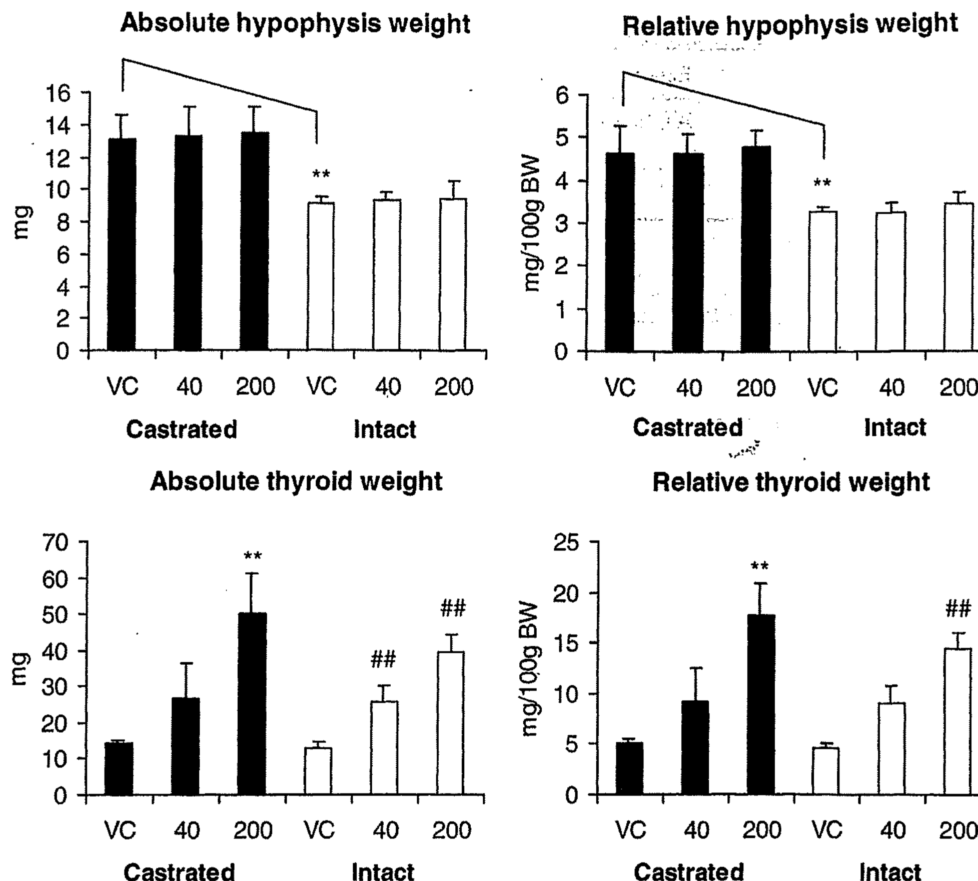

Fig. 2 Hypophyseal and thyroid weights of AT-treated castrated and intact male rats. **Significantly different from the castrated vehicle control at $p < 0.01$. ## Significantly different from the intact vehicle control at $p < 0.01$

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

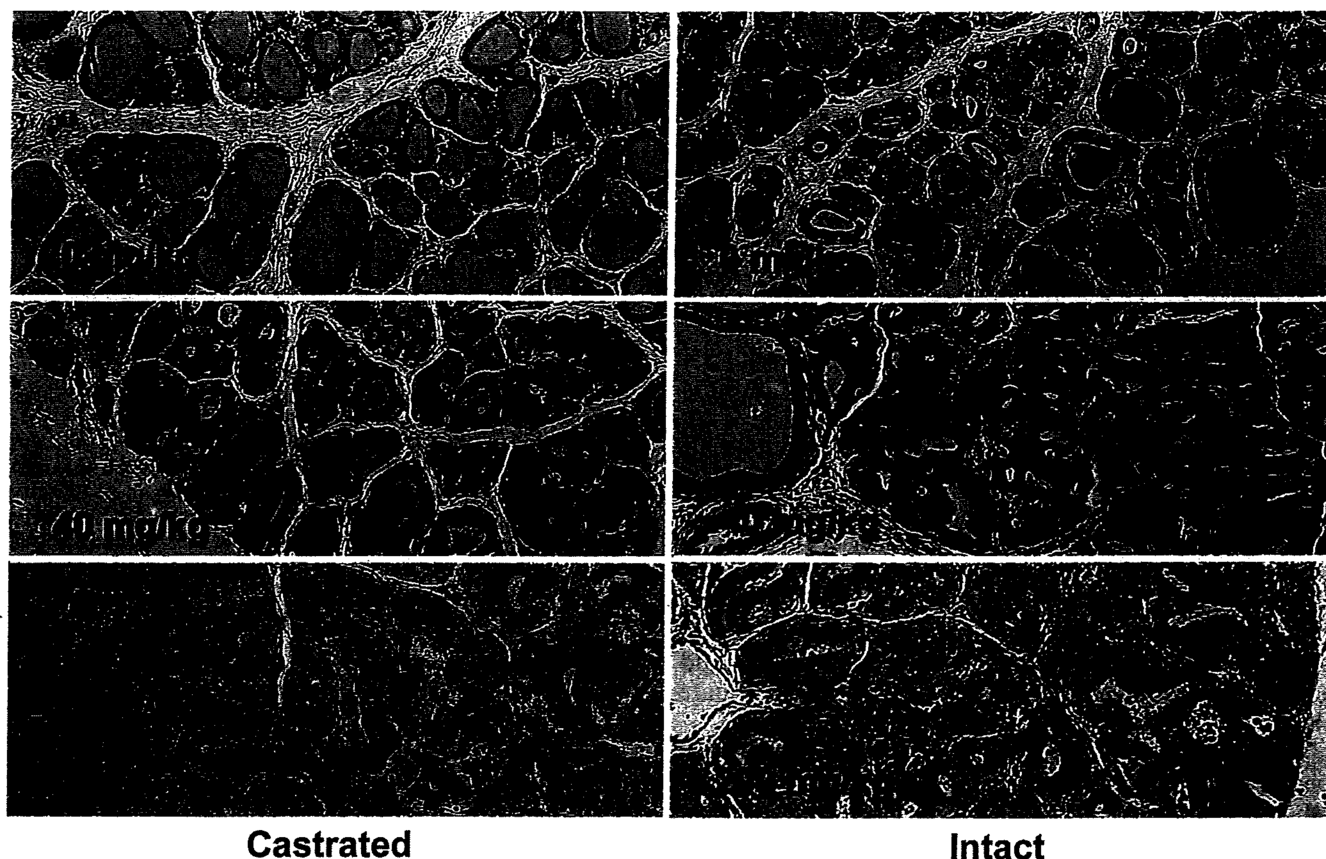

Fig. 3 Micrographs of the thyroid gland of an AT-treated castrated rat and an AT-treated intact male rat. Magnification (125 x)

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

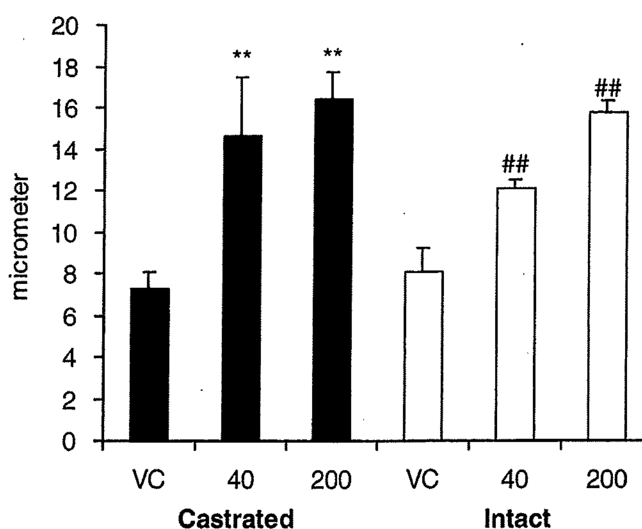

Fig. 4 Height of thyroid follicular epithelial cells of an AT-treated castrated rat and an AT-treated intact male rat. **Significantly different from the castrated vehicle control at $p<0.01$. ## Significantly different from the intact vehicle control at $p < 0.01$

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.

Acknowledgements This study was supported by a Grant-in-Aid from the Ministry of Economy, Trade and Industry, Japan.

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

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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.
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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

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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

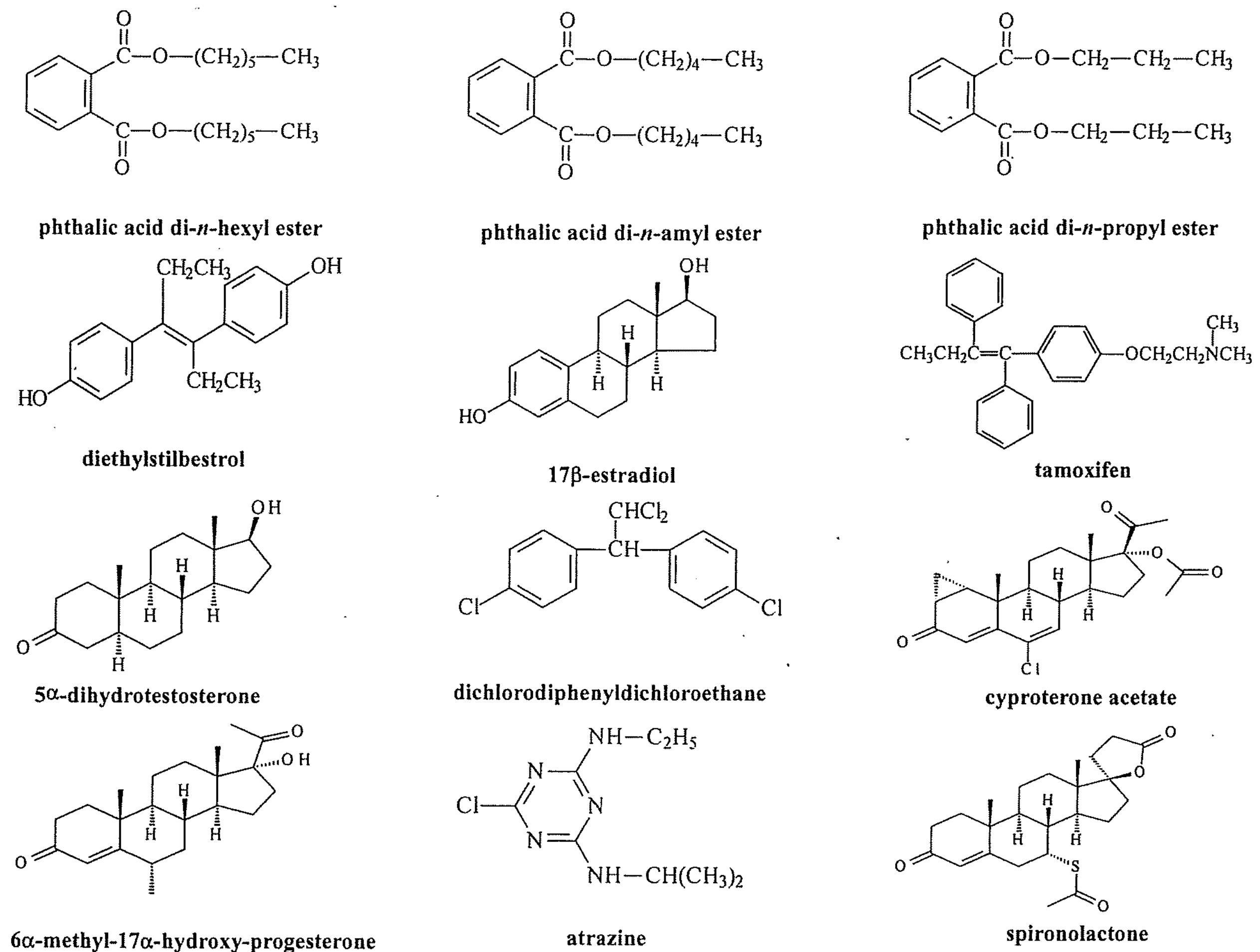

Fig. 1. Chemical structures of text chemicals.

