*	83.9 ± 4.3	73.2 ± 10.0*	90.2 ± 6.1	60.8 ± 3.4*
	Cowper's glands (mg)	8.3 ± 2.8*	35.6 ± 6.5	37.5 ± 11.1	33.4 ± 5.3	9.3 ± 2.5*	23.5 ± 5.9*	20.9 ± 4.6*	34.8 ± 6.7	12.0 ± 3.8*
	Total of five organs (mg)	326.9 ± 21.3*	1266.0 ± 151.6	1163.1 ± 217.1	1161.6 ± 84.1	345.0 ± 57.2*	934.7 ± 130.1*	584.2 ± 90.7*	1028.0 ± 110.7*	410.7 ± 61.4*

V.C., vehicle control; TP, testosterone propionate. Each substance was coadministered with 0.2 mg/kg TP. $n = 6$ rats/group/Lab.
* Significantly different from TP group at $P < 0.05$.

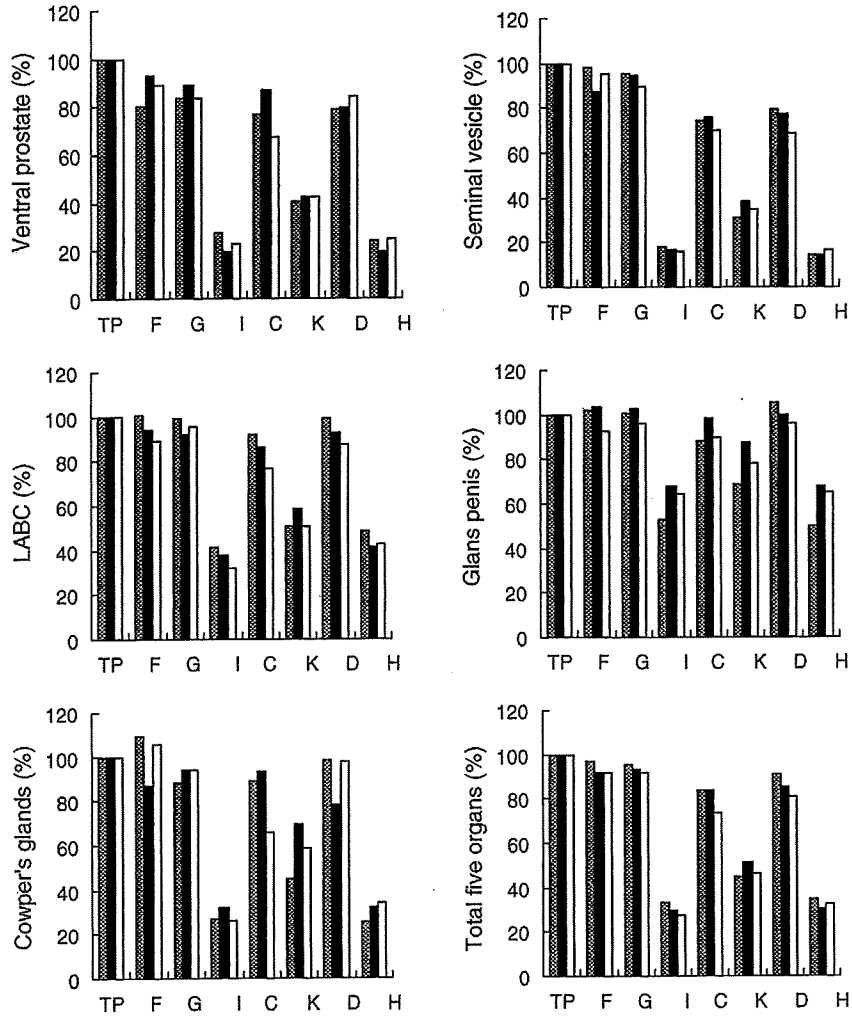

Fig. 2. Organ weights in antagonistic version. Values from each laboratory were normalized to the value of TP group set equal to 100%. LABC: levator ani and bulbocavernosus muscle; F, G, I, C, K, D, and H: coded chemicals; TP: testosterone propionate. $n = 6$ rats/group/Lab (▩, Lab 1; ■, Lab 2; □, Lab 3).

plus TP and D plus TP groups. No changes were detected in rats given coded substances F and G. These findings demonstrate that coded substances I, C, K, and D had antagonistic properties and coded substances F and G had no antagonistic properties. We accepted the information; substances F and G were negative compounds, I, C, K, and D were weak antagonistic compounds, and H was a positive control compound, flutamide; the groups of substances I and C, or K and D were the same compound, and dose levels of I and K were higher than those of C and D. We also received the information that C and I were *p,p'*-DDE and D and K were linurone. The ventral prostate and glans penis in Lab 1, the seminal vesicle and LABC in Lab 2, and the ventral prostate, LABC and Cowper's glans in Lab 3 were significantly affected in the rats given coded substance C plus TP. In addition, the ventral prostate in Lab 1 and seminal vesicle in Labs 2 and 3 were significantly affected in rats given coded substance D plus TP. The differential effects across laboratories were observed in rats given coded C plus TP and D plus TP. We found that the most sensitive organ among the five accessory sex organs was the prostate and/or seminal vesicle in our previous validation Phase 2 study, and in the Hershberger assays using various chemicals (Yamasaki et al., 2003a,b). The ventral prostate and/or seminal vesicle were responded with or without significant differences in rats given coded substances C plus TP and D plus TP, so we determined that coded C and D have androgen antagonistic properties. On the other hand, the LABC weight in rats given coded substance G plus TP was significantly lower than that in the TP group in Lab 2, but the normalized change of this organ was not so apparent. Therefore, the Japanese data in this study demonstrated that the Hershberger assay

is considered to be a good screening assay for detecting the androgen antagonistic effects of chemicals. The findings that some animals died in rats given coded substance I and decreased body weights were detected in rats given K and I, and the liver weights increased in rats given I means the coded substances I and K were at a toxic dose level. In addition, the liver weights increased in rats given C in one laboratory, so a dose of C may be at a toxic level. The general toxicity is considered to be important for this assay, because a 10% change in terminal weight is suggested to affect some Hershberger assay endpoints (Marty et al., 2003).

