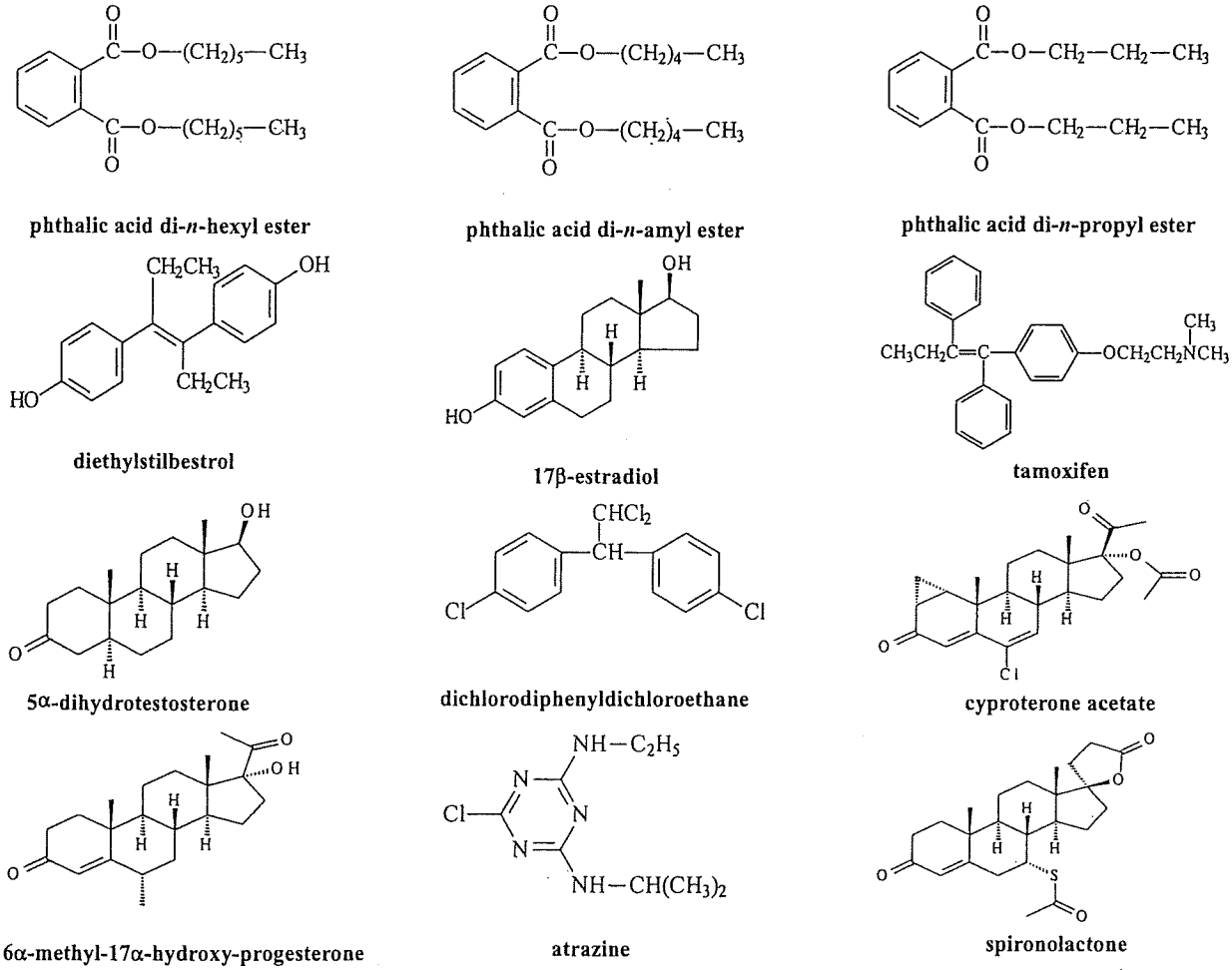

Fig. 1. Chemical structures of text chemicals.

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.

		VCZ (mg/kg/day)				
Lab		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 $p < 0.05$.