acetate, 6α-methyl-17α-hydroxy-progesterone, and spironolactone have an androgenic steroid structure and are known to have androgen antagonistic properties; and atrazine is a reference endocrine disruptor (Friedmann, 2002; Gray et al., 2001; Laws et al., 2000). All chemicals were dissolved in olive oil (Fujimi Pharmaceutical, Company, Osaka, Japan) before use.

2.1.2. Animals

Male Brl Han: WIST Jcl (GALAS) rats castrated at 42-days of age were purchased from Clea Japan, Inc. (Shizuoka, Japan) and housed three per cage in stainless steel wire-mesh cages throughout the study. After allowing 14-days to recover from the operation, the rats were weighed, weight-ranked, and randomly assigned to each of the experimental and control groups. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water automatically and given ad libitum access to a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan). The animal room was maintained at a temperature of 23 ± 2 °C and relative humidity of 55 ± 5%, and was artificially illuminated with fluorescent light on a 12-h light/dark cycle (0600–1800 h). 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.

2.1.3. Study design

Each chemical was orally administered via a stomach tube for 10 consecutive days beginning on postnatal day 56. A vehicle control group given only olive oil was also established. Testosterone propionate (TP,

CAS No. 57-63-6, 98% purity, Sigma Chemical Co.), 0.2 mg/kg/day, was administered to some rats by subcutaneous injection in the back after oral administration of each chemical, and a positive control injected with TP was also established. A group given the androgen antagonist flutamide, 10 mg/kg/day, plus TP was established to confirm the reliability of the study. Each group consisted of six rats. The doses of each chemical were selected based on the results of a preliminary study. In the preliminary study, each chemical was orally administered to castrated rats of the same strain for 7-days beginning on postnatal day 56, and the no observed adverse effect level (NOAEL) in the preliminary study was selected as the highest dosage in the 10-day administration study. However, increased body weight gain was observed in rats given some chemicals in the 10-day administration study. The volume of the olive oil solution containing TP was 0.2 ml/kg/day, and the volume of the olive oil solution containing the chemical was 5 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 h after the final dose. The ventral prostate with fluid, seminal vesicle with fluid, bulbocavernosus/levator ani muscle (BC/LA), glans penis, and Cowper's gland were carefully dissected free of adhering fat and weighed.

2.1.4. Statistical analysis

Differences in body weight and organ weight between the vehicle group and each of the chemical groups and between the vehicle-plus-TP group and each of the chemicals plus-TP groups were assessed for statistical significance by the two-tailed Student's *t*-test.

2.2. Receptor binding assay

A recombinant human androgen receptor ligand binding domain (hAR-LBD), which expressed in *Escherichia coli* as a fused protein with maltose binding protein, was purchased from Toyobo Co., Ltd (Tokyo, Japan). It was supplied as a solution of 5.13 nM, determined from binding affinity of tritium-labeled mibolerone, in 50 mM pipes (piperazine-1,4-bis(2-ethanesulfonic acid)) buffer (pH 7.4).

The stock solutions (10 mM) of the test substance and dihydrotestosterone as a standard ligand were prepared with DMSO and diluted to 10 times higher concentration than test solution with tris–HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM leupeptin (binding buffer). Each 10 μl of the test substance and radiolabeled ligand ([1,2,4,5,6,7-3H]dihydrotestosterone, Amersham Biosciences K.K.) solutions and 30 μl of the hAR-LBD solution were mixed with 50 μl of the binding buffer. After the solution was incubated for 1 h at 25 °C, free radiolabeled ligand was removed by incubation with 100 μl of 0.4% dextran-coated charcoal (SIGMA-Aldrich Co., USA) suspension in the binding buffer for 10 min at 4 °C, followed by filtration. The radioactivity in the filtrate was measured using liquid scintillation counter. More than four final concentrations were set in the suitable range in 10^{-11}–10^{-4} M to determine the IC_{50} value for each test substance depending on the binding affinity. The final concentration of hAR-LBD was 1.5 nM.

The percent ratio (B/B_0 (%)) of radiolabeled ligand bound to the receptor was calculated from the radioactivities of the solutions with (B) and without (B_0) the test substance subtracting the radioactivity due to nonspecifically bound ligand. The B/B_0 value versus concentration of test substance curve was fit to the logistic equation and IC_{50} value of each chemical was calculated by least-squares method using the computer program GraphPad Prism®. The binding abilities of test chemicals to the receptor were evaluated by relative binding affinity (RBA), ratio of IC_{50} values to standard ligand (dihydroteststeron).

3. Results

3.1. Hershberger assay

3.1.1. Clinical signs and body weights

Final body weights are shown in Table 2.

No abnormal clinical signs were detected in any of the rats.

A significant decrease in body weight gain was observed in rats given 0.05 mg/kg diethylstilbestrol and 0.05 mg/kg diethylstilbestrol plus TP, 2.0 mg/kg 17β-estradiol and 2.0 mg/kg 17β-estradiol plus TP, 0.1 mg/kg tamoxifen and 0.1 mg/kg tamoxifen plus TP, 10 mg/kg cyproterone acetate plus TP, 100 mg/kg

Table 2
Body weight and absolute accessory sex organ weight

Chemicals	RAB (% of DHT)	Dosages (mg/kg/day)	Body weight (g)	Vental prostate (mg)	Seminal vesicle (mg)	BC/LA (mg)	Glans penis (mg)	Cowper's gland (mg)
Phthalic acid di-*n*-hexyl ester	not binding	Vehicle control	270.8 ± 9.0	16.4 ± 2.7	22.4 ± 3.7	147.9 ± 24.7	39.8 ± 3.2	4.7 ± 1.3
		40	274.4 ± 11.2	12.0 ± 4.5	26.1 ± 6.1	126.1 ± 21.7	40.5 ± 4.7	4.6 ± 2.1
		200	268.2 ± 9.6	17.1 ± 6.3	25.3 ± 3.2	146.4 ± 17.1	42.7 ± 1.8	4.7 ± 0.4
		1000	275.0 ± 9.7	14.8 ± 2.6	24.1 ± 4.5	130.8 ± 22.7	41.7 ± 5.4	4.2 ± 1.1
		Vehicle + TP	282.5 ± 12.7	111.0 ± 18.6	216.8 ± 59.2	321.5 ± 39.1	71.7 ± 6.2	20.3 ± 4.8
		40 + TP	281.6 ± 11.0	114.6 ± 26.3	271.6 ± 44.8	325.9 ± 39.8	67.4 ± 3.7	22.2 ± 4.0
		200 + TP	284.1 ± 13.9	105.6 ± 25.4	212.0 ± 45.1	337.2 ± 16.0	71.5 ± 7.0	20.0 ± 2.0
		1000 + TP	277.8 ± 12.6	94.1 ± 23.2	178.2 ± 31.0	315.0 ± 20.1	68.2 ± 6.0	19.8 ± 1.1
		FT + TP	279.2 ± 12.2	16.9 ± 2.9**	26.0 ± 4.7**	146.5 ± 25.6**	41.4 ± 4.3**	4.7 ± 0.7**
Phthalic acid di-*n*-amyl ester	not binding	Vehicle control	271.8 ± 10.5	13.1 ± 2.8	21.9 ± 4.5	145.7 ± 31.8	37.7 ± 4.5	3.6 ± 1.1
		40	269.4 ± 10.6	15.3 ± 3.8	25.0 ± 3.2	135.7 ± 19.1	41.3 ± 3.9	4.1 ± 0.8
		200	268.1 ± 10.3	13.1 ± 4.1	22.9 ± 4.5	143.6 ± 30.8	39.7 ± 3.7	4.2 ± 1.4
		1000	275.2 ± 13.2	13.0 ± 2.4	24.8 ± 3.5	136.9 ± 21.3	41.2 ± 3.6	4.3 ± 1.7
		Vehicle + TP	274.7 ± 15.3	108.3 ± 10.5	189.9 ± 25.6	343.2 ± 32.4	72.1 ± 5.0	25.2 ± 5.2
		40 + TP	280.5 ± 11.2	98.8 ± 21.7	190.6 ± 36.3	315.6 ± 49.9	69.4 ± 6.2	17.1 ± 3.4**
		200 + TP	282.0 ± 6.2	108.3 ± 19.0	158.0 ± 28.1	297.4 ± 27.5*	68.0 ± 4.3	20.4 ± 2.0
		1000 + TP	279.7 ± 8.3	76.7 ± 15.2**	147.6 ± 13.3**	285.9 ± 20.9**	70.7 ± 4.6	18.4 ± 4.7*
		FT + TP	271.4 ± 15.8	16.8 ± 3.9**	23.2 ± 4.1**	154.8 ± 21.6**	40.6 ± 4.1**	4.3 ± 0.9**
Phthalic acid di-*n*-propyl ester	not binding	Vehicle control	262.4 ± 11.6	14.0 ± 3.0	28.9 ± 5.8	134.1 ± 23.3	37.4 ± 3.7	4.2 ± 1.5
		40	261.4 ± 11.6	11.3 ± 3.5	24.0 ± 6.3	127.7 ± 10.5	36.8 ± 1.1	3.7 ± 0.9
		200	261.0 ± 11.2	14.5 ± 4.6	23.4 ± 5.1	139.8 ± 16.9	37.4 ± 2.7	4.3 ± 1.1
		1000	262.0 ± 14.9	11.7 ± 3.9	24.2 ± 5.4	115.9 ± 9.2	37.1 ± 3.3	3.8 ± 0.8
		Vehicle + TP	265.0 ± 6.0	87.9 ± 12.8	140.6 ± 22.0	298.4 ± 39.5	65.3 ± 5.6	16.6 ± 2.7
		40 + TP	270.1 ± 16.1	92.0 ± 17.2	140.3 ± 27.6	321.6 ± 62.1	64.6 ± 4.3	19.7 ± 4.9
		200 + TP	267.9 ± 10.3	92.4 ± 13.5	168.2 ± 31.7	338.8 ± 31.7	68.7 ± 4.9	21.4 ± 0.9**
		1000 + TP	272.2 ± 7.8	88.7 ± 22.1	156.0 ± 46.4	284.2 ± 20.1	67.4 ± 5.8	17.4 ± 3.3
		FT + TP	270.2 ± 9.9	15.9 ± 2.7**	28.3 ± 5.4**	139.3 ± 23.1**	39.6 ± 2.2**	3.9 ± 1.1**
Diethylstilbestrol	0.00539	Vehicle control	267.7 ± 12.0	13.2 ± 1.9	24.7 ± 3.1	134.3 ± 17.3	38.5 ± 4.6	4.3 ± 1.0
		0.002	268.4 ± 9.3	14.9 ± 1.8	27.2 ± 4.1	150.9 ± 21.1	38.3 ± 4.8	5.1 ± 0.9
		0.01	259.9 ± 6.7	14.4 ± 2.2	24.6 ± 4.3	143.9 ± 26.8	40.1 ± 4.2	4.0 ± 0.7
		0.05	248.2 ± 11.9****	14.6 ± 3.4	31.0 ± 4.0****	122.6 ± 18.9	41.6 ± 4.3	5.4 ± 1.1
		Vehicle + TP	280.2 ± 8.8	105.8 ± 19.9	221.6 ± 27.0	341.9 ± 42.3	68.8 ± 5.9	21.2 ± 3.1
		0.002 + TP	273.8 ± 12.1	109.8 ± 15.4	239.8 ± 37.0	338.6 ± 43.7	71.2 ± 6.1	22.8 ± 3.7
		0.01 + TP	269.2 ± 14.6	102.5 ± 16.6	226.5 ± 31.8	328.5 ± 16.4	70.9 ± 3.4	21.3 ± 4.7
		0.05 + TP	252.1 ± 12.0**	76.4 ± 10.2**	209.0 ± 50.9	289.1 ± 48.0	67.7 ± 5.8	19.3 ± 3.5
		FT + TP	269.3 ± 11.7	20.1 ± 2.2**	34.6 ± 8.3**	141.5 ± 18.1**	43.7 ± 6.1**	5.7 ± 1.3**