In conclusion, we performed the OECD validation study Phase 3 using coded chemicals. All five accessory sex organs responded with statistically significant changes in weight within a narrow window in the agonistic and antagonistic versions, and no false positive or false negative results were observed in this study. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

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REGULATORY TOXICOLOGY

Shuji Noda · Takako Muroi · Saori Takakura
Satoko Sakamoto · Mineo Takatsuki · Kanji Yamasaki
Susumu Tateyama · Ryoji Yamaguchi

Ability of the Hershberger assay protocol to detect thyroid function modulators

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Abstract In vivo screening methods for detection of thyroid function modulators are now under development in many research laboratories. We assessed the applicability of the Hershberger assay protocol to screen for thyroid function modulators. In experiment 1, castrated male BrlHan WIST@Jcl (GALAS) rats were administered a potent thyroid peroxidase inhibitor, 3-amino-1,2,4-triazole (AT), in doses of 0, 40, 200, and 1,000 mg/kg/day with gravimetric endpoint, and in experiment 2, castrated and intact male rats were administered in doses of 0, 40, and 200 mg/kg/day, with quantification of the extent of hypertrophy of the thyroid epithelium, to assess the effects of castration, by gavage to 8-week-old for 10 consecutive days. At necropsy of both experiments, the thyroid glands and hypophysis were collected and fixed with 10% neutral-buffered formalin. To avoid crushing during weighing because of their fragility, the thyroid glands and hypophysis were weighed approximately 24 h after fixation with 10% neutral-buffered formalin. All animals were sacrificed approximately 24 h after the final dose. In experiment 2, the thyroid glands of all animals were stained with hematoxylin and eosin for histological examination and morphometry of follicular epithelial height. In experiment 1, absolute and relative thyroid weights in all of the AT groups were statistically increased in a dose-dependent manner, regardless of the testosterone propionate (TP)-injection. In experiment 2, the results showed a significant increase in thyroid weight in the 200 mg/kg groups of both castrated and intact rats. Hypophyseal weight was unaltered by AT, but comparison of vehicle-treated groups showed that the hypophyseal weight of the castrated rats was greater than that of the intact rats. Enlarged thyroid glands were observed in the AT-treated rats at necropsy. Histological examination of the thyroid glands of all the AT-treated animals showed hypertrophy and hyperplasia of the follicular epithelial cells, and the height of follicular epithelium of the thyroid glands increased in a dose-dependent manner in both the castrated and intact rats. In experiment 1, assessment of the (anti-) androgenic action of AT in seminal vesicle weight revealed a significant increase in the 200 and 1,000 mg/kg + TP groups in a dose-dependent manner. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol and may be useful for screening for thyroid function modulators regardless of whether the animals have been castrated.

Keywords Hershberger assay · Thyroid function modulators · 3-amino-1,2,4-triazole · Castrated rat · Intact rat

S. Noda (✉) · T. Muroi · S. Takakura · S. Sakamoto
M. Takatsuki · K. Yamasaki
Chemicals Assessment Center, Chemicals Evaluation and Research Institute, 822, 3-chome, Ishii-machi, Hita-shi, Oita, 877-0061, Japan
E-mail: noda-shuji@ceri.jp
Tel.: +81-973-24-7211
Fax: +81-973-23-9800

S. Tateyama · R. Yamaguchi
Department of Veterinary Pathology, Faculty of Agriculture, Miyazaki University, Miyazaki-shi, Miyazaki, 889-2155, Japan

Introduction

Currently, there is a great deal of concern that certain environmental chemicals may have the ability to impair normal sexual differentiation and development in humans and wildlife (McLachlan 1993; McLachlan and Korach 1995). Endocrine Disruptor Screening and Testing Committee (EDSTAC) is developing a tiered chemical screening and testing program to evaluate endocrine disrupting effects (EDSTAC 1998). In this program, high priority chemicals will be evaluated for hormonal activity in the early screening stage, and chemicals positive in the screening stage will be tested for hazard in a definitive test (EDSTAC 1998; Gray et al. 2002).

A Hershberger assay in surgically castrated male rats was proposed by the Organization for Economic

Cooperation and Development (OECD) and EDSTAC of the US Environmental Protection Agency as an in vivo screening method to detect the (anti-) androgenic activity of the chemicals acting mainly via androgen receptor (AR)-mediated mechanisms (Hershberger et al. 1953; Dorfman 1969; OECD 1997, 2001, 2003; EDSTAC 1998). Focusing on the disruption of androgenic system, the proposed endpoints of this assay are the weight of the ventral prostate, seminal vesicles, bulbocavernosus/levator ani (BC/LA) muscles, glans penis, Cowper's glands, and liver. The gravimetric endpoint has the advantage of enabling the detection of (anti-) androgenic action of a chemical at low cost in a short time. The Hershberger assay will be used in the early stage of a chemical screening and testing program after the in vitro screening assays, such as the AR binding assay and the AR reporter gene assay (Gray et al. 2002).

In vitro and in vivo screening methods for the detection of thyroid function modulators are now under development in many research laboratories, and a variety of in vivo screening methods have been proposed, e.g., enhanced TG407 and male or female pubertal assay. But they have the disadvantage such as long experimental periods or low cost-performance, and there have been no breakthroughs thus far. Yamada et al. (2004) first reported that the enhanced Hershberger assay evaluation of thyroid histopathology and weights, and determination of serum hormone levels, appears to be reliable for screening thyroid modulators with a single dose of propylthiouracil (PTU), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) and phenobarbital (PB). The original objective of the Hershberger assay was to screen for (anti-) androgenic activity of the chemicals in male rats subjected to castration, which is essential for the detection of androgenic activities. If the Hershberger assay is capable of detecting the (anti-) androgenic activity and thyroid hormone modulating activity of the chemicals at the same time, it will be possible to screen for hormonal activity of the chemicals rapidly and with better cost-performance.

