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Antiandrogenic Effects *in Vitro* and *in Vivo* of the Fungicide Prochloraz

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The commonly used imidazole fungicide prochloraz was tested for antiandrogenic effects *in vitro* and *in vivo*. Prochloraz, but not the metabolites 2,4,6-trichlorophenoxyacetic acid or 2,4,6-trichlorophenol, inhibited the R1881-induced response in an androgen receptor reporter gene assay. In the Hershberger assay, prochloraz exposure at all dose levels (50, 100, and 200 mg/kg) given orally to castrated testosterone (T)-treated males markedly reduced weights of ventral prostate, seminal vesicles, musc. levator ani/bulbocavernosus, and bulbourethral gland. These effects were accompanied by an increase in LH and a reduction of the T_4 and TSH level. The effects on seminal vesicles, LH, T_4, and TSH were also evident in intact prochloraz-exposed young adult rats. Body weights were unaffected whereas liver weights were increased in prochloraz-treated animals. Changes in androgen-regulated gene expression were determined in ventral prostates by real-time RT-PCR. A pronounced decrease of ornithin decarboxylase and PBP C3 mRNA levels was observed for both prochloraz and flutamide. These results indicate that prochloraz antagonizes the peripheral androgen receptors resulting in decreased growth of androgen-dependent tissues and that it antagonizes central androgen receptors blocking the negative feed-back mechanism of testosterone resulting in increased LH secretion from the pituitary. The antiandrogenic effects of prochloraz were in many ways qualitatively comparable, although weaker, to the effects of flutamide. However, differential effects on levels of FSH, T_4, and TSH indicate that other modes of action apart from the pure AR antagonism might play a role *in vivo*.

Key Words: **antiandrogen; prochloraz; rat; AR reporter gene assay; reproductive organs; hormone levels; gene expression.**

Attention to xenobiotic actions on hormone-sensitive tissues as significant risks to human and wildlife reproduction increased markedly during the 1990s. Maternal transfer of environmental contaminants during early development with potential perturbation of steroid hormone receptor-mediated tissue differentiation is of great concern. Interactions of xenobiotics with estrogen receptors were initially identified, but research in recent years has demonstrated that some pesticides may also exert their effects by interfering with the androgen receptor (AR) (Gray *et al.*, 1999b; Kelce *et al.*, 1998). One of the first chemicals reported to be an antiandrogen was the fungicide vinclozolin (Gray *et al.*, 1994; Kelce *et al.*, 1994). Since then several other pesticides have been demonstrated to possess antiandrogenic activity, e.g., *p,p'*-DDE, procymidone, linuron, methoxychlor, and fenitrothion *in vitro* (Kelce *et al.*, 1995; Maness *et al.*, 1998; Ostby *et al.*, 1999; Sohoni and Sumpter, 1998; Vinggaard *et al.*, 1999) and *in vivo* (Gray *et al.*, 1999a; Hosokawa *et al.*, 1993; Kelce *et al.*, 1997; Lambright *et al.*, 2000; Ostby *et al.*, 1999; Monosson *et al.*, 1999; Tamura *et al.*, 2001). In general, most antiandrogenic compounds identified so far are pesticides, many of which are abandoned in the Western part of the world.

Prochloraz is an imidazole fungicide that is widely used in the Western world within horticulture and agriculture. The action of imidazoles used as fungicides or antimycotic drugs (e.g., ketoconazole) is based on the inhibition of the cytochrome P450-dependent 14α-demethylase activity required in the conversion of lanosterol to ergosterol (Henry and Sisler, 1984), an essential component of fungal cell membranes. The molecular basis of this inhibition is the presence of an imidazole moiety that interacts strongly with the iron atom of cytochrome P450. The binding is fairly unspecific and thus imidazole fungicides also inhibit the activities of a broad spectrum of other cytochrome P450-dependent enzymes, including key enzymes involved in biosynthesis and metabolism of steroids as for instance CYP19 aromatase (see references in Laignelet *et al.*, 1992). Apart from inhibition, prochloraz is also capable of inducing some cytochrome P450 enzymes (Laignelet *et al.*, 1989; Needham *et al.*, 1992).