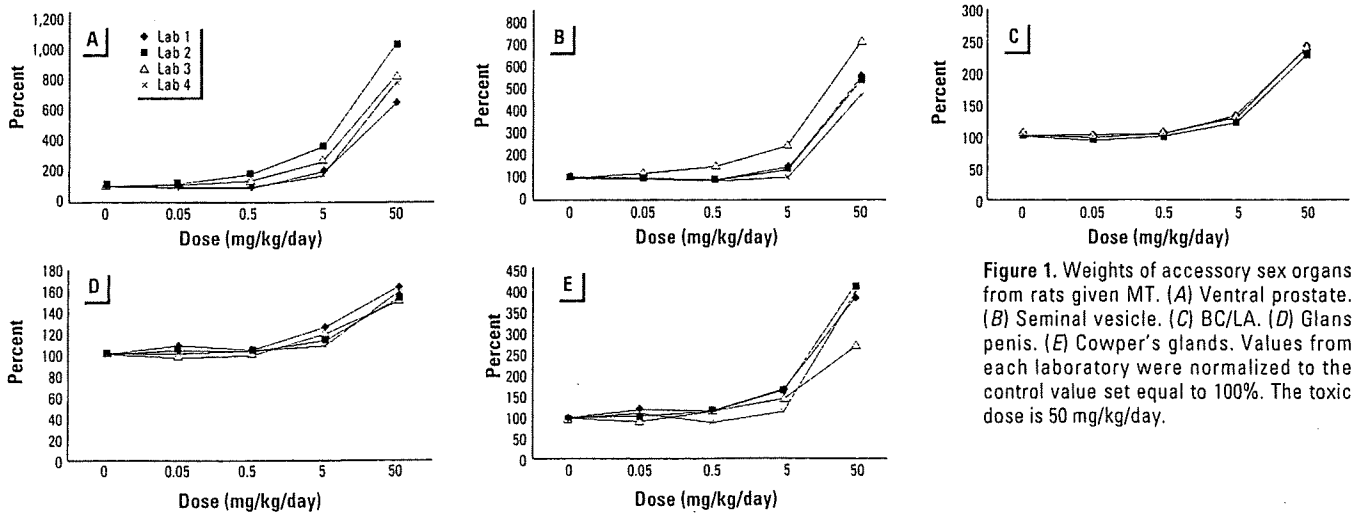

Figure 1. Weights of accessory sex organs from rats given MT. (*A*) Ventral prostate. (*B*) Seminal vesicle. (*C*) BC/LA. (*D*) Glans penis. (*E*) Cowper's glands. Values from each laboratory were normalized to the control value set equal to 100%. The toxic dose is 50 mg/kg/day.

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.