Table 2 (*Continued*)

Chemicals	RAB (% of DHT)	Dosages (mg/kg/day)	Body weight (g)	Vental prostate (mg)	Seminal vesicle (mg)	BC/LA (mg)	Glans penis (mg)	Cowper's gland (mg)
17β-Estradiol	2.56	Vehicle control	275.3 ± 14.1	14.3 ± 4.5	25.8 ± 3.6	150.9 ± 19.3	40.4 ± 3.4	4.0 ± 0.7
		0.1	276.3 ± 15.1	15.7 ± 2.4	26.9 ± 4.6	138.6 ± 11.3	41.2 ± 4.9	4.9 ± 1.1
		0.4	271.3 ± 9.2	15.3 ± 4.2	28.9 ± 8.6	145.4 ± 22.4	39.6 ± 1.4	5.0 ± 0.8****
		2.0	253.2 ± 8.2***	16.3 ± 1.7	28.1 ± 3.3	126.4 ± 11.6****	41.2 ± 5.4	4.1 ± 0.9
		Vehicle + TP	283.8 ± 13.8	121.8 ± 21.9	258.0 ± 39.7	349.2 ± 27.8	73.2 ± 3.1	23.9 ± 5.3
		0.1 + TP	285.1 ± 10.3	122.0 ± 9.9	255.2 ± 45.8	350.0 ± 31.4	71.1 ± 1.3	22.3 ± 4.8
		0.4 + TP	277.3 ± 9.9	102.3 ± 11.2	232.5 ± 31.5	365.3 ± 50.6	70.7 ± 7.6	21.0 ± 3.3
		2.0 + TP	257.0 ± 13.7**	99.0 ± 22.2	208.1 ± 61.3	294.0 ± 59.8	64.0 ± 4.2**	19.4 ± 6.7
		FT + TP	277.9 ± 13.5	18.1 ± 2.3**	24.7 ± 5.7**	150.0 ± 16.8**	38.9 ± 2.6**	4.6 ± 0.8**
Tamoxifen	0.0129	Vehicle control	279.4 ± 17.8	14.0 ± 3.6	26.2 ± 5.1	132.0 ± 18.9	42.1 ± 3.7	3.7 ± 1.6
		0.004	275.8 ± 13.8	16.8 ± 3.2	27.8 ± 6.9	139.7 ± 29.9	41.1 ± 5.2	4.8 ± 1.6
		0.02	265.0 ± 12.7	14.8 ± 4.5	23.7 ± 5.2	135.0 ± 11.0	38.5 ± 4.3	4.5 ± 1.4
		0.1	253.2 ± 11.5****	14.0 ± 3.0	22.6 ± 1.8	125.9 ± 21.0	39.5 ± 4.0	5.0 ± 0.8
		Vehicle + TP	289.3 ± 14.9	110.8 ± 14.5	237.0 ± 53.1	349.4 ± 48.9	68.2 ± 3.2	22.5 ± 3.1
		0.004 + TP	288.9 ± 14.9	120.9 ± 10.8	244.3 ± 35.9	349.2 ± 39.8	70.9 ± 6.3	23.6 ± 3.4
		0.02 + TP	279.2 ± 14.9	123.9 ± 13.2	250.3 ± 39.5	337.6 ± 36.5	71.3 ± 6.0	21.2 ± 5.6
		0.1 + TP	258.6 ± 7.9**	112.6 ± 22.2	268.7 ± 50.7	318.5 ± 50.8	71.0 ± 6.0	22.4 ± 3.3
		FT + TP	289.8 ± 10.2	18.1 ± 5.7**	23.1 ± 3.8**	150.8 ± 19.3**	42.5 ± 3.6**	4.7 ± 0.5**
5α-Dihydrotestosterone	100	Vehicle control	262.3 ± 17.2	16.2 ± 1.5	26.8 ± 5.9	141.2 ± 20.0	40.4 ± 2.0	5.0 ± 1.3
		8	261.0 ± 11.0	16.1 ± 1.9	23.5 ± 3.7	125.6 ± 8.9	40.9 ± 3.2	4.5 ± 0.8
		40	258.3 ± 9.1	27.9 ± 9.9****	27.4 ± 6.3	143.7 ± 19.1	44.3 ± 6.4	4.4 ± 1.1
		200	252.2 ± 19.1	63.6 ± 18.9***	90.8 ± 44.3****	230.3 ± 59.0****	58.2 ± 6.4***	11.9 ± 2.7***
		Vehicle + TP	266.6 ± 11.1	102.5 ± 16.5	231.6 ± 42.4	362.7 ± 28.2	70.2 ± 4.4	25.3 ± 3.7
		8 + TP	272.8 ± 13.4	110.1 ± 20.5	237.8 ± 56.6	325.0 ± 33.1	67.4 ± 1.8	20.3 ± 3.5*
		40 + TP	270.2 ± 14.9	108.6 ± 6.1	235.9 ± 43.1	333.2 ± 49.5	70.5 ± 3.7	21.5 ± 3.5
		200 + TP	266.3 ± 18.0	105.9 ± 17.8	274.0 ± 33.5	357.6 ± 33.4	73.5 ± 4.3	26.9 ± 4.3
		FT + TP	267.2 ± 14.4	20.4 ± 2.9**	27.8 ± 5.3**	147.8 ± 17.8**	42.1 ± 4.5**	5.1 ± 2.3**
Dichlorodiphenyl-dichloroethane	0.0139	Vehicle control	268.3 ± 14.5	19.7 ± 5.8	28.9 ± 7.6	131.8 ± 13.3	42.1 ± 4.5	4.9 ± 1.5
		8	270.9 ± 16.7	14.4 ± 2.8	26.2 ± 10.0	144.8 ± 14.3	37.6 ± 4.2	4.6 ± 0.5
		40	271.3 ± 12.7	14.5 ± 2.3	23.2 ± 3.7	144.4 ± 12.9	39.0 ± 3.0	3.8 ± 0.7
		200	263.6 ± 14.3	15.9 ± 3.1	26.1 ± 5.7	122.9 ± 11.7	40.2 ± 3.0	4.4 ± 0.5
		Vehicle + TP	276.8 ± 15.6	106.8 ± 14.4	235.0 ± 34.9	328.7 ± 53.4	71.3 ± 3.4	23.2 ± 4.0
		8 + TP	281.1 ± 13.0	121.5 ± 14.4	231.2 ± 63.0	349.3 ± 44.7	72.0 ± 5.7	23.1 ± 3.5
		40 + TP	272.7 ± 11.3	99.2 ± 23.6	202.7 ± 31.7	305.5 ± 39.4	69.5 ± 4.1	22.0 ± 2.0
		200 + TP	270.0 ± 11.3	95.9 ± 10.0	155.0 ± 19.1**	272.7 ± 24.7*	68.1 ± 2.3	21.5 ± 3.4
		FT + TP	268.3 ± 18.9	17.9 ± 1.8**	24.0 ± 2.3**	136.1 ± 29.8**	40.2 ± 3.3**	4.3 ± 1.6**