In a recent study, in which 25 commonly used pesticides were tested *in vitro* for estrogenic and androgenic effects as well as effects on aromatase activity, prochloraz reacted as both an estrogen and androgen receptor antagonist as well as a potent aromatase inhibitor (Andersen *et al.*, 2002). Furthermore, prochloraz was able to activate the Ah receptor (unpublished data). The purpose of this study was to further characterize the *in vitro* antiandrogenic effects and to determine if prochloraz also acts as an antiandrogen *in vivo*. The effects of prochloraz in intact and castrated testosterone (T)-treated rats

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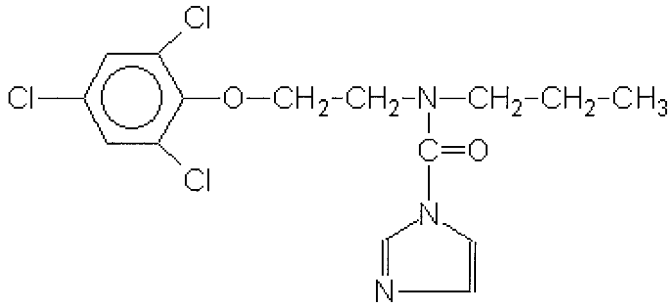

FIG. 1. Chemical structure of prochloraz (N-propyl-N-(2-(2,4,6-trichlorophenoxy)ethyl)-Imidazole-1-carboxamide).

on reproductive organs, hormone levels, and gene expression was investigated in the Hershberger assay.

After binding of ligand, the intracellular androgen receptor regulates transcription of specific genes either by increasing or suppressing their expression (Chang *et al.*, 1995). It is well known that lack of androgens results in decrease in prostate specific binding protein polypeptide C3 (PBP C3) expression in the ventral prostate of castrated rats (Bettuzzi *et al.*, 1989; Bossyns *et al.*, 1986). As examples of androgen-responsive genes we have chosen to investigate the expression of PBP C3 (Bossyns *et al.*, 1986) and ornithin decarboxylase (ODC; Betts *et al.*, 1997; Crozat *et al.*, 1992) in the ventral prostate of the rat. PBP C3 has previously been shown to be affected by antiandrogens such as vinclozolin and *p,p′*-DDE (Kelce *et al.*, 1997; Nellemann *et al.*, 2001).

The results reported here demonstrate that prochloraz in many ways acts similarly to other antiandrogens. The compound blocks the AR *in vitro* and *in vivo*; it reduces the weight of the reproductive organs in castrated testosterone-treated male rats; it increases the LH level and reduces PBP C3 and ODC mRNA levels. Compared to flutamide, however, the FSH serum level is not affected by prochloraz. Furthermore prochloraz attenuates the serum T_4 and TSH levels, hormones that are not affected by flutamide. Whether prochloraz that is a well-known aromatase inhibitor has to be considered as a classical antiandrogen is discussed.

MATERIALS AND METHODS

Test compounds. Prochloraz (Fig. 1), 99.4% pure, was from Riedel de Häen, Seelze, Germany (no. 35987). 2,4,6-Trichlorophenoxyacetic acid (lot no. F1E01) and 2,4,6-trichlorophenol (lot no. FHG01) were from TCI (Tokyo, Japan). Testosterone propionate (CAS no. 319491, lot no. 257211) was from UniKem, Denmark, and flutamide (CAS no. F-9397) was from Sigma-Aldrich (St. Louis, MO).

AR reporter gene assay. Effects on androgen receptor activity were tested in a recently developed reporter gene assay (Vinggaard *et al.*, 1999). Chinese Hamster Ovary cells (CHO K1) were maintained in DMEM/F12 (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO) and 10% fetal bovine serum (BioWhitaker, Walkersville, MD). The cells were seeded in white 96-well microtiter plates (PerkinElmer Life Sciences, Packard) at a density of 7000 cells per well in DMEM/F12 containing 10% charcoal-treated fetal bovine serum (BioWhitaker) and incubated at 37°C in a humidified atmosphere of 5% CO_2/air. After 24 h cells were transfected for 5 h with a total of 75 ng DNA per well consisting of the expression vector pSVAR0 and the MMTV-LUC reporter plasmid (both provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam) in a ratio of 1:100 using 300 nl of the transfection reagent FuGene (Boehringer Mannheim, Germany). The ratio of DNA (μg) to Fugene (μl) was kept at 0.25. The chemicals to be tested were added with or without 0.01 nM of the AR agonist R1881 (NEN, Boston, MA). The test solutions were prepared from 20 mM stock solutions in ethanol. Hydroxyflutamide was included in every experiment as a positive control. After incubation for 20 h, the media was aspirated and the cells were lysed by adding 20 μl per well of a lysis buffer containing 25 mM trisphosphate, pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM DTT, and 8 mM $MgCl_2$, followed by shaking at room temperature for 10 min. Luciferase activity was measured directly using a BioOrbit Galaxy luminometer by automatic injection of 40 μl substrate containing 1 mM luciferin (Amersham Int., Buckinghamshire, UK) and 1 mM ATP (Boehringer Mannheim, Germany) in lysis buffer and the chemiluminiscense generated from each well was measured over a 1 s interval after an incubation time of 2 s.

Cytotoxicity experiments were performed as described above except that the pSVAR0 expression vector was replaced by the constitutively active androgen receptor expression vector, pSVAR13 (a gift from Brinkmann), which lacks the ligand binding domain of the receptor. The ratio between pSVAR13 and MMTV-LUC was 2:100.