Because of the well-known presence of androgen receptors in the thyroid gland of mammalians (Pellietier 2000; Banu et al. 2002), the thyroid gland is speculated to be one of the target organs of androgenic compounds. Moreover, testosterone has a stimulatory effect of on the expression of the TSH mRNA (Ross 1990), and testosterone administration results in a significant decrease in serum T3 and T4 levels in 15-day intact male assays (O'Connor et al. 2000). Thus, if we intend to screen chemicals for (anti-) androgenic activity based on their thyroid hormone modulating activity, effect of castration of male rats on the thyroid should be evaluated. It appears rather unlikely that thyroid-specific endpoints can be used to specifically detect (anti-) androgenicity. However, it is necessary to evaluate whether thyroid-active compounds affect the classical parameters of the Hershberger assay and thus confound the detection of (anti-) androgenicity.

We performed two experiments. Experiment 1 was designed to assess whether thyroid hormone modulating activity of 3-amino-1,2,4-triazole (AT), which is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey et al. 1984; Krauss and Eling 1987; Reader et al. 1987; Masuda and Goto 1994; Santini et al. 2003), could be detected by the thyroid gravimetric method, and whether the dose-dependent AT-induced effect on thyroid gland would be confirmed by the Hershberger assay in castrated male rats. Experiment 2 was designed to assess the influence of castration on the AT-induced effect on thyroid gland, and thus, both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

Materials and methods

Chemicals

AT (CAS No. 61-82-5, 99% pure) was obtained from Tokyo Kasei Kougyou (Tokyo, Japan), and olive oil was obtained from Fujimi Pharmaceutical Company (Osaka, Japan). The physical stability of AT was assessed with a Fourier transform infrared spectrophotometer (FTS-135, Nippon Bio-Rad Laboratories K.K., Tokyo, Japan), and the stability, homogeneity, and concentration of each AT suspension prepared for administration were confirmed by HPLC.

Animals

Seven-week-old BrlHan WIST@Jcl (GALAS) rats castrated at 6 weeks of age, and 7-week-old intact BrlHan WIST@Jcl (GALAS) rats were purchased from Clear Japan Inc. (Fuji, Japan). The animals were housed, three per cage, in stainless steel, wiremesh cages throughout the study. The rats were weighed, weight-ranked, and randomly assigned to one of the treatment or control groups. Body weight and clinical signs were recorded daily throughout the study. Rats were given ad libitum access to tap water and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and ad libitum access to water from an automatic dispenser. The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and it was artificially illuminated with fluorescent light on a 12-h light/dark cycle (0600–1800 hours). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

Study design

Experiment 1: Hershberger assay

The experimental design of our study followed the OECD validation of the rodent Hershberger bioassay: phase-2 protocol (OECD 2002). Six castrated rats/group

were given AT orally at doses of 40, 200, or 1,000 mg/kg daily via a stomach tube for 10 consecutive days beginning at 9 weeks of age with or without s.c. injection of 0.2 mg/kg testosterone propionate (TP). The volume of the olive oil solutions of AT was 5 ml/kg for oral administration and 5 ml/kg for s.c. injection. A vehicle control group given olive oil alone was also established. The dosage was adjusted daily for body weight change. Approximately 24 h after the final dose, the animals were killed by bleeding from the abdominal vein under deep ether anesthesia. At necropsy, the ventral prostate, seminal vesicles, BC/LA muscles, glans penis, Cowper's gland, and liver were removed and weighed. The thyroid glands and hypophysis were collected and fixed with 10% neutral-buffered formalin, and to avoid crushing during weighing because of their fragility, they were weighed approximately 24 h after fixation.

Experiment 2: quantification of the extent of hypertrophy of the thyroid epithelium

The same experimental procedures, as described below, were performed on intact rats and castrated rats.

The rats were given AT in doses of 40 or 200 mg/kg daily. Administration and necropsy were executed according to the same procedure as experiment 1. At necropsy, the thyroid glands and hypophysis were removed and fixed with 10% neutral-buffered formalin. To avoid crushing during weighing because of its fragility, the thyroid glands and hypophysis were weighed approximately 24 h after fixation, stained with hematoxylin and eosin, and examined under a light microscope. Morphometry of the thyroid epithelium was performed to quantify the extent of hypertrophy. The height of the epithelial cells of all rats was measured under a microscope with Scion Image image analysis software (Scion Corporation, MA, USA). Fifty points/rat (25 left, 25 right) of the thyroid epithelium were measured.

Statistical analyses

Body weight, organ weights, and height of thyroid follicular epithelium of the rats in the experimental groups and corresponding vehicle control groups were compared, i.e., data obtained from the castrated group were compared with data from the castrated control group. Data from the castrated control group and intact control group were also compared. Each endpoint was analyzed by Bartlett's test for homogeneity of variance. If the variances were homogeneous at the 5% level of significance, one-way analysis of variance was performed, and if it revealed a significant difference, the difference between the control group and each of the experimental groups was analyzed by Dunnett's test. If the variances were not homogeneous, the Kruskal–Wallis test was used, and if it showed a significant difference, the difference between the control group and each of the experimental groups was analyzed by the nonparametric Dunnett's test.

Results

Experiment 1: Hershberger assay

General observation revealed no abnormal findings in the AT-treated castrated rats. Irrespective of TP-treatment, the body weight of the AT-treated castrated rats remained normal throughout the study (Table 1).

Organ weight changes are shown in Tables 1 and 2. Absolute and relative thyroid weights in all the AT groups were statistically increased in a dose-dependent manner, regardless of the TP-injection, and increases were statistically significant.

In the classical parameters of the Hershberger assay, absolute seminal vesicle weights were significantly higher in the 200 mg/kg + TP group, and absolute and relative seminal vesicle weights were significantly higher in the 1,000 mg/kg + TP group when compared to each corresponding vehicle control group. In the 1,000 mg/kg + TP group, relative liver weights were significantly higher. Besides, absolute seminal vesicle weights were significantly lower in the 1,000 mg/kg group, absolute and relative weights were significantly higher in the 40 mg/kg group, absolute glans penis weights and relative BC/LA weights were significantly lower, and absolute and relative liver weights were significantly higher in the 40 mg/kg + TP group, and absolute and relative BC/LA weights were significantly higher in the 200 mg/kg + TP group when compared to each corresponding vehicle control group.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group, in all rats in the 200 and 1,000 mg/kg group, in 5/6 rats in the 40 mg/kg + TP group, and in all rats in the 200 and 1,000 mg/kg + TP group (Table 3).