Cyproterone acetate	11.5	Vehicle control	270.0 ± 16.2	13.8 ± 3.9	24.9 ± 4.0	142.8 ± 8.2	39.3 ± 3.3	4.5 ± 1.0
		0.4	261.8 ± 14.3	15.8 ± 2.2	25.4 ± 1.9	131.0 ± 16.8	39.2 ± 3.7	4.7 ± 1.0
		2	250.7 ± 14.9	16.6 ± 2.1	26.8 ± 5.5	114.9 ± 17.2***	39.1 ± 2.4	4.6 ± 0.8
		10	242.3 ± 19.0	13.6 ± 1.8	22.3 ± 3.1	120.7 ± 8.5***	38.7 ± 1.4	3.1 ± 0.8****
		Vehicle + TP	271.3 ± 18.8	91.9 ± 11.0	153.2 ± 25.6	315.8 ± 35.8	67.3 ± 2.9	21.4 ± 3.6
		0.4 + TP	269.7 ± 15.8	74.3 ± 9.2*	119.1 ± 30.7	261.6 ± 20.7**	59.6 ± 3.0**	17.2 ± 1.6*
		2 + TP	252.4 ± 13.2	41.4 ± 7.7**	64.0 ± 10.2**	175.7 ± 28.7**	54.7 ± 9.0**	9.5 ± 1.7**
		10 + TP	248.1 ± 10.6**	23.8 ± 2.4**	32.2 ± 5.9**	129.2 ± 15.3**	42.3 ± 6.5**	5.4 ± 1.4**
		FT + TP	272.6 ± 19.0	20.2 ± 1.8**	25.2 ± 2.6**	133.4 ± 26.5**	41.2 ± 3.4**	5.0 ± 0.7**
6α-Methyl-17α-hydroxy-progesterone	5.86	Vehicle control	274.3 ± 14.9	17.2 ± 1.7	25.2 ± 3.0	140.2 ± 11.6	39.0 ± 2.9	5.8 ± 0.7
		20	260.8 ± 13.6	16.5 ± 0.9	26.9 ± 2.9	128.6 ± 21.7	41.5 ± 2.6	5.5 ± 2.1
		100	253.2 ± 13.2****	14.4 ± 2.3****	23.2 ± 2.6	119.1 ± 21.8	37.5 ± 1.6	4.4 ± 1.2****
		500	257.3 ± 14.0	16.5 ± 2.5	25.2 ± 4.0	120.9 ± 12.4****	39.9 ± 2.3	4.7 ± 1.2
		Vehicle + TP	276.6 ± 6.6	104.8 ± 8.2	201.4 ± 32.7	328.7 ± 43.7	70.3 ± 2.0	22.8 ± 3.2
		20 + TP	271.5 ± 15.8	96.8 ± 12.3	200.0 ± 23.6	308.9 ± 28.1	70.2 ± 3.5	21.4 ± 5.1
		100 + TP	271.0 ± 11.2	83.3 ± 17.8*	188.3 ± 39.7	259.6 ± 35.3*	65.1 ± 5.5	18.0 ± 2.0*
		500 + TP	262.1 ± 12.8*	61.9 ± 15.0**	105.8 ± 19.9**	219.7 ± 51.1**	60.3 ± 4.5**	14.9 ± 4.9**
		FT + TP	280.8 ± 14.0	19.7 ± 1.4**	27.6 ± 3.5**	147.6 ± 22.0**	43.3 ± 3.0**	5.0 ± 0.4**
Atrazine	not binding	Vehicle control	277.2 ± 16.5	15.1 ± 2.6	25.2 ± 4.5	141.9 ± 37.7	38.6 ± 3.0	5.2 ± 1.5
		3.2	273.5 ± 19.9	16.4 ± 1.8	26.7 ± 3.5	134.1 ± 20.5	38.8 ± 3.0	5.0 ± 1.8
		16	276.2 ± 13.2	17.2 ± 2.6	26.1 ± 3.0	147.9 ± 23.3	41.1 ± 3.4	4.6 ± 0.6
		80	258.5 ± 10.2****	15.1 ± 2.6	24.4 ± 2.3	127.2 ± 16.3	40.3 ± 3.1	4.9 ± 0.9
		Vehicle + TP	284.5 ± 11.0	108.5 ± 29.2	211.2 ± 73.4	314.0 ± 39.9	69.8 ± 6.2	21.9 ± 3.4
		3.2 + TP	292.3 ± 16.8	107.8 ± 14.0	206.3 ± 26.8	328.9 ± 25.4	70.2 ± 8.4	22.1 ± 2.2
		16 + TP	276.3 ± 12.2	103.2 ± 13.4	218.1 ± 44.9	319.3 ± 45.0	69.4 ± 4.7	23.8 ± 4.2
		80 + TP	272.4 ± 14.3	97.5 ± 8.1	231.1 ± 70.3	320.7 ± 42.0	69.5 ± 3.4	20.1 ± 3.6
		FT + TP	293.6 ± 12.5	17.5 ± 4.4**	25.3 ± 2.8**	153.6 ± 16.7**	41.1 ± 3.1**	4.7 ± 1.0**
Spironolactone	2.70	Vehicle control	275.2 ± 14.0	17.0 ± 2.4	29.3 ± 8.5	155.8 ± 26.3	41.0 ± 4.2	5.3 ± 2.0
		8	276.0 ± 11.1	16.5 ± 1.4	27.7 ± 3.4	158.8 ± 30.6	39.4 ± 2.1	5.2 ± 1.4
		40	276.6 ± 11.9	16.7 ± 2.6	26.8 ± 5.4	141.2 ± 13.7	40.2 ± 2.5	4.6 ± 0.5
		200	263.3 ± 14.2	17.5 ± 2.2	27.5 ± 4.9	144.9 ± 35.1	41.3 ± 2.4	4.8 ± 1.1
		Vehicle + TP	280.1 ± 16.6	109.7 ± 12.0	231.1 ± 39.8	326.3 ± 40.2	69.7 ± 4.7	22.6 ± 5.0
		8 + TP	276.7 ± 14.4	89.8 ± 9.9*	192.3 ± 32.9	305.2 ± 43.9	67.2 ± 4.2	18.8 ± 2.7
		40 + TP	275.9 ± 16.8	69.9 ± 12.1**	146.8 ± 36.0**	277.6 ± 24.1*	65.0 ± 2.4	17.6 ± 1.9
		200 + TP	269.4 ± 16.5	34.2 ± 7.6**	49.0 ± 14.3**	184.3 ± 13.8**	51.3 ± 4.2**	8.7 ± 1.6**
		FT + TP	279.8 ± 12.6	17.0 ± 2.2**	24.3 ± 4.2**	152.5 ± 13.8**	40.0 ± 2.9**	5.2 ± 2.2**

RBA, receptor binding affinity; DHT, dihydrotestosterone; TP, testosterone propionate; FT, flutamide; BC/LA, Bulbocavernosus and levator ani muscles.

* Significantly different from vehicle control + TP at $P < 0.05$.

** Significantly different from vehicle control + TP at $P < 0.01$.

*** Significantly different from vehicle control at $P < 0.01$.

**** Significantly different from vehicle control at $P < 0.05$.

6α-methyl-17α-hydroxy-progesterone and 500 mg/kg 6α-methyl-17α-hydroxy-progesterone plus TP, and 80 mg/kg atrazine.

3.1.2. Organ weight

Absolute accessory sex organ weights are shown in Table 2, and these were essential the same as the relative organ weight changes.The weight of all accessory sex organs increased significantly in the rats given 5α-dihydrotestosterone, but they decreased significantly in the rats given cyproterone acetate, 6α-methyl-17α-hydroxy-progesterone, and spironolactone. The weight of the accessory sex organs except the glans penis decreased significantly in rats given phthalic acid di-*n*-amyl ester, and the weight of some accessory sex organs decreased significantly in the rats given dichlorodiphenyldichloroethane.

Seminal vesicle weight increased significantly in the high diethylstilbestrol dosage group.

Despite the significant decrease in absolute ventral prostate weight in the high diethylstilbestrol dosage plus TP group, decrease in absolute BC/LA weight in the high 17β-estradiol dosage group, decrease in absolute weight of glans penis in the high 17β-estradiol dosage plus TP group, decrease in absolute BC/LA weight in the middle and high cyproterone acetate dosage groups, decrease in absolute weight of the Cowper's gland in the high cyproterone acetate dosage group, and decrease in absolute BC/LA in the high 6α-methyl-17α-hydroxy-progesterone dosage group, no relative weight changes in these organs were detected within the same dosage group (relative weight changes not shown). The decreases in absolute weight of some accessory sex organs in response to some chemicals without any change in the relative organs were considered to be attributable to the decreases in body weight gain induced by these chemicals.

No organ weight changes or dose-related changes were detected in the rats given phthalic acid di-*n*-hexyl ester, phthalic acid di-*n*-propyl ester, tamoxifen, and atrazine.

3.2. Receptor binding assay

The results of the receptor binding assays are shown in Table 2.

Eight of 12 chemicals, diethylstilbestrol, 17β-estradiol, tamoxifen, 5α-dihydrotestosterone, dichlorodiphenyldichloroethane, cyproterone acetate, 6α-methyl-17α-hydroxy-progesterone, and spironolactone, were positive in the androgen receptor binding assay.

4. Discussion

The OECD proposed the Hershberger assay as a screening method to detect the androgenic properties of endocrine disrupting chemicals, and this has been reported to be useful in this regard (OECD, 2000, 2003). In the present study, we performed Hershberger assays of 12 various chemicals. The weights of the accessory sex organs of rats given TP were higher than those of rats given the vehicle alone, and the organ weights of rats given flutamide plus TP were lower than those of rats given TP alone, thereby confirming the reliability of this study.

The weight of all or some of the accessory sex organs decreased significantly in rats given cyproterone acetate plus TP, spironolactone plus TP, 6α-methyl-17α-hydroxy-progesterone plus TP, and dichlorodiphenyldichloroethane plus TP, and thus these chemicals are thought to have androgen antagonist affinity. The antagonist affinity of cyproterone acetate and spironolactone was higher than that of the other chemicals. The receptor binding affinity of 6α-methyl-17α-hydroxy-progesterone and spironolactone was 5.86 and 2.70, respectively, and weight changes in accessory sex organs were detected at 100 mg/kg/day 6α-methyl-17α-hydroxy-progesterone and at 8 mg/kg/day spironolactone. These findings indicate that (anti)androgen potency according to the receptor binding assay does not completely correspond to potency according to the Hershberger assay. Metabolism and other unknown factors present in the in vivo tests are thought to be related to this phenomenon, and differences in pharmaco/toxicokinetics between the in vitro and in vivo tests are also suggested. Hershberger assay and receptor binding assay using many chemicals are needed to clarify the relationship between two assays.

Increased seminal vesicle weight was detected in the high estrogenic compound "diethylstilbestrol" group in this study. However, whether diethylstilbestrol has an androgenic effect remains uncertain. In our previous Hershberger assay, the weight of some male accessory sex organs increased in rats given the

typical estrogenic compounds ethinyl estradiol, equilin, norgestrel, or estrone, but we could not determine whether these chemicals exhibit androgen agonistic properties (Yamasaki et al., 2003), because estrogen and androgen receptors are said to be present in the accessory sex organs of male rats and mice (Re et al., 2001; Weihua et al., 2001; Williams et al., 2001). On the other hand, the receptor binding affinity of other estrogenic compounds, i.e., 17β-estradiol and tamoxifen, was higher than that of diethylstilbestrol, but no androgenic properties of these two estrogenic compounds were detected in the Hershberger assays. The androgenic affinity detected in the Hershberger assay may not be present in diethylstilbestrol.