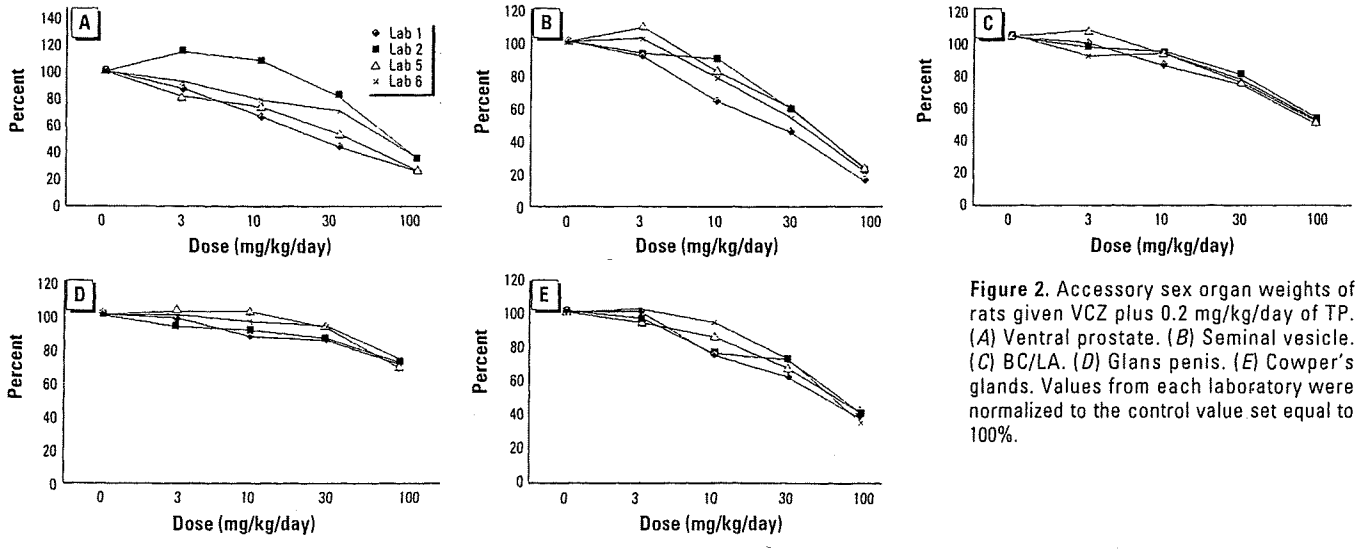

Figure 2. Accessory sex organ weights of rats given VCZ plus 0.2 mg/kg/day of TP. (*A*) Ventral prostate. (*B*) Seminal vesicle. (*C*) BC/LA. (*D*) Glans penis. (*E*) Cowper's glands. Values from each laboratory were normalized to the control value set equal to 100%.

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.

		p,p'-DDE (mg/kg/day)				
Lab		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 $p < 0.05$.

penis exhibited the least sensitive end point, based on benchmark dose estimates (OECD 2001). When the overall dose–response curves for agonistic TP were compared, the glans penis was the most sensitive and the seminal vesicle was the least sensitive (OECD 2001). In the present study, it was difficult to select a particularly sensitive organ from among the five tissues examined in the androgen agonistic MT and antagonistic VCZ and *p,p′*-DDE assays. In the Hershberger assay using MT, the CV for the glans penis was smaller than that of the other organs, but the TRT of the ventral prostate was the highest among the values measured in the study. On the other hand, the LAB values of the ventral prostate and Cowper's glands were smaller than the values of the other organs, and the percentage weight change relative to the control value at the highest dose was the greatest in the ventral prostate. These findings demonstrate that the ventral prostate was particularly sensitive based on the TRT, LAB, and increasing percentage of organ weight, whereas the glans penis was sensitive based on the CV values. Similarly, the seminal vesicle was sensitive based on the TRT, LAB, and decreasing percentage of organ weight, whereas the glans penis was sensitive based on the CV values in the assays using antagonistic VCZ and *p,p′*-DDE.

The CV values for the ventral prostate, seminal vesicle, and Cowper's gland were higher than those for the glans penis and BC/LA in the assays for all three chemicals. These organs contain fluid, and the dissection of these organs is technically difficult, compared with that of the glans penis and BC/LA. These technical issues may have influenced the varied CV values obtained for these organs. Furthermore, we did not confirm whether preputial separation had occurred in the rats before castration. Preputial separation has been reported to occur between days 39 and 44 in SD rats (Yamasaki et al. 2001); in this study, the castration was performed between days 40 and 46. Thus, the rats used in this study were likely a mixture of animals with or without preputial separation. The castration times may also have influenced the variation in the CV values for each organ.

In the assay using the androgen antagonistic chemicals, slight differences in the normalized response curves for low doses in the *p,p′*-DDE assay were observed among the laboratories, but the response curves for each organ in the VCZ assay were similar. The fact that the percentages of organ weight relative to the control at high doses in the *p,p′*-DDE assay were lower than those in the VCZ assay suggests that the androgen antagonistic affinity of *p,p′*-DDE is weaker than that of VCZ. On the other hand, the organ weights of the rats given only TP varied among the laboratories. The slight variation in responses among the laboratories for the low dose in the *p,p′*-DDE assay may have been affected by the relationship between the agonistic affinity of TP and the weak antagonistic affinity of *p,p′*-DDE.

In the phase 1 validation study using TP, the OECD reported that no essential differences were observed when the weights of the fresh and fixed organs were compared (OECD 2001). Lab 4 weighed the prostate, seminal vesicle, and Cowper's glands after fixation, whereas the other laboratories measured the weights of fresh organs; the changes in organ weight among the laboratories were essentially similar. Therefore, the difference in the weighing method (fresh vs. fixed organs) did not appear to affect the results of the assay. Although the terminal body weights were different between SD and Wistar rats, the responsiveness of these rats to VCZ and *p,p′*-DDE did not differ in this study. This finding demonstrates that no significant differences exist regarding the use of SD and Wistar rats in the Hershberger assay for the detection of androgen antagonists.