Experiment 2: quantification of the extent of hypertrophy of the thyroid epithelium

Castrated rats

General observation revealed no abnormal findings in the AT-treated castrated rats. The body weight of the AT-treated castrated rats remained normal throughout the study (Fig. 1).

Organ weight changes are shown in Fig. 2. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group. No statistically significant differences were detected in hypophysis weight.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group and in all rats in the 200 mg/kg group (Table 3).

Histopathological findings in the thyroid glands of the AT-treated castrated rats are shown in Fig. 3 (left panel); the thyroid glands of all AT-treated rats

Table 1 Absolute organ weight in AT-treated castrated male rats

Dose (mg/kg/day)	Thyroid (mg)	Ventral prostate (mg)	Seminal vesicle (mg)	Bulbocavernosus/ levator ani muscles (mg)	Glans penis (mg)	Cowper's galnd (mg)	Liver (g)	Final body weight (g)
VC	16.4 ± 5.9	15.1 ± 1.7	29.3 ± 4.2	146.0 ± 17.1	39.0 ± 3.0	3.9 ± 1.5	11.0 ± 0.6	281.2 ± 13.3
40	27.8 ± 8.8*	14.1 ± 6.1	27.1 ± 3.0	148.4 ± 19.2	41.3 ± 4.2	5.3 ± 0.9	12.0 ± 0.8	12.0 ± .08*
200	50.2 ± 11.0**	17.1 ± 1.9	25.3 ± 2.8	144.2 ± 23.1	38.2 ± 5.3	4.5 ± 0.8	11.6 ± 0.9	11.6 ± 0.9
1,000	56.5 ± 19.1**	15.9 ± 2.5	24.0 ± 4.1*	139.8 ± 24.6	39.6 ± 4.4	4.9 ± 1.0	11.2 ± 1.1	11.2 ± 1.1
VC + TP	14.8 ± 2.3	108.2 ± 9.4	232.2 ± 30.2	368.7 ± 23.8	78.5 ± 6.4	23.7 ± 3.8	11.0 ± 0.9	11.0 ± 0.9
40 + TP	27.1 ± 6.2##	118.9 ± 14.2	275.5 ± 66.1	344.7 ± 28.0	70.5 ± 6.4	24.3 ± 3.2	12.6 ± 1.2#	12.6 ± 1.2#
200 + TP	45.8 ± 16.8##	109.0 ± 13.8	285.4 ± 45.0#	317.5 ± 14.8##	71.4 ± 5.1	23.3 ± 3.8	11.7 ± 1.1	11.7 ± 1.1
1,000 + TP	45.4 ± 9.3##	113.3 ± 25.6	307.5 ± 39.5##	348.9 ± 32.2	73.3 ± 3.4	26.3 ± 2.3	12.2 ± 1.5	12.2 ± 1.5

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.
*,** Significantly different from VC at $p < 005$ and $p < 0.01$, respectively.
#,## Significantly differently from VC + TP at $p < 0.05$ and $p < 0.01$, respectively.

Table 2 Relative organ weight in AT-treated castrated male rats

Dose (mg/kg/day)	Thyroid (mg/100 g b.w.)	Ventral prostate (mg/100 g b.w.)	Seminal vesicle (mg/100 g b.w.)	Bulbocavernosus/ levator ani muscles (mg/100 g b.w.)	Glans penis (mg/100 g b.w.)	Coeper's gland (mg/100 g b.w.)	Liver (mg/100 g b.w.)
VC	5.8 ± 1.7	5.4 ± 0.8	10.5 ± 1.8	52.0 ± 6.7	13.9 ± 1.3	1.4 ± 0.5	3.9 ± 0.2
40	9.9 ± 3.3*	4.9 ± 2.0	9.5 ± 0.9	51.9 ± 4.6	14.5 ± 1.2	1.8 ± 1.2	4.2 ± 0.2*
200	17.7 ± 3.2**	6.1 ± 0.8	9.0 ± 1.4	50.9 ± 6.0	13.6 ± 2.3	1.6 ± 0.3	4.0 ± 0.2
1,000	20.1 ± 5.5**	5.7 ± 0.8	8.6 ± 1.2	50.1 ± 6.0	14.4 ± 2.3	1.8 ± 0.4	1.8 ± 0.2
VC + TP	5.2 ± 0.9	37.7 ± 4.9	81.0 ± 14.6	127.9 ± 9.2	27.3 ± 3.3	8.2 ± 1.4	3.8 ± 0.2
40 + TP	9.1 ± 1.9##	39.9 ± 5.7	92.2 ± 21.5	115.5 ± 9.3#	23.7 ± 2.4	8.2 ± 1.4	4.2 ± 0.3#
200 + TP	15.9 ± 5.2##	38.0 ± 4.9	99.6 ± 16.3	110.8 ± 6.6##	24.9 ± 1.8	8.1 ± 1.1	4.1 ± 0.2
1,000 + TP	15.8 ± 3.6##	39.3 ± 9.4	106.5 ± 13.9#	120.7 ± 10.6	25.4 ± 1.7	9.1 ± 0.9	4.2 ± 0.3#

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.
*,** Significantly different from VC at $p < 0.05$ and $p < 0.01$, respectively.
#,## Significantly different from VC + TP at $p < 0.05$ and $p < 0.01$, respectively.

Table 3 Gross pathological findings in AT-treated rats (No. of animals = 6)

	Experiment 1								Experiment 2					
	AT (mg/kg/day)				AT + TP (mg/kg/day)				Castrated (mg/kg/day)			Intact (mg/kg/day)		
	VC	40	200	1,000	VC	40	200	1,000	VC	40	200	VC	40	200
No abnormalities detected	6	2	0	0	6	1	0	0	6	2	0	6	0	0
Thyroid enlargement	0	4	6	6	0	5	6	6	0	4	6	0	6	6

AT, 3-amino-1,2,4-triazole; VC, Vehicle control; Tp, testosterone propionate.