Di(*n*-butyl)phthalate has been reported to inhibit the endocrine mediated effects in male rats after in gestational and lactational exposure without the androgen receptor affinity (Mylchreest et al., 1999). In the present study, the Hershberger assays of three phthalates, phthalic acid di-*n*-hexyl ester, phthalic acid di-*n*-amyl ester, and phthalic acid di-*n*-propyl ester, were performed at the same dosages, and the results showed weak androgen antagonistic affinity only in the assay of phthalic acid di-*n*-amyl ester. We did not investigate why only phthalic acid di-*n*-amyl ester has androgen antagonist affinity. The chemical structure of these compounds is similar, and only their number of CH_2 groups differs. Phthalic acid di-*n*-amyl ester contains many more CH_2 groups than phthalic acid di-*n*-propyl ester and fewer than phthalic acid di-*n*-hexyl ester, and there may be some relationship between the number of CH_2 groups and androgen antagonistic affinity. On the other hand, it is noteworthy that no receptor binding affinity was detected in these three phthalates. The positive result of phtalic acid di-*n*-amyl ester in the Hershberger assay may be related to the testosterone metabolism in the liver.

A clear androgen agonistic effect was detected in 5α-dihydrotestosterone in the present study. Androgen agonistic affinity of androgen derivatives testosterone enanthate and methyltestosterone were also detected in the Hershberger assay in our previous study (Yamasaki et al., 2003). Therefore, it is suggested that the androgen agonistic effect in androgenic chemicals is detected in the Hershberger assay.

Atrazine has been reported to inhibit testosterone production in rat males following peripubertal exposure (Friedmann, 2002), and atrazine is said to affect the pituitary/hypothalamic axis (USEPA, 2002). Thus, it was not surprising that no endocrine disrupter property of atrazine was detected in the Hershberger assay in castrated rats.

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OECD Validation of the Hershberger Assay in Japan: Phase 2 Dose Response of Methyltestosterone, Vinclozolin, and *p,p'*-DDE

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The Organisation for Economic Co-operation and Development has initiated the development of new guidelines for the screening and testing of potential endocrine disruptors. 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, the 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 program employs a range of additional androgen agonists and antagonists as well as 5α-reductase inhibitors. Seven Japanese laboratories have contributed phase 2 validation studies of the Hershberger assay using methyltestosterone, vinclozolin, and 2,2-bis (4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE). The methyltestosterone doses were 0, 0.05, 0.5, 5, and 50 mg/kg/day, and the vinclozolin and *p,p'*-DDE doses were 0, 3, 10, 30, and 100 mg/kg/day. All chemicals were orally administered by gavage for 10 consecutive days. In the antagonist version of the assay using vinclozolin and *p,p'*-DDE, 0.2 mg/kg/day of testosterone propionate was coadministered by subcutaneous injection. All five accessory sex preproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects. *Key words:* Hershberger assay, methyltestosterone, OECD validation, *p,p'*-DDE, vinclozolin. *Environ Health Perspect* 111:1912–1919 (2003). doi:10.1289/ehp.6357 available via *http://dx.doi.org/* [Online 10 September 2003]

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 Organisation 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, 2001, 2003).

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 co-workers, and a number of accessory sex organs and tissues were shown to be useful by these and other investigators, including the ventral prostate (Deanesly and Parkes 1936; Dingemanse et al. 1935; Korenchevsky 1932; Korenchevsky et al. 1932, 1933a, 1933b), the seminal vesicles and coagulating glands (Deanesly and Parkes 1936; Dingemanse et al. 1935; Korenchevsky 1932; Korenchevsky et al. 1932, 1933a, 1933b), the preputial glands (Bülbring and Burn 1935; Korenchevsky 1932; Korenchevsky et al. 1932, 1933a, 1933b), the 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, 1933b). In the 1940s, it was discovered that the levator ani and bulbocavernosus muscles also responded to androgens, but in a different way from the other tissues (Eisenberg and Gordan 1950; Eisenberg et al. 1949; Wainman and Shipounoff 1941). 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 that were also administered a set of doses of the purported antagonist. 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 U.S. Endocrine Disruptor Screening and Testing Advisory Committee (U.S. EPA 1998) and the OECD Endocrine Disrupter Testing and Assessment Working Group (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 ventral prostate, seminal vesicles with coagulating glands, levator ani and bulbocavernosus muscle complex, Cowper's glands, and glans penis was successfully tested against a reference androgen compound, testosterone propionate (TP), and a reference antagonist, flutamide (OECD 2001). Therefore, the OECD proposed a phase 2 validation program using additional androgen agonists and antagonists as the next step to validate the assay.

In phase 2, the selected androgens were methyltestosterone (MT) and trenbolone; the selected antagonists were vinclozolin (VCZ), procymidone, linurone, and 2,2-bis (4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE); and the 5α-reductase inhibitor was finasteride. These test substances will be used to investigate the reliability of the assay, including a demonstration of the protocol's

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transferability among laboratories and the reproducibility of the protocol's results. Seven Japanese laboratories participated in the phase 2 validation study that used three of the selected compounds: MT, VCZ, and *p,p*′-DDE. The participation of the laboratories in the OECD phase 2 validation study was performed as part of a national validation program in Japan.

Materials and Methods

Laboratories. The seven participating Japanese laboratories were the Chemicals Evaluation and Research Institute (CERI); the Food Drug Safety Center; the Institute of Environmental Toxicology; the Japan Bioassay Research Center; Mitsubishi Chemical Safety Institute; Panapham Co., Ltd.; and Sumitomo Chemical Company Ltd. Each laboratory performed in compliance with principles of good laboratory practice.

Test substances. The test substances were methyltestosterone (MT; CAS No. 58-18-4; 99.8% pure; Fluka Production GmbH, St. Louis, MO, USA), vinclozolin (VCZ; CAS No. 50471-44-8; 99% pure; Kanto Chemical Co., Tokyo, Japan), and *p,p*′-DDE (CAS No. 72-55-9; 99.5% pure; Sigma-Aldrich Co., St. Louis, MO, USA). Testosterone propionate (TP; CAS No. 57-85-2; 97% pure; Fluka) was used as a reference positive chemical control and was coadministered with VCZ and *p,p*′-DDE to detect androgen antagonistic effects. MT, *p,p*′-DDE, and TP were obtained from a centralized chemical repository at TNO (Zeist, the Netherlands) and distributed through CERI to each laboratory; VCZ was obtained by CERI and distributed to each laboratory in the study. All laboratories used corn oil as the vehicle. The test substances used in each laboratory are shown in Table 1.

Animals. Laboratory details regarding rat strain, age at castration, number of postoperative acclimation days, age at autopsy, animal diet, and the number of animals housed per cage are summarized in Table 1. Five laboratories used Crj:CD (SD) (Sprague-Dawley) castrated rats from Charles River Japan, Inc. (Kanagawa/Shiga, Japan) between the ages of 40 and 46 days, and the test substances were administered 7–11 days after castration. Two laboratories used Brl Han: WIST Jcl (GALAS) castrated rats from Japan Clea, Inc. (Tokyo, Japan) between the ages of 40 and 43 days, and the test substances were administered 6 or 7 days after castration. In all of the laboratories, the rats were weighed, weight-ranked, and assigned to each of the experimental and control groups after they had recovered from castration. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water and a commercial diet (MF or CRF-1, Oriental Yeast Co., Tokyo, Japan) *ad libitum.* The animals were kept under specific-pathogen–free conditions. The animal room was maintained at a temperature of 23 ± 2°C, a relative humidity of 55 ± 15%, and artificial illumination with fluorescent light on a 12-hr light/dark cycle. 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 (1992).

Chemical administration. Each test chemical 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 all cases. For the androgen antagonists (VCZ and *p,p*′-DDE), 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. In these cases, a positive control group of animals received TP injections alone. We selected the dose of TP on the basis of OECD recommendations and published data (OECD 2001; Sunami et al. 2000). The group size in all cases was six rats. For the TP and corn oil solutions containing each of the test chemicals, the volume of corn oil was 5 mL/kg. The MT doses were 0.05, 0.5, 5, and 50 mg/kg/day, and the VCZ and *p,p*′-DDE doses were 3, 10, 30, and 100 mg/kg/day. All doses were selected based on the results of preliminary, range-finding studies. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 hr after receiving their final dose. The five mandatory tissues—the ventral prostate and

Table 2. Weights of optional organs from rats given MT, VCZ, or *p,p*′-DDE.

Chemical	Lab	Animal/tissue	Dose				
MT							
MT (mg/kg/day)			0	0.05	0.5	5	50
	1	Terminal body wt (g)	309.2	318.5	313.4	315.6	317.0
		Liver (g)	12.5	13.5	13.1	12.7	13.9
		Adrenals (mg)	50.5	60.4	56.1	48.1	45.3
		Kidneys (g)	2.2	2.4	2.3	2.3	2.5
	3	Terminal body wt (g)	282.7	288.6	293.1	290.4	288.7
		Liver (g)	12.0	12.0	11.7	11.3	12.3
	4	Terminal body wt (g)	287.3	286.0	281.4	293.4	294.2
		Liver (g)	12.3	12.4	11.7	13.0	13.8
		Adrenals (mg)	52.8	46.7	49.0	49.0	41.1*
		Kidneys (g)	2.0	2.2	2.0	2.2	2.4*
VCZ							
TP (mg/kg/day)			0.2	0.2	0.2	0.2	0.2
VCZ (mg/kg/day)			0	3	10	30	100
	1	Terminal body wt (g)	338.4	344.7	340.3	347.4	334.0
		Liver (g)	14.5	14.3	14.5	15.2	15.5
		Adrenals (mg)	54.9	56.0	60.8	66.9	72.9*
		Kidneys (g)	2.4	2.4	2.4	2.5	2.5
p,p′-DDE							
TP (mg/kg/day)			0.2	0.2	0.2	0.2	0.2
p,p′-DDE (mg/kg/day)			0	3	10	30	100
	4	Terminal body wt (g)	292.7	291.0	290.4	293.2	289.4
		Liver (g)	12.8	12.4	14.3	16.5*	20.1*
		Adrenals (mg)	47.2	46.8	45.7	47.6	51.2
		Kidneys (g)	2.2	2.1	2.2	2.3	2.3
	7	Terminal body wt (g)	240.9	241.4	241.4	238.0	235.8
		Kidneys (g)	2.1	2.1	2.2	2.1	2.1

*Significantly different from control at $p < 0.05$.

Table 1. Laboratory details for test compounds and animals.

Laboratory	Test compound(s)	Rat strain	Age at castration (days)	Postoperative acclimation days	Age at autopsy (days)	Diet	No. per cage
1	MT, VCZ	Crj:CD (SD)[a]	41–44	11	62–65	CRF-1	1
2	MT, VCZ, *p,p*′-DDE	Crj:CD (SD)[a]	40–44	8	59–63	MF	1
3	MT	Crj:CD (SD)[b]	41–43	7	59–61	MF	3
4	MT, *p,p*′-DDE	Crj:CD (SD)[b]	42–44	7	59–61	CRF-1	2
5	VCZ, *p,p*′-DDE	Crj:CD (SD)[b]	43–46	7	61–64	MF	2
6	VCZ, *p,p*′-DDE	Brl Han: WIST Jcl (GALAS)	41–43	7	59–61	MF	3
7	*p,p*′-DDE	Brl Han: WIST Jcl (GALAS)	40–42	6	57–59	MF	3

[a]Facility in Kanagawa, Japan. [b]Facility in Shiga, Japan.