Among the optional organs measured in this study, the weight of the adrenal glands increased significantly in rats given 100 mg/kg/day of VCZ and decreased in rats given 50 mg/kg/day MT. The decrease in adrenal weight may be suppressed by a high dose of

Table 8. Overall mean organ weights, R^2, and CV in rats given 0.2 mg/kg/day TP and *p,p′*-DDE: data and log-transformed data.

	R^2 (%)			*p,p′*-DDE (mg/kg/day)				
Overall means	TRT	LAB	CV (%)	0 (*n* = 30)	3 (*n* = 30)	10 (*n* = 30)	30 (*n* = 30)	100 (*n* = 30)
Overall								
Ventral prostate (mg)	36	32	20	120.6	110.9	117.2	94.3	57.3
Seminal vesicles (mg)	45	31	19	300.8	278.0	296.5	227.0	109.2
BC/LA (mg)	37	42	10	460.0	465.8	442.9	412.3	283.0
Glans penis (mg)	30	37	7	72.8	71.9	71.5	71.1	59.8
Cowper's glands (mg)	33	29	19	27.7	26.4	26.4	24.4	15.6
Log-transformed								
Ventral prostate (mg)	48	23	4.4	2.1	2.0	2.1	2.0	1.7
Seminal vesicles (mg)	59	20	3.7	2.5	2.4	2.5	2.3	2.0
BC/LA (mg)	40	43	1.7	2.7	2.7	2.6	2.6	2.4
Glans penis (mg)	31	35	1.8	1.9	1.9	1.9	1.9	1.8
Cowper's glands (mg)	40	25	6.3	1.4	1.4	1.4	1.4	1.2

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

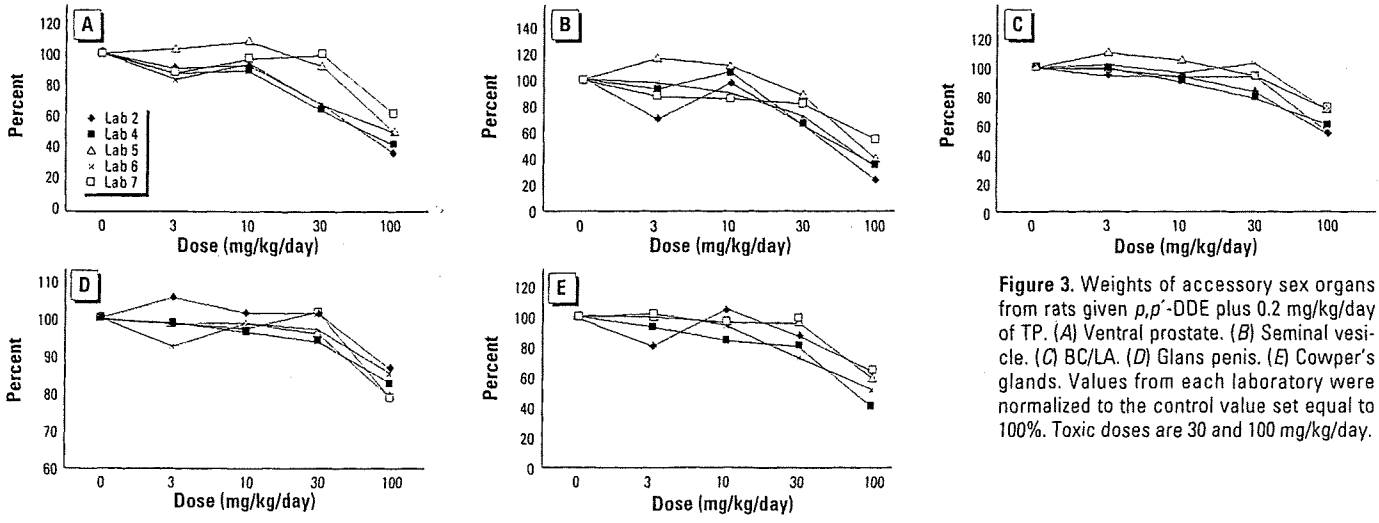

Figure 3. Weights of accessory sex organs from rats given *p,p′*-DDE plus 0.2 mg/kg/day of TP. (*A*) Ventral prostate. (*B*) Seminal vesicle. (*C*) BC/LA. (*D*) Glans penis. (*E*) Cowper's glands. Values from each laboratory were normalized to the control value set equal to 100%. Toxic doses are 30 and 100 mg/kg/day.