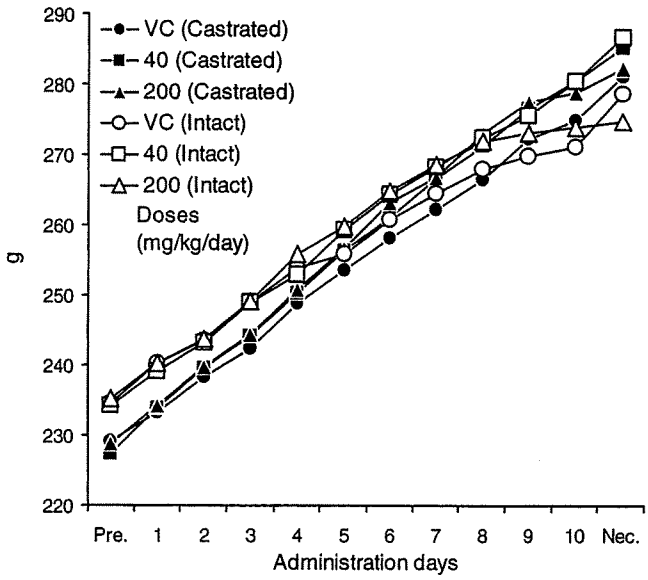

Fig. 1 Body weight changes in AT-treated castrated and intact male rats

exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The follicular epithelial cell heights are shown in Fig. 4. The height of the follicular epithelial cell in all AT-treated groups was increased in a dose-dependent manner by AT (40 mg/kg group, 200.8% of the control; 200 mg/kg group, 225.7% of the control), and the increase were statistically significant.

Intact rats

General observation revealed no abnormal findings in the AT-treated intact rats. The body weight of the AT-treated rats remained normal throughout the study (Fig. 1).

Organ weight changes are shown in Fig. 2. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group, and absolute thyroid weight in the 40 mg/kg group. No statistically significant differences in hypophyseal weights were detected. Comparison between the VC groups, however, revealed significantly decreased absolute and relative hypophyseal weights in the intact VC group.

Gross examination revealed enlargement of the thyroid glands in all rats in all the AT-treated groups (Table 3). Histopathological findings in the thyroids of the AT-treated intact rats are shown in Fig. 3 (right panel). The thyroid glands of all AT-treated animals exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The height of the follicular epithelial cell is shown in Fig. 4. Follicular epithelial cell height in all AT-treated groups was significantly increased in a dose-dependent manner by AT (40 mg/kg group, 149.6% of the control; 200 mg/kg group, 195.0% of the control).

Discussion

We examined the applicability of the Hershberger assay 10-day administration protocol to screening of chemicals for a thyroid hormone modulator. The thyroid peroxidase inhibitor, AT, a widely used herbicide found to produce thyroid and liver tumors in rodents and classified as possibly carcinogenic to humans, was investigated to acquire further information about its mechanism of action (Mattioli et al. 1994). In this study, AT was used as the test substance to determine whether the Hershberger assay can detect thyroid hormone modulation caused by a mechanism different from that of PTU, *p, p'*-DDE, and PB, such as by the mechanisms of modulation of iodination of thyroglobulin and of the coupling reaction and metabolism enhancers. Both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

The organ weight changes showed that thyroid weight increased in all AT-treated groups in a dose-dependent manner, and thyroid gland enlargement in the AT-treated rats. Histologically, the thyroid glands of all AT-treated rats exhibited hypertrophy and hyperplasia of the follicular epithelial cells, and their height increased in a dose-dependent manner in both the castrated and intact rats. AT is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey et al. 1984; Krauss and Eling 1987; Reader et al. 1987; Masuda and Goto 1994; Santini et al. 2003). It has been reported that the administration of AT to rats is followed by enlargement of the thyroid

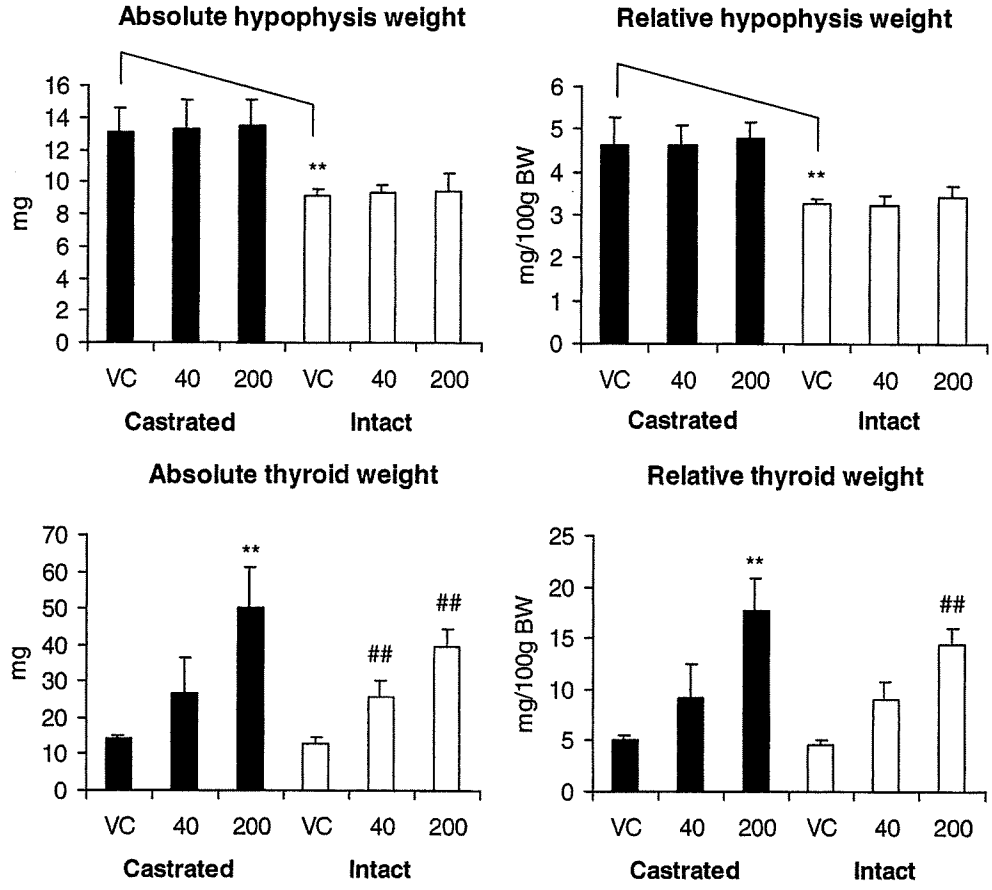

Fig. 2 Hypophyseal and thyroid weights of AT-treated castrated and intact male rats. **Significantly different from the castrated vehicle control at $p < 0.01$.## Significantly different from the intact vehicle control at $p < 0.01$