Table 3. Mean body weights and mean organ weights in rats given MT: data and log-transformed data.

Lab		MT (mg/kg/day) 0	0.05	0.5	5	50
1	Starting body wt (g)	260.0	259.8	259.3	249.8	258.9
	Terminal body wt (g)	309.2	318.5	313.4	315.6	317.0
	Ventral prostate (mg)	21.1	20.4	18.7	40.6	135.0*
	Seminal vesicles (mg)	45.2	43.7	43.3	65.7	248.2*
	BC/LA (mg)	192.3	198.0	198.1	253.2*	460.5*
	Glans penis (mg)	51.4	55.6	53.4	64.8*	83.2*
	Cowper's glands (mg)	6.5	7.8	7.6	10.8*	25.3*
2	Starting body wt (g)	227.5	227.3	226.9	228.0	226.7
	Terminal body wt (g)	297.6	291.2	292.6	294.6	299.1
	Ventral prostate (mg)	12.6	14.4	21.4	45.3	128.3*
	Seminal vesicles (mg)	52.5	50.2	48.1	70.7	278.5*
	BC/LA (mg)	236.3	218.9	228.7	287.3	533.8*
	Glans penis (mg)	48.9	50.8	49.9	55.0*	73.3*
	Cowper's glands (mg)	6.5	6.8	7.6	11.0	27.2*
3	Starting body wt (g)	218.4	218.8	219.3	219.4	218.2
	Terminal body wt (g)	282.7	288.6	293.1	290.4	288.7
	Ventral prostate (mg)	19.3	22.1	26.2	51.1*	158.2*
	Seminal vesicles (mg)	44.2	52.9	66.2	108.0*	312.6*
	BC/LA (mg)	201.5	203.6	212.6	254.2	482.0*
	Glans penis (mg)	62.6	60.8	61.9	74.4	94.7*
	Cowper's glands (mg)	8.1	7.5	9.5	11.8*	22.1*
4	Starting body wt (g)	227.4	227.4	227.8	228.5	227.3
	Terminal body wt (g)	287.3	286.0	281.4	293.4	294.2
	Ventral prostate (mg)	19.2	20.8	19.5	32.3*	150.9*
	Seminal vesicles (mg)	39.7	37.3	34.8	41.2	184.4*
	BC/LA (mg)	206.3	199.8	203.6	243.8	487.1*
	Glans penis (mg)	52.8	53.1	53.7	56.8	83.5*
	Cowper's glands (mg)	6.5	7.4	5.8	7.6	26.3*
Log-transformed data						
1	Ventral prostate (mg)	1.3	1.3	1.3	1.6*	2.1*
	Seminal vesicles (mg)	1.7	1.6	1.6	1.8*	2.4*
	BC/LA (mg)	2.3	2.3	2.3	2.4*	2.7*
	Glans penis (mg)	1.7	1.7	1.7	1.8*	1.9*
	Cowper's glands (mg)	0.8	0.9	0.9	1.0	1.4*
2	Ventral prostate (mg)	1.1	1.1	1.3*	1.6*	2.1*
	Seminal vesicles (mg)	1.7	1.7	1.7	1.8	2.4*
	BC/LA (mg)	2.4	2.3	2.4	2.5*	2.7*
	Glans penis (mg)	1.7	1.7	1.7	1.7	1.9*
	Cowper's glands (mg)	0.8	0.8	0.9	1.0*	1.4*
3	Ventral prostate (mg)	1.3	1.3	1.4	1.7*	2.2*
	Seminal vesicles (mg)	1.6	1.7	1.8	2.0*	2.5*
	BC/LA (mg)	2.3	2.3	2.3	2.4*	2.7*
	Glans penis (mg)	1.8	1.8	1.8	1.9*	2.0*
	Cowper's glands (mg)	0.9	0.9	1.0	1.1*	1.3*
4	Ventral prostate (mg)	1.3	1.3	1.3	1.5*	2.2*
	Seminal vesicles (mg)	1.6	1.6	1.5	1.6	2.3*
	BC/LA (mg)	2.3	2.3	2.3	2.4	2.7*
	Glans penis (mg)	1.7	1.7	1.7	1.8	1.9*
	Cowper's glands (mg)	0.8	0.9	0.8	0.9	1.4*

*Significantly different from vehicle control at $p < 0.05$.

Table 4. Overall mean organ weights, R^2, and CV in rats given MT: data and log-transformed data.

Overall means	R^2 (%) TRT	LAB	CV (%)	MT (mg/kg/day) 0 (n = 22–24)	0.05 (n = 23–24)	0.5 (n = 22–24)	5 (n = 22–24)	50 (n = 24)
Overall								
Ventral prostate (mg)	90	1	25	18.0	19.4	21.5	42.3	143.1
Seminal vesicles (mg)	81	4	20	45.4	46.0	48.1	71.4	255.9
BC/LA (mg)	83	6	12	209.1	205.0	210.7	259.6	490.9
Glans penis (mg)	78	19	7	54.0	55.2	54.8	63.3	83.7
Cowper's glands (mg)	81	0	22	6.9	7.4	7.6	10.3	25.2
Overall log-transformed								
Ventral prostate (mg)	87	2	7.7	1.2	1.3	1.3	1.6	2.1
Seminal vesicles (mg)	83	6	4.7	1.7	1.6	1.7	1.8	2.4
BC/LA (mg)	87	2	2.2	2.3	2.3	2.3	2.4	2.7
Glans penis (mg)	77	21	1.8	1.7	1.7	1.7	1.8	1.9
Cowper's glands (mg)	78	2	10.3	0.8	0.9	0.9	1.0	1.4

Abbreviations: TRT, R^2 values for effects of treatments; LAB, R^2 values for effects among laboratories.

fluid, seminal vesicle and fluid, bulbocavernosus/levator ani muscle (BC/LA), glans penis, and Cowper's gland—were carefully dissected free of adhering fat and weighed to the nearest 0.1 mg. Six of the laboratories weighed the wet organs. One laboratory (Lab 4) weighed the prostate, seminal vesicle, Cowper's glands, and adrenal glands after approximately 24 hr fixation in 10% formalin solution, following the procedure of Yamada et al. (2000). The liver, paired kidneys, and paired adrenal glands were weighed as optional organs in some laboratories in each assay described in Table 2.

Statistical analysis. Body weight and organ weight data were tested using Bartlett's test for homogeneity of variance. When the variances were homogeneous at the 5% significance level, one-way analysis of variance (ANOVA) was performed. If it yielded significant differences, the differences between the vehicle control group and each of the MT groups or the positive control group and each of the VCZ and p,p'-DDE groups were analyzed by Dunnett's test. When the variances were not homogeneous, the Kruskal-Wallis test was used. If it yielded significant differences, the differences between each group and the corresponding control group were analyzed by the nonparametric Dunnett's test. Log-transformed organ-weight data were also tested by the same method. The coefficient of variance (CV) and R^2 values for the different effects of each compound were also calculated by dividing the sums of the squares of the ANOVA scores for an effect by the total sum of the squares. This calculation provides an estimate of the strength of an effects association with an end point. Data for each end point were also analyzed using a two-way ANOVA, with dosage and laboratory as the main effects, so that the magnitude of the overall dosage and laboratory effects could be determined. For graphic presentation, the sex accessory organ data were normalized to visually compare the shapes of the dose–response curves produced by each laboratory. For this normalization, the control value was set to 100% in the MT assay, and 100% in the TP without VCZ or p,p'-DDE assays. ANOVA was performed on the data from each laboratory and for the pooled laboratory data; these normalized values were not analyzed statistically.

Results

Methyltestosterone. Body weights, clinical observations, and organ weights. The weight changes in optional organs and the body weights on the first day of dosing and at necropsy are shown in Tables 2 and 3. No significant differences in body weight were observed between the vehicle control group and the MT group in each laboratory. No abnormal clinical signs were observed in any

of the rats that were treated with MT. The paired kidney weights increased significantly at 50 mg/kg/day MT in Lab 4, and adrenal weights decreased at the same dose in Lab 4.

Accessory sex organ weights. Accessory sex organ weight changes and overall means are shown in Tables 3 and 4, and normalized organ weight changes are shown in Figure 1.

For the ventral prostate, the normalized dose–response curves produced by the four laboratories were similar, and the weight change at 50 mg/kg/day MT relative to the vehicle control ranged from 641% to 1,022%. This was the largest weight change observed in any of the examined organs. The R^2 values for effects of treatments (TRT) in the ventral prostate was higher than the respective TRT values for other organs.

The normalized dose–response curves produced by the four laboratories were similar for the seminal vesicle; the weight change ranged from 465% to 707% at 50 mg/kg/day MT relative to the vehicle control.

For BC/LA, the normalized dose–response curves produced by the four laboratories were almost the same, and the weight change at 50 mg/kg/day MT relative to the vehicle control ranged from 226% to 240%.

The normalized dose–response curves produced by the four laboratories were similar for the glans penis, and the weight change at 50 mg/kg/day MT relative to the vehicle control ranged from 150% to 162%. Although the range between the low and high relative weight changes in animals receiving 50 mg/kg/day MT was narrow, the relative weight increase at this dose was the smallest of the weight changes in all of the accessory sex organs that were examined. The average CV for the glans penis was the lowest of all the average values obtained for the other organs. The R^2 values for effects among laboratories (LAB) for the glans penis was the highest value obtained among the accessory sex organs examined in this study.

Table 5. Mean body weights and mean organ weights in rats given 0.2 mg/kg/day TP and VCZ: data and log-transformed data.