androgen in the form of MT, and the adrenal glands may be hypertrophied in response to a high level of antagonist. Increased kidney weights in rats given 50 mg/kg/day of MT and increased liver weights in rats given 30 and 100 mg/kg/day of *p,p′*-DDE suggested toxic effects. On the other hand, a significant decrease or a tendency to decrease of the body weights in the *p,p′*-DDE assay was observed by two out of five laboratories; this response was also considered to be a toxic effect of *p,p′*-DDE.

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Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals

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Abstract

We performed an immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals to assess the relationship between the results of two assays. The chemicals tested by the uterotopic assay were 4-n-amylphenol, p-dodecyl-phenol, p-(tert-pentyl)phenol, 4-cyclohexylphenol, 4-(1-adamantyl)phenol, 4,4′-thiobis-phenol, diphenyl-p-phenylenediamine, 4-hydroxyazobenzene, 4-(phenylmethyl)phenol, 4,4′-(hexafluoroisopropylidene)diphenol, 2,2-bis(4-hydroxyphenyl)-4-methyl-n-pentane, 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol, 4,4′-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 4-hydroxybenzophenone, 2,4,4′-trihydroxybenzophenone, testosterone enanthate, and methyltestosterone. The chemicals tested by the Hershberger assay were the 18 chemicals tested in the uterotrophic assay plus the following: 17alpha estradiol, estrone, equilin, norethindrone, norgestrel, ethynyl estradiol, bisphenol A, bisphenol B, bisphenol F, 4-tert-octylphenol, p-cumyl phenol, and nonylphenol. All chemicals examined in this study were positive in a reporter gene assay for ER-alpha. In the immature rat uterotrophic assay, all chemicals induced uterotrophy and p-(tert-pentyl)phenol, 4,4′-thiobis-phenol, 4-(phenylmethyl)phenol, 4,4′-(hexafluoroisopropylidene)diphenol, 2,2-bis(4-hydroxyphenyl)-4-methyl-n-pentane, 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol, 4,4′-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 4-hydroxybenzophenone, and 2,4,4′-trihydroxybenzophenone exerted both estrogen agonistic effect and reduced the estrogenic effect of ethynylestradiol. In the Hershberger assay, a clear androgen agonistic effect was detected in the androgen derivatives testosterone enanthate and methyltestosterone.

Keywords: Androgenic effect; Endocrine; Estrogenic effect; Hershberger assay; Rat; Uterotrophic assay

1. Introduction

There is concern that certain chemicals may have the potential to interfere with normal sexual differentiation and development in animals and humans (McLachlan, 1993; McLachlan and Korach, 1995), and the Organisation for Economic

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Cooperation and Development (OECD) has proposed the uterotrophic assay and the Hershberger assay as screening tests to detect estrogenic and androgenic properties of potentially endocrine disrupting chemicals (OECD, 2001). In the uterotrophic assay, the test method that chemical compounds are injected subcutaneously to immature female rats for 3 consecutive days is one of option among four standardized protocols, and validation studies were already finished. In the Hershberger assay, on the other hand, chemicals are orally administration to castrated rats for 10 days, and validation studies have been performed. Although there have been reports of estrogenic compounds displaying androgenic effects in toxicological studies (Atanassova et al., 1999; Yamasaki et al., 2002a), the relationship between the agonistic and antagonistic effects of the same estrogenic chemical or between the estrogenic and androgenic effects of the same chemical have never been adequately studied. We, therefore, performed uterotrophic assays and the Hershberger assay of 18 chemicals and Hershberger assay of 12 chemicals identified as having estrogenic properties in our previous study (Yamasaki et al., 2002b).

2. Materials and methods

The studies were performed under Good Laboratory Practice guidelines.