gland, a decrease in colloid content, and proliferation of the follicular epithelium (Masuda and Goto 1994). The results of the present study showed that the Hershberger assay 10-day administration protocol clearly detected thyroid hormone modulating effect of AT on the thyroid glands. Moreover, the reproducibility of the results was demonstrated by experiments 1 and 2. Yamada and colleagues (2004) first reported that the Hershberger assay enhanced by evaluation of thyroid histopathology and weights and serum hormone levels appears to be a reliable method of screening for thyroid modulators. PTU is not only an inhibitor of type I deiodinase inhibitor, but also an inhibitor of iodination of thyroglobulin and of the coupling reaction, whereas peroxidase inhibition seems not to occur under in vivo inhibition (Shiroozu et al. 1983; Moura et al. 1990; Taurog et al. 1995). *p,p'*-DDE and PB are the thyroid hormone metabolism enhancers (McClain et al. 1989; Barter and Klaassen 1994; Capen 1997). Thus, the thyroid inhibiting effects of several thyroid modulators have already been detected by the Hershberger assay. It therefore seems possible to use the Hershberger assay to assess the potency of anti-thyroid chemicals and perhaps to bioassay them.

The workshop on Screening Methods for Chemicals that Alter Thyroid Hormone Action, Function and Homeostasis (DeVito et al. 1997, 1999) concluded that at least a 2–6-week dosing period is necessary to be able to observe consistent thyroid responses. Other assays, such as the male and female pubertal assays (Goldman et al. 2000; Stoker et al. 2000) and enhanced TG 407 (OECD 1999), require a 20–30-day administration period and are not convenient screening methods for evaluating large numbers of chemicals for the thyroid hormone modulating activity. If the Hershberger assay is used to screen for thyroid function modulating activity and (anti-) androgenic compounds at the same time, more chemicals can be screened for its (anti-) androgenic activity and/or thyroid hormone modulating activity.

It was reported that castration induced a dramatic increase in the size and proportion of LH-immunoreactive cells present within the adenohypophysis of control rats (Kirby et al. 1997) and the castration induces an immediate increase in the serum levels and pituitary content of the gonadotropins, luteinizing hormone, and follicle-stimulating hormone as well as a concomitant rise in the steady state levels of the messenger RNAs directing their synthesis (Emanuele et al. 1996; Valenti et al. 1997). It is speculated that an increased hypophysis weight in castrated rats may be a result of the removal of testicular negative feedback. Histologically, however, the thyroid glands of all AT-treated rats exhibited follicular epithelial cell hypertrophy and hyperplasia, and the height of the follicular epithelium of the thyroid glands increased in a dose-dependent manner in both the