Animal Experiments

Test species. Male Wistar rats were acquired from M&B (Eiby, Denmark). In the first experiment 12 intact male rats (67–72 days old at the dosing start) and 18 rats, castrated at an age of 4 weeks 14 days prior to study start, were used. In the second experiment 6 intact male rats (42 days at the dosing start) and 36 Wistar males, castrated at the same age and time as described above, were used. All animals were delivered one week prior to study start and upon arrival rats were housed in Bayer Makrolon type 3118 cages (Type: 80-III-420-H-MAK, Techniplast), three per cage with Tapvai bedding. They were fed Syn 8.IT (a diet known to be free of phytoestrogens) and were provided with acidified tap water *ad libitum*. Animal rooms were maintained on a 12-h light/dark cycle, a temperature of 22 ± 1°C, and a relative humidity of 55 ± 5%. Rats were weighed and divided by randomization into treatment groups so that there were no statistically significant differences among group body weight means. During testing rats were weighed daily and visually inspected for health effects twice a day.

Testing of intact and castrated animals (Experiment 1). Two groups of intact Wistar male rats were included, the one served as controls and the other was given prochloraz (250 mg/kg) that was dosed orally each day for 7 days. Three groups (n = 6/group) of castrated Wistar rats were treated with testosterone propionate (0.5 mg/kg/day sc) with or without flutamide given orally (75 mg/kg) or prochloraz given orally at a dose of 250 mg/kg.

Experiment 2. One group of intact animals and six groups of castrated male Wistar rats, which were 42 days at the dosing start, were included in the study (n = 6 per group). The intact rats and one group of castrated rats served as negative controls and were given peanut oil. One group was dosed testosterone propionate (0.5 mg/kg/day sc) and the positive control group received testosterone propionate plus flutamide (20 mg/kg sc). The last three groups received testosterone propionate plus prochloraz given orally each day for 7 days at doses of 50, 100, and 200 mg/kg.

For both experiments all compounds were dissolved in peanut oil. Sterile oil (the Royal Veterinary Agriculture Pharmacy, Copenhagen, Denmark) was used for testosterone propionate and flutamide solutions. All compounds were administered in a dosing volume of 2 ml/kg body weight and the dosing period was 7 days for all animals. The testosterone dose was always given a few minutes after the test compound and the last dosing was performed in the morning at the day of killing the animals. Body weights were recorded and

animals were euthanized using CO_2/O_2 followed by exsanguinations. All the animals from each group underwent a thorough autopsy. The testis (for intact animals), both lobes of the ventral prostata, combined seminal vesicles and coagulating glands including fluids, musc. levator ani/bulbocavernosus, paired bulbourethral glands, pituitary, liver, and paired kidneys were dissected and weighed. Organ weights were calculated relative to body weights. The ventral prostates from Experiment 2 were put in 0.5 ml RNAlater (Ambion) and stored at –20°C until gene expression analysis. Blood was collected by exsanguinations in plain glass tubes from Experiments 1 and 2 and serum was prepared and stored at –80°C until measurement of hormones.

Hormone analysis. rLH, rFSH, T_4 and testosterone concentrations were analyzed in serum using the technique of time-resolved fluorescense (Delfia, Wallac). LH and FSH were analyzed by Pirjo Pakarinen, Turku University, Finland. Rat FSH immunoreactivity was determined by a two-site immunofluorometric assay (IFMA; van Casteren *et al.*, 2000). A monoclonal mouse antibody against human recombinant FSHβ (Mab ahFSHβ FG 5020, N.V. Organon, Oss, The Netherlands) was used as a capture antibody coated to the walls of 96-well plates (Maxisorp 4-73709A, Nunc, Denmark). As signal antibody biotin-labeled rabbit polyclonal antibody against human recombinant FSHα (R-ahFSH-Biotin R93-2705, N.V. Organon) was used. Finally, europium-labelled streptavidin was bound to the biotin-labelled antibody. Time-resolved fluorescence evoked by a europium label was used for signal detection with Wallac Victor^2 1420 Multilabel Counter (PerkinElmer Life Sciences, Wallac Finland Oy, Turku, Finland). The standard used was a NIDDK standard FSH-RP-2 obtained from the National Hormone and Pituitary Program, NIH, Rockville, MD. Buffers and washing solutions used throughout the assay were Delfia® reagents obtained from Wallac.

Rat LH was measured using the time-resolved fluorimetric assay (IFMA, Delfia, Wallac OY, Turku, Finland) as described by Haavisto *et al.* (1993). The standard rLH RP-3 was kindly provided by NIDDK, NIH (Baltimore, MD).