2.1. Uterotrophic assay

2.1.1. Chemicals

The chemicals tested in the uterotrophic assay are listed in Table 1, and chemical structures of test compounds are shown in Fig. 1. All chemicals were dissolved in olive oil (Fujimi Pharmaceutical, Osaka, Japan) before use. All of chemicals tested in the uterotrophic assay in this study were positive in the reporter gene assay for ER-alpha based on their PC10 values. Chemicals tested in this assay were selected based on the list of suspected endocrine disrupters published by the EU (Commission of the European Communities, 2001).

2.1.2. Animals

Crj:CD (SD) rats, dams and their 10-day-old pups, were purchased from Charles River Japan (Shiga, Japan). The dams and pups were housed in polycarbonate pens until weaning. All pups were weaned at 17 days of age and subsequently individually housed in stainless steel wire-mesh cages throughout the study. The immature rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with tap water and a commercial diet (CRF-1, Oriental Yeast, Tokyo, Japan) ad libitum before weaning, and with water automatically and a commercial diet (MF, Oriental Yeast) ad libitum after weaning. The animal room was maintained at a temperature of 23 ± 2 °C and a relative humidity of $55 \pm 5\%$, and it was artificially illuminated with fluorescent light on a 12-h light/dark cycle (06:00–18:00 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 subcutaneously injected on 3 consecutive days into the back of 19-day-old rats. The doses of each chemical are shown in Table 4. In some rats, ethynyl estradiol (EE, CAS No. 57-63-6, 98% purity, Sigma Chemical) in olive oil was also subcutaneously injected into the back at a dose of 0.6 μg/kg per day on 3 consecutive days after the administration of each chemical at the same doses. A vehicle control group was injected with olive oil alone, and a positive control group was injected with EE after administration of olive oil. A group injected with the estrogen antagonist chemical tamoxifen at a dose of 1 mg/kg per day plus EE was also established to confirm the reliability of this study. Each group consisted of six rats. The doses of each chemical were based on the results of a preliminary study. The volume of the olive oil solution containing the chemical or EE for subcutaneous injections was 2 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 h after the final dose. At necropsy, the

Table 1
Chemicals tested in the uterotrophic assay

Chemicals	CAS no.	Source	Purity (%)
4-n-Amylphenol	14938-35-3	Tokyo Kasei Kogyo, Co.	99.2
p-Dodecyl-phenol	104-43-8	Kanto Chemical Co.	Unknown
p-(Tert-pentyl)phenol	80-46-6	Wako Pure Chemicals	100.0
4-Cyclohexylphenol	1131-60-8	Tokyo Kasei Kogyo Co.	99.7
4-(1-Adamantyl)phenol	29799-07-3	Aldrich Co.	97
4,4′-Thiobis-phenol	2664-63-3	Tokyo Kasei Kogyo Co.	99.8
Diphenyl-p-phenylenediamine	74-31-7	Wako Pure Chemicals	97.2
4-Hydroxyazobenzene	1689-82-3	Wako Pure Chemicals	96
4-(Phenylmethyl)phenol	101-53-1	Tokyo Kasei Kogyo, Co.	99.8
4,4′-(Hexafluoroisopropylidene)diphenol	1478-61-1	Aldrich Co.	98.8
2,2-Bis(4-hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	Wako Pure Chemicals	100.0
4,4′-(Octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol	1943-97-1	Across Organics	99.5
4,4′-Dihydroxybenzophenone	611-99-4	Wako Pure Chemicals	98.5
2,2′,4,4′-Tetrahydroxybenzophenone	131-55-5	Wako Pure Chemicals	98.2
4-Hydroxybenzophenone	1137-42-4	Sigma Chemical Co.	98.0
2,4,4′-Trihydroxybenzophenone	1470-79-7	Aldrich Co.	95
Testosterone enanthate	315-37-7	Wako Pure Chemicals	99.6
Methyltestosterone	58-18-4	Wako Pure Chemicals	100.0

uteri were carefully dissected free of adhering fat and mesentery 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-EE group and each of the chemical plus EE groups were assessed for statistical significance by the two-tailed Student's t-test.

2.2. Hershberger assay

2.2.1. Chemicals

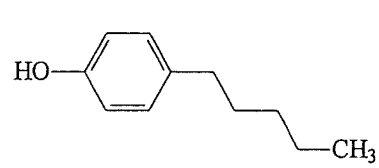
The chemicals tested in the Hershberger assay are listed in Table 2, and chemical structures of test compounds are shown in Fig. 1. All chemicals were dissolved in olive oil (Fujimi Pharmaceutical, Osaka, Japan) before use. Chemicals tested in this assay were selected based on the list of suspected endocrine disrupters published by the EU (Commission of the European Communities, 2001).