Lab		VCZ (mg/kg/day) 0	3	10	30	100
1	Starting body wt (g)	273.4	273.6	273.3	274.8	273.3
	Terminal body wt (g)	338.4	344.7	340.3	347.4	334.0
	Ventral prostate (mg)	136.5	118.8	91.3	60.7*	36.4*
	Seminal vesicles (mg)	393.5	358.5	248.7*	174.5*	60.7*
	BC/LA (mg)	533.9	511.5	441.9*	381.8*	257.8*
	Glans penis (mg)	91.1	88.9	79.8*	76.8*	64.0*
	Cowper's glands (mg)	32.7	32.7	24.3*	20.2*	12.4*
2	Starting body wt (g)	241.6	241.5	240.1	241.1	241.0
	Terminal body wt (g)	326.8	327.7	320.8	319.6	319.1
	Ventral prostate (mg)	97.2	111.6	105.1	79.4	34.1*
	Seminal vesicles (mg)	361.7	335.9	321.0	210.8*	71.8*
	BC/LA (mg)	537.7	500.5	485.2	416.0*	275.2*
	Glans penis (mg)	81.7	75.9	73.7	69.8	58.5*
	Cowper's glands (mg)	28.0	26.8	21.1*	20.1*	11.2*
5	Starting body wt (g)	247.6	247.6	248.3	248.0	247.7
	Terminal body wt (g)	340.6	337.0	338.8	333.5	335.3
	Ventral prostate (mg)	183.6	149.7*	136.7*	98.2*	51.0*
	Seminal vesicles (mg)	420.8	458.7	344.3	247.7	96.4*
	BC/LA (mg)	590.4	608.8	529.3	430.7*	308.6*
	Glans penis (mg)	76.4	78.0	77.7	70.2	52.7*
	Cowper's glands (mg)	38.6	36.0	32.9	25.9*	16.2*
6	Starting body wt (g)	229.5	229.6	229.7	229.4	229.2
	Terminal body wt (g)	291.1	289.9	286.2	286.3	289.2
	Ventral prostate (mg)	106.4	98.9	84.1	75.3	38.9*
	Seminal vesicles (mg)	216.7	221.6	168.4*	116.2*	47.3*
	BC/LA (mg)	361.3	320.6	323.9	268.0*	181.8*
	Glans penis (mg)	70.1	69.9	67.3	64.7	51.4*
	Cowper's glands (mg)	20.8	21.1	19.5	15.1	7.4*
Log-transformed data						
1	Ventral prostate (mg)	2.1	2.1	1.9*	1.8*	1.5*
	Seminal vesicles (mg)	2.6	2.6	2.4*	2.2*	1.8*
	BC/LA (mg)	2.7	2.7	2.6	2.6*	2.4*
	Glans penis (mg)	2.0	1.9	1.9*	1.9*	1.8*
	Cowper's glands (mg)	1.5	1.5	1.4*	1.3*	1.1*
2	Ventral prostate (mg)	1.9	2.0	2.0	1.9	1.5*
	Seminal vesicles (mg)	2.6	2.5	2.5	2.3*	1.8*
	BC/LA (mg)	2.7	2.7	2.7	2.6*	2.4*
	Glans penis (mg)	1.9	1.9	1.9	1.8*	1.8*
	Cowper's glands (mg)	1.4	1.4	1.3	1.3	1.0*
5	Ventral prostate (mg)	2.3	2.2	2.1*	2.0*	1.7*
	Seminal vesicles (mg)	2.6	2.7	2.5*	2.4*	2.0*
	BC/LA (mg)	2.8	2.8	2.7	2.6*	2.5*
	Glans penis (mg)	1.9	1.9	1.9	1.8	1.7*
	Cowper's glands (mg)	1.6	1.5	1.5	1.4*	1.2*
6	Ventral prostate (mg)	2.0	2.0	1.9	1.9	1.6*
	Seminal vesicles (mg)	2.3	2.3	2.2	2.1*	1.7*
	BC/LA (mg)	2.6	2.5	2.5	2.4*	2.3*
	Glans penis (mg)	1.8	1.8	1.8	1.8	1.7*
	Cowper's glands (mg)	1.3	1.3	1.3	1.2*	0.9*

*Significantly different from vehicle control at $p < 0.05$.

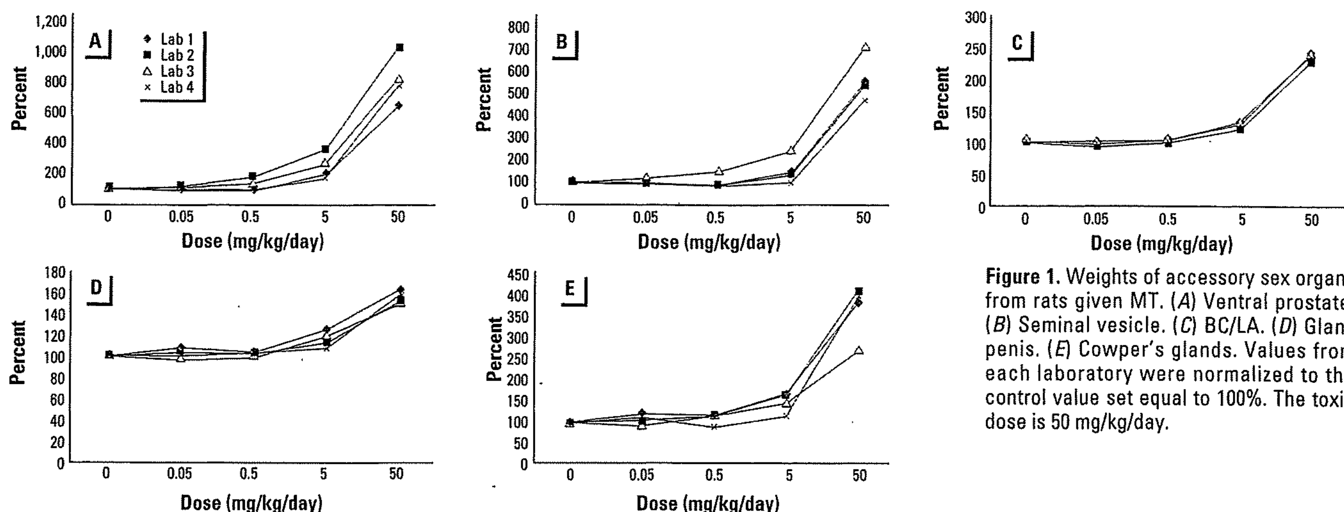

Figure 1. Weights of accessory sex organs from rats given MT. (*A*) Ventral prostate. (*B*) Seminal vesicle. (*C*) BC/LA. (*D*) Glans penis. (*E*) Cowper's glands. Values from each laboratory were normalized to the control value set equal to 100%. The toxic dose is 50 mg/kg/day.

For the Cowper's glands, the normalized dose–response curves produced by the four laboratories were similar, and the weight change ranged from 273% to 417% at 50 mg/kg/day MT relative to the vehicle control.

Vinclozolin. **Body weights, clinical general observations, and organ weights.** The weight changes in optional organs and the body weight changes for VCZ-treated rats are shown in Tables 2 and 5. No significant differences in body weight were observed between the positive control group that received TP injections alone and the VCZ group in any of the laboratories. No abnormal clinical signs were observed in any of the rats treated with VCZ plus TP. Weight of the paired adrenal glands increased significantly at 100 mg/kg/day, and no other significant changes were detected in the liver and paired kidneys.

Accessory sex organ weights. Weight changes in accessory sex organs and overall means are shown in Tables 5 and 6, and normalized organ weight changes are shown in Figure 2.

For the ventral prostate, the normalized dose–response curves produced by the four laboratories were similar. The ventral prostate weight changes at 100 mg/kg/day VCZ relative to the positive control ranged from 27% to 37%.

The normalized dose–response curves produced by the four laboratories were similar for seminal vesicles. The weight changes at 100 mg/kg/day VCZ relative to the positive control were similar, ranging from 15% to 23%. These values were the lowest of all the values for the accessory sex organs, and the decreasing dose–response curve for the seminal vesicle was sharper than the curves for the other organs.

For the BC/LA, the normalized dose–response curves produced by the four laboratories were similar, and the weight change at 100 mg/kg/day VCZ relative to the positive control were similar, ranging from 48% to 52%.

The normalized dose–response curves produced by the four laboratories were similar for the glans penis, and the weight change at 100 mg/kg/day VCZ relative to the positive control were similar, ranging from 69% to 73%. The overall CV value was the lowest among the values for the examined accessory sex organs.

The normalized dose–response curves produced by the four laboratories were similar for Cowper's glands. The weight change ranged from 36% to 42% at a dose of 100 mg/kg/day VCZ relative to the positive control.

p,p′-DDE. **Body weights, clinical observations, and organ weights.** The weight changes in optional organs and the body weight changes for *p,p′*-DDE–treated rats are shown in Tables 2 and 7. The body weight decreased significantly in the 100 mg/kg/day group of Lab 5, and a similar (but not significant) tendency was also observed in the 100 mg/kg/day group of Lab 2. No abnormal clinical signs were detected in any of the rats treated with *p,p′*-DDE plus TP. The liver weights increased significantly at 30 and 100 mg/kg/day in Lab 4. No significant changes were observed in other organs.

Accessory sex organ weights. Weight changes in accessory sex organs and overall means are shown in Tables 7 and 8, and normalized organ weight changes are shown in Figure 3.

For the ventral prostate, the normalized dose–response curves produced by the five laboratories were very similar, except for the curve produced by Lab 7 because of the value at 30 mg/kg/day *p,p′*-DDE. The weight change at a dose of 100 mg/kg/day relative to the positive control ranged from 37% to 62%.

The normalized dose–response curves produced by the laboratories were similar at 10, 30, and 100 mg/kg/day *p,p′*-DDE for seminal vesicle. The weight change of the seminal vesicles at 100 mg/kg/day relative to the positive control ranged from 23% to 54%. The dose–response curve for the seminal vesicle was the sharpest of the various curves produced for the

Table 6. Overall mean organ weights, R^2, and CV in rats given 0.2 mg/kg/day TP and VCZ: data and log-transformed data.

	R^2 (%)			VCZ (mg/kg/day)				
Overall means	TRT	LAB	CV (%)	0 (*n* = 24)	3 (*n* = 24)	10 (*n* = 24)	30 (*n* = 24)	100 (*n* = 24)
Overall								
Ventral prostate (mg)	60	16	19	130.9	119.8	104.3	78.4	40.1
Seminal vesicles (mg)	64	19	19	348.2	343.7	270.6	187.3	69.1
BC/LA (mg)	50	34	12	505.8	485.3	445.1	374.1	255.9
Glans penis (mg)	67	29	7	79.8	78.2	74.7	70.4	56.7
Cowper's glands (mg)	51	26	19	30.0	29.2	24.5	20.3	11.8
Overall log-transformed								
Ventral prostate (mg)	69	11	4.8	2.1	2.1	2.0	1.9	1.6
Seminal vesicles (mg)	77	14	3.8	2.5	2.5	2.4	2.2	1.8
BC/LA (mg)	54	34	2.0	2.7	2.7	2.6	2.6	2.4
Glans penis (mg)	70	27	1.8	1.9	1.9	1.9	1.8	1.8
Cowper's glands (mg)	59	22	6.6	1.5	1.5	1.4	1.3	1.0

Abbreviations: TRT, R^2 values for effects of treatments; LAB, R^2 values for effects among laboratories.