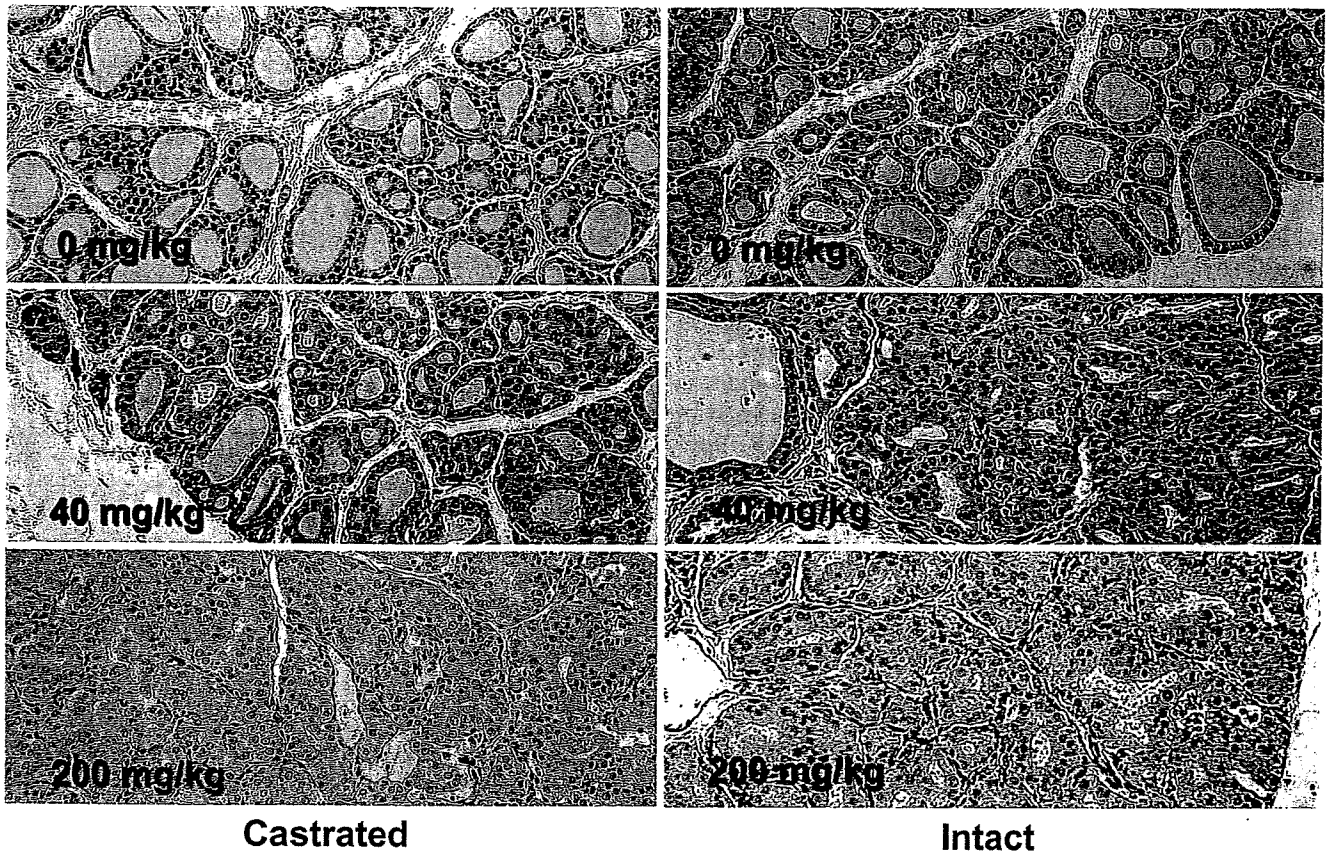

Fig. 3 Micrographs of the thyroid gland of an AT-treated castrated rat and an AT-treated intact male rat. Magnification (125 x)

castrated and intact rats. Moreover, results of morphometry of the thyroid epithelium showed almost same sensitivity in both the castrated rats and intact rats. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol regardless of castration. The other assays to detect the thyroid modulating effect, such as male or female pubertal assay (Goldman et al. 2000; Stoker et al. 2000), and enhanced TG 407 (OECD 1999) are used in intact rats. Our results emphasize the possibility of applying the Hershberger assay protocol to the detection of thyroid modulators. At the same time, the 10-day oral administration protocol in intact male rats may provide a new approach to screening for thyroid hormone modulators. In terms of prevention of surgical intervention of animals, it is valuable to develop screening methods that do not involve surgical invasion, such as castration, ovariectomy, etc. Thus, further study is needed, including evaluation of other compounds with mechanisms of action different from those of AT, such as thyroid hormone metabolism enhancers and thyroid hormone antagonist etc.

In addition, assessment of the (anti-) androgenic action of AT in seminal vesicle weight revealed a significant increase in the 200 and 1,000 mg/kg + TP groups in a dose-dependent manner. The mechanisms and biological or toxicological significance of the increase of seminal vesicle weight in AT + TP groups were unclear and there were no AT-related changes in other androgen-sensitive organs, but this seminal vesicle weight change was not considered to be disregarded

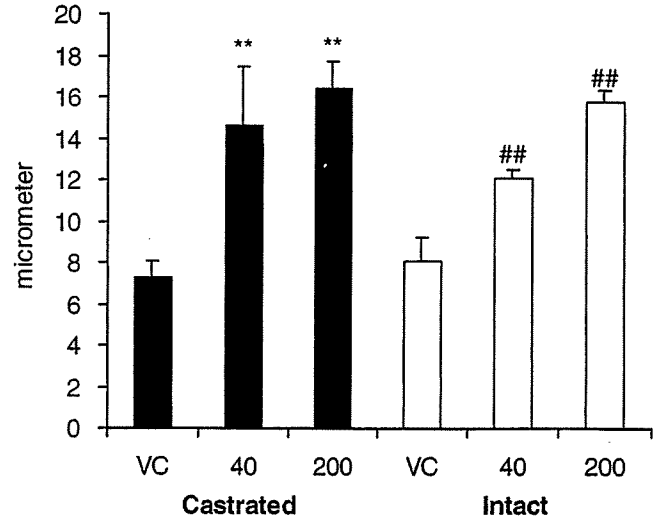

Fig. 4 Height of thyroid follicular epithelial cells of an AT-treated castrated rat and an AT-treated intact male rat. **Significantly different from the castrated vehicle control at $p < 0.01$. ## Significantly different from the intact vehicle control at $p < 0.01$

because dose dependency was confirmed, although the study to determine the reproducibility of the data will be needed to confirm these findings in the present study. Other organ weight changes, excluding the seminal vesicle weight changes in the AT + TP groups, were judged to be of no toxicological significance, since there were no dose relationships. Based on this finding, it might be appropriate to conclude that the androgen action of TP is reinforced or synergized. The original Hershberger assay is a screening method that evaluates the hormonal activity of chemicals based on changes in accessory sex organs alone (Hershberger et al. 1953; Dorfman 1969; OECD 1997; Gray et al. 2002), and it is impossible to speculate on the mechanism of changes in the accessory sex organs. We performed the AR binding assay and AR reporter gene assay of AT, and AT showed lack of binding affinity to AR and did not exhibit any agonist or antagonist activity in the reporter gene assay in these in vitro assays (data not shown). These results point to the existence of an endocrine-disrupting mechanism other than the AR-mediated mechanism in the Hershberger assay and suggest the importance of the in vitro assay to evaluate the androgen action by the Hershberger assay.

In conclusion, we wish to emphasize the that Hershberger assay has the ability to be used to assess the potency, and possibly to bioassay not only of the (anti-) androgenic activity of chemicals but of thyroid hormone modulators, although further studies will be needed to confirm the reproducibility of the findings in the present study, and evaluated another endpoints, such as hormonal evaluation, and other chemicals with thyroid hormone modulating effects to evaluate the value of the Hershberger assay protocol as a method to screen thyroid function modulators. Moreover, 10-day oral administration protocol in intact male rats may enable a new approach to screening for thyroid hormone modulators, although other compounds with different mechanisms of action than those of AT, such as thyroid hormone metabolism enhancers and thyroid hormone antagonists should be tested.