2.2.2. Animals

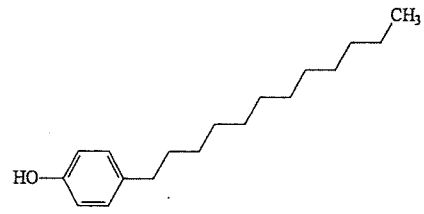
Seven-week-old castrated male Brl Han: WIST Jcl (GALAS) rats were purchased from Clea Japan (Shizuoka, Japan) and housed three per cage in stainless steel wire-mesh cages throughout the study. After allowing 14 days to recover from the operation, the rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups. Other housing conditions were essentially the same as in the uterotrophic assay.

2.2.3. Study design

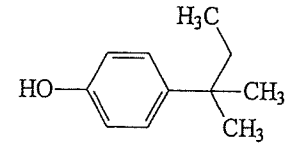
Each chemical was orally administered via a stomach tube for 10 consecutive days beginning on postnatal day 56. A vehicle control group given only olive oil was also established. Testosterone propionate (TP, CAS No. 57-63-6, 98% purity, Sigma), 0.2 mg/kg per day, was also administered to some rats by subcutaneous injection into the back after oral administration of each chemical, and a positive control injected with TP was also established. A group given the androgen antagonist chemical flutamide, 10 mg/kg per day, plus TP was established to confirm the reliability of this 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 non-castrated rats for 7 days beginning on postnatal day 56. The volume of the olive oil solution



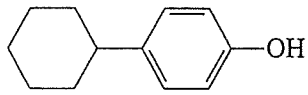
4-n-Amylphenol



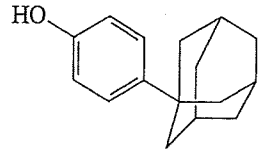
p-Dodecyl-phenol



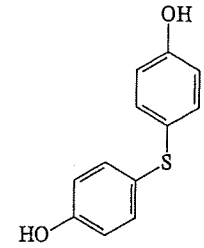
p-(Tert-pently)phenol



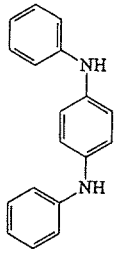
4-Cyclohexylphenol



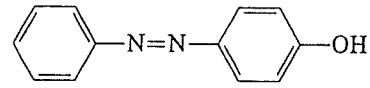
4-(1-Adamantyl)phenol



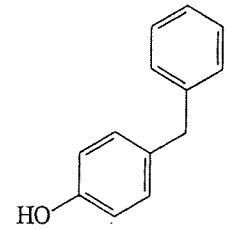
4,4'-Thiobis-phenol



Diphenyl-p-phenylenediamine



4-Hydroxyazobenzene



4-(Phenylmethyl)-phenol

Fig. 1. Chemical structures of test compounds.

containing TP was 1 ml/kg, 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.

Because toxic signs, including death and/or decrease in body weight gain, were observed when animals were administered in this study with the high doses of 4-n-amylphenol, p-(tert-pentyl)phenol, 4-cyclohexylphenol, 4-(phenylmethyl)phenol, 4,4′-(hexafluoroisopropylidene)diphenol, 2,2-bis(4-hydroxyphenyl)-4-methyl-n-pentane, 2,2′,4,4′-tetrahydroxybenzophenone, estrone, equilin, and 4-tert-octylphenol, the maximum dose of these compounds were reduced.

2.2.4. Statistical analysis

The analytical method was essentially the same as in study 1.

3. Results

3.1. Uterotrophic assay

3.1.1. Clinical signs and body weight

Body weights are shown in Table 4. No clinical abnormalities were observed in any of the groups, and body weight increased normally in all groups.

3.1.2. Uterine weight

Uterine blotted weights are shown in Table 4. Watery uterine contents were grossly detected in all rats given EE, 800 mg/kg 4-n-amylphenol, 200 mg/kg p-dodecyl-phenol, 40 and 200 mg/kg 4-(1-