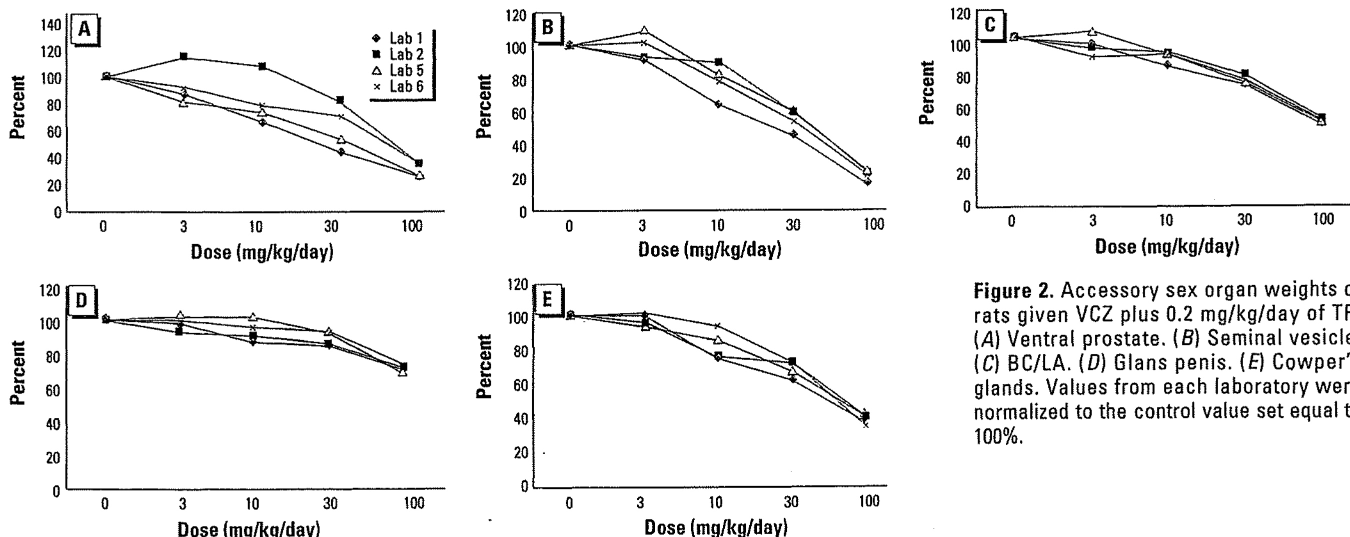

Figure 2. Accessory sex organ weights of rats given VCZ plus 0.2 mg/kg/day of TP. (*A*) Ventral prostate. (*B*) Seminal vesicle. (*C*) BC/LA. (*D*) Glans penis. (*E*) Cowper's glands. Values from each laboratory were normalized to the control value set equal to 100%.

accessory sex organs for *p,p*′-DDE. The TRT in the seminal vesicle was the highest value among the accessory sex organs measured in this study.

For BC/LA, the normalized dose–response curves produced by four laboratories were similar. The weight change ranged from 55% to 72% at 100 mg/kg/day *p,p*′-DDE relative to the positive control.

The normalized dose–response curves were similar in glans penis above a dose of 30 mg/kg/day *p,p*′-DDE. The weight change at 100 mg/kg/day *p,p*′-DDE relative to the positive control ranged from 79% to 86%, and this percentage was the highest among the values for the accessory sex organs receiving *p,p*′-DDE. The CV of the glans penis and the BC/LA were smaller than the values for the other organs. The TRT for the glans penis was the smallest of the values observed among the accessory sex organs in *p,p*′-DDE–treated rats.

For Cowper's glands, the normalized dose–response curves produced by the laboratories were similar above a dose of 30 mg/kg/day *p,p*′-DDE. The weight change at 100 mg/kg/day relative to the positive control ranged from 41% to 65%.

Discussion

Seven Japanese laboratories performed the Hershberger assay using MT, VCZ, and *p,p*′-DDE as part of a national validation program. The weights of all the accessory sex organs from the experimental animals in all the laboratories exhibited significant dose-related changes in the assays using agonistic MT or antagonistic VCZ and *p,p*′-DDE; the normalized dose–response curves showed that all five tissues reacted in a similar manner for each compound. Furthermore, the weights of all the tissues treated with middle and/or high doses in each assay fell within narrow ranges. Therefore, we consider the Hershberger assay, as proposed by the OECD, to be a good screening assay for detecting the androgen agonistic and antagonistic effects of chemicals.

The OECD proposed TP doses of 0.2 mg/kg/day and 0.4 mg/kg/day to detect antagonistic effects of chemicals based on the data from the OECD phase 1 validation of the Hershberger assay (OECD 2001). In the previous study, we used the 0.2 mg/kg/day dose of TP in Hershberger assays of 30 chemicals based on the OECD draft protocol and found that the accessory sex organ weights of the castrated rats were lower than those of castrated rats given TP, and the weights of these organs in rats given 10 mg/kg/day flutamide plus TP were also lower than in castrated rats given TP (Yamasaki et al. 2003). In addition, the weights of the accessory sex organs of the castrated rats were lower than those of castrated rats given 0.4 mg/kg/day TP, and their weights were also lower in noncastrated rats than in castrated rats given TP (Yamasaki et al. 2002). We selected the 0.2 mg/kg/day dose in this study, however, a dose of 0.4 mg/kg/day was used in the phase 2 validation studies except in Japan (OECD 2003). The sensitivity of this assay of antagonistic chemicals at the 0.2 mg/kg/day and 0.4 mg/kg/day doses needs to be compared.

The OECD phase 1 validation of the Hershberger assay using antagonistic flutamide reported that the seminal vesicle exhibited the most sensitive end point and that the glans

Table 7. Mean body weights and mean organ weights in rats given 0.2 mg/kg/day TP and *p,p*′-DDE: data and log-transformed data.

Lab		*p,p*′-DDE (mg/kg/day) 0	3	10	30	100
2	Starting body wt (g)	229.7	228.9	230.4	230.7	228.4
	Terminal body wt (g)	313.4	313.4	313.0	317.3	303.3
	Ventral prostate (mg)	137.8	125.7	128.9	93.6*	51.5*
	Seminal vesicles (mg)	387.2	272.3*	377.0	256.0*	88.3*
	BC/LA (mg)	549.9	521.9	519.4	458.5*	300.4*
	Glans penis (mg)	73.3	76.5	73.5	73.6	63.0*
	Cowper's glands (mg)	27.2	21.8	28.3	23.7	17.4*
4	Starting body wt (g)	224.6	223.8	223.6	222.5	224.0
	Terminal body wt (g)	292.7	291.0	290.4	293.2	289.4
	Ventral prostate (mg)	115.0	101.3	103.3	74.7*	48.7*
	Seminal vesicles (mg)	237.2	219.5	251.4	156.8*	82.7*
	BC/LA (mg)	495.5	496.6	450.9	395.1*	301.5*
	Glans penis (mg)	81.0	80.1	77.6	76.2	66.7*
	Cowper's glands (mg)	30.2	28.1	25.5	24.2	12.3*
5	Starting body wt (g)	233.2	232.6	231.8	233.0	232.8
	Terminal body wt (g)	319.4	326.9	322.9	323.6	307.3*
	Ventral prostate (mg)	153.3	158.4	165.9	141.8	77.6*
	Seminal vesicles (mg)	371.1	432.6	411.8	326.1	148.1*
	BC/LA (mg)	518.7	574.3	547.0	490.9	291.3*
	Glans penis (mg)	77.3	75.9	76.5	74.0	60.8*
	Cowper's glands (mg)	33.9	33.8	32.8	32.6	20.2*
6	Starting body wt (g)	217.1	217.1	216.2	217.0	218.2
	Terminal body wt (g)	276.6	270.2	272.6	274.7	273.0
	Ventral prostate (mg)	106.2	89.8	100.0	71.7*	52.5*
	Seminal vesicles (mg)	225.7	219.4	202.4	164.5	75.1*
	BC/LA (mg)	300.3	305.7	290.3	309.0	209.9*
	Glans penis (mg)	67.0	62.3	66.3	65.0	56.6*
	Cowper's glands (mg)	21.0	21.5	19.8	15.4*	11.0*
7	Starting body wt (g)	173.8	173.7	174.0	174.2	173.8
	Terminal body wt (g)	240.9	241.4	241.4	238.0	235.8
	Ventral prostate (mg)	90.6	79.1	88.1	89.6	56.5*
	Seminal vesicles (mg)	282.8	246.2	240.0	231.6	152.0*
	BC/LA (mg)	435.7	430.3	407.0	408.2	311.9*
	Glans penis (mg)	65.5	64.9	63.8	66.9	52.0*
	Cowper's glands (mg)	26.2	26.7	25.4	25.9	17.0*
Log-transformed						
2	Ventral prostate (mg)	2.1	2.1	2.1	2.0	1.7*
	Seminal vesicles (mg)	2.6	2.4	2.6	2.4	1.9*
	BC/LA (mg)	2.7	2.7	2.7	2.7*	2.5*
	Glans penis (mg)	1.9	1.9	1.9	1.9	1.8*
	Cowper's glands (mg)	1.4	1.3	1.4	1.4	1.2*
4	Ventral prostate (mg)	2.1	2.0	2.0	1.9*	1.7*
	Seminal vesicles (mg)	2.4	2.3	2.4	2.2*	1.9*
	BC/LA (mg)	2.7	2.7	2.7	2.6*	2.5*
	Glans penis (mg)	1.9	1.9	1.9	1.9	1.8*
	Cowper's glands (mg)	1.5	1.4	1.4	1.4	1.1*
5	Ventral prostate (mg)	2.2	2.2	2.2	2.1	1.9*
	Seminal vesicles (mg)	2.6	2.6	2.6	2.5	2.2*
	BC/LA (mg)	2.7	2.8	2.7	2.7	2.5*
	Glans penis (mg)	1.9	1.9	1.9	1.9	1.8*
	Cowper's glands (mg)	1.5	1.5	1.5	1.5	1.3*
6	Ventral prostate (mg)	2.0	2.0	2.0	1.8*	1.7*
	Seminal vesicles (mg)	2.3	2.3	2.3	2.2*	1.9*
	BC/LA (mg)	2.5	2.5	2.5	2.5	2.3*
	Glans penis (mg)	1.8	1.8	1.8	1.8	1.7*
	Cowper's glands (mg)	1.3	1.3	1.3	1.2*	1.0*
7	Ventral prostate (mg)	2.0	1.9	1.9	1.9	1.7*
	Seminal vesicles (mg)	2.4	2.4	2.4	2.4	2.2*
	BC/LA (mg)	2.6	2.6	2.6	2.6	2.5*
	Glans penis (mg)	1.8	1.8	1.8	1.8	1.7*
	Cowper's glands (mg)	1.4	1.4	1.4	1.4*	0.2*

*Significantly different from vehicle control at $p < 0.05$.