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Comparison of the Hershberger assay and androgen receptor binding assay of twelve chemicals

Kanji Yamasaki*, Masakuni Sawaki, Shoji Noda, Takako Muroi, Saori Takakura, Hideo Mitoma, Satoko Sakamoto, Makoto Nakai, Yoshikuni Yakabe

Chemicals Assessment Center, Chemicals Evaluation and Research Institute, 3-822, Ishii, Hita, Oita 877-0061, Japan

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Abstract

We performed the Hershberger assay of 12 chemicals based on the OECD draft protocol. The chemicals tested by the Hershberger assay were phthalic acid di-*n*-hexyl ester, phthalic acid di-*n*-amyl ester, phthalic acid di-*n*-propyl ester, diethylstilbestrol, 17β-estradiol, tamoxifen, 5α-dihydrotestosterone, dichlorodiphenyldichloroethane, cyproterone acetate, 6α-methyl-17α-hydroxy-progesterone, atrazine, and spironolactone. Phthalic acid di-*n*-hexyl ester, phthalic acid di-*n*-amyl ester, and phthalic acid di-*n*-propyl ester are phthalates; diethylstilbestrol and 17β-estradiol are estrogenic chemicals; tamoxifen is partial estrogen receptor antagonist with mainly estrogenic properties; 5α-dihydrotestosterone is an androgen derivatives; dichlorodiphenyldichloroethane is a reference androgen antagonistic chemical; cyproterone acetate, 6α-methyl-17α-hydroxy-progesterone, and spironolactone have an androgenic steroid structure and are known as androgen antagonistic chemicals; and atrazine is a reference endocrine disruptor. We also subjected these chemicals to the receptor binding assay for androgen.

A clear androgen agonistic effect was detected in 5α-dihydrotestosterone, and an androgen antagonistic effect was observed in five chemicals: cyproterone acetate, spironolactone, 6α-methyl-17α-hydroxy-progesterone, phthalic acid di-*n*-amyl ester, and dichlorodiphenyldichloroethane. By contrast, diethylstilbestrol, 17β-estradiol, tamoxifen, 5α-dihydrotestosterone, dichlorodiphenyldichloroethane, cyproterone acetate, 6α-methyl-17α-hydroxy-progesterone, and spironolactone were positive in the receptor binding assay for androgen. Three estrogenic chemicals, diethylstilbestrol, 17β-estradiol, and tamoxifen, were negative in the Hershberger assay with receptor binding affinity. On the other hand, the Hershberger assays of three phthalates were performed at the same dosages, and the results showed androgen antagonistic affinity only in the assay of phthalic acid di-*n*-amyl ester without receptor binding affinity.

Keywords: Androgenic effect; Castration; Endocrine; Hershberger assay; OECD draft protocol; Rat; Receptor binding assay

1. Introduction

Because of concern that certain chemicals may have the potential to interfere with the normal sexual differentiation and development of animals and humans (McLachlan, 1993; McLachlan and Korach, 1995), the Organization for Economic Co-operation and

* Corresponding author. Tel.: +81-973-24-7211; fax: +81-973-23-9800.
E-mail address: yamasaki-kanji@ceri.jp (K. Yamasaki).

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Development (OECD) has initiated a project to revise existing guidelines and develop new screening and testing guidelines to aid in the identification and assessment of such toxicants (OECD, 1998, 2000, 2003).

Assays to detect androgenic properties have been developed since the 1930s (Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b; Bülbring and Burn, 1935; Dingemanse et al., 1935; Deanesly and Parkes, 1936; Wainman and Shipounoff, 1941; Eisenberg et al., 1949; Eisenberg and Gordan, 1950; Di Salle et al., 1994), and the capabilities of the assays were demonstrated in 1953 by Hershberger et al. when they analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones (Hershberger et al., 1953).

In the 1970s and 80s, the discovery of the androgen receptor and the first compounds that acted as antagonists of the receptor, such as cyprotone acetate, was followed by modification of the assay to address antagonistic activity. Briefly, a fixed dose of a reference agonist was administered to several groups of animals that were also administered a set of doses of the purported antagonist. This modified system was successfully used by several investigators to assay androgen antagonists (Peets et al., 1973; Raynaud et al., 1980, 1984; Wakeling et al., 1981).

Based on the recommendations of scientific workshops, both the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) (USEPA, 1998) and the OECD Endocrine Disrupter Testing and Assessment Group (EDTA) of the OECD (OECD, 2000) have proposed this assay as a Tier-1 screen to identify possible reproductive and developmental toxicants acting through androgen agonist and antagonist mechanisms.

We performed the Hershberger assay on 30 chemicals having estrogenic properties in our previous study (Yamasaki et al., 2003). In the present study, we performed the Hershberger assay on 12 chemicals based on the OECD draft protocol and assessed the androgen receptor binding assay for the same chemicals to confirm the usefulness of the assay.

2. Materials and methods

2.1. Hershberger assay

The studies were performed under Good Laboratory Practice guidelines.

2.1.1. Chemicals

The chemicals tested in the Hershberger assay are listed in Table 1, and their chemical structures are shown in Fig. 1. Phthalic acid di-*n*-hexyl ester, phthalic acid di-*n*-amyl ester, and phthalic acid di-*n*-propyl ester are phthalates; diethylstilbestrol and 17β-estradiol are estrogenic chemicals; tamoxifen is partial estrogen receptor antagonist with mainly estrogenic properties; 5α-dihydrotestosterone is an androgen derivative; dichlorodiphenyldichloroethane is a reference androgen antagonistic chemical;cyproterone

Table 1
Chemicals tested in this study

Chemicals	CAS No.	Purity (%)	Source
Phthalic acid di-*n*-hexyl ester	84-75-3	99.3	Tokyo Kasei Kogyo, Co.
Phthalic acid di-*n*-amyl ester	131-18-0	99.5	Tokyo Kasei Kogyo, Co.
Phthalic acid di-*n*-propyl ester	131-16-8	98.4	Tokyo Kasei Kogyo, Co.
Diethylstilbestrol	56-53-1	99.8	Wako Pure Chemicals
17β-Estradiol	50-28-2	99.2	Wako Pure Chemicals
Tamoxifen	10540-29-1	>99	Sigma Chemical Co.
5α-Dihydrotestosterone	521-18-6	99	Tokyo Kasei Kogyo, Co.
Dichlorodiphenyldichloroethane	72-54-8	97.2	Aldrich Co.
Cyproterone acetate	427-51-0	100.3	Sigma Chemical Co.
6α-Methyl-17α-hydroxy-progesterone	520-85-4	97.4	Sigma Chemical Co.
Atrazine	1912-24-9	99.9	Kanto Chemical Co.
Spironolactone	52-01-7	99.5	Across Organics