Testosterone was extracted from rat serum by solid-phase extraction using IST Isolute C18 SPE columns of 100 mg (Mid Glamorgan, UK). The serum samples (200 μl) were diluted two-fold with purified water and applied to columns preconditioned and rinsed with methanol and water, respectively. Interfering substances were eluted with 2 ml methanol:water (20:80 v/v) and steroids were eluted with 2 $\times$ 1.2 ml methanol. The solvent in these fractions was evaporated and samples were resuspended in 100 μl diluent based on human serum (PerkinElmer Life Sciences, Wallac). Testosterone in these extracts was measured using commercially available FIA kits from PerkinElmer Life Sciences, Wallac. Kits from the same supplier were used for T_4 determination.

Thyroid stimulating hormone (TSH) was analyzed using the enzyme immunoassay (Biotrak™) developed by Amersham. Serum samples were diluted 20 times in assay buffer and 50 μl of this was analysed as recommended by the manufacturer.

RNA isolation and cDNA production. Ventral prostates were weighed and homogenized in RLT buffer (RNeasy Mini-kit, QIAGEN) by an Ultra Turrax rotor-stator homogenizer. Subsequent extraction of total RNA was performed using the RNeasy Mini-kit (QIAGEN) following the manufacturer's instructions. The quantity and quality of the purified RNA was evaluated by spectrophotometry. cDNA was produced from 0.5–1.0 μg of total RNA using display-THERMO-RT kit and the manufacturer's instructions (Display Systems Biotech, Kem-en-tec, Denmark).

Real-time RT-PCR. Real-time RT-PCR with online detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche) was employed. We used hybridization probes (TIB MolBiol, Berlin, Germany) to monitor the amount of specific target sequence produced. Quantitative results were obtained by the cycle threshold value where a signal rises above background level. Expression of the genes coding for ODC and PBP C3 was compared to the steady expression of 18S rRNA. PCR was performed with 5mM $MgCl_2$ for ODC and 18S RNA and with 4 mM $MgCl_2$ for PBP C3. 0.5 μM primers (5′-ACGAACCAGAGCGAAAGCAT-3′, 5′-GGACATCTAAGGGCATCACAGAC-3′) and 0.2 μM probes (FL530: 5′-TCGGAACTACGACGGTATCTGATCGTC-3′, LC640: 5′-CGAACCTCCGACTTTCGTTCTTGAT-3′) for 18S rRNA, 0.3 and 0.2 μM of primers (5′-TTGCTGCTATGCCAGTGGTT-3′, 5′-CCTCCATCATCACGCTAACATT-3′) and probes (FL530: 5′-AGGCTGTGAAGCAATTCAAGCAGTGT-3′, LC640: 5′-TTCTAGATCAGACCGACAAGACTCTGGAAA-3′), respectively, for PBP C3 and 0.6 and 0.2 μM of primers (5′-CAGATGCCCGCTGTGTCTT-3′, 5′-TGACTCATCTTCATCGTCCGAG-3′) and probes (FL530: 5′-CCAGTGTAATCAACCCAGCTCTGGAC-3, LC640: 5′-GTACTTCCCATCGGACTCTGGAGTGA-3′), respectively, for ODC.

The PCR program followed the manufacturer's instructions (LightCycler-DNA Master Hybridization Probes, Roche) except that Taq start antibody (Clontech; 0.16 μl per 20 μl reaction) was incubated with the DNA Master Hybridization Probes mix at room temperature for 5 min prior to addition of the rest of the components. The program "Lightcycler Relative Quantification Software" version 1.0 (Roche) was employed to calculate the relative gene expressions. Gene expressions were analyzed at least three times for each animal in the same cDNA preparation.

Histopathalogy and androgen receptor immunohistochemistry. The right testis from the intact males were fixed overnight in formalin and embedded in paraffin. One section was stained with haematoxylin and eosin and another section was immunostained with an antibody against the human AR. The staining protocol for AR immunohistochemistry was kindly provided by K. J. Turner, MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh, U.K. Briefly, paraffin sections (5 μm) were deparaffinized, and heat-induced epitope retrieval in a microwave oven was performed for 2 $\times$ 5 min in 0.01M citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Nonspecific background staining was eliminated with 10% normal swine serum. The tissue sections were incubated overnight at 4°C with the primary rabbit polyclonal antibody (N20:sc-816, Santa Cruz Biotechnology). This was followed by incubation with secondary biotinylated swine anti-rabbit antibody (DAKO). Sections were incubated with the avidin biotinylated horseradish peroxidase complex and visualized by the chromogenic substrate 3-amino-9-ethylcarbazole (Sigma). Tissue sections were counter-stained with haematoxylin, mounted with Aquamount (Gurr®), and examined by light microscopy. For validation of the immunostaining, three control slides were stained simultaneously: on one testis slide the primary antibody was omitted, on another testis slide the primary antibody was replaced with normal rabbit serum, and on a third slide a rat prostate, known to express the androgen receptor, was examined. The intensity of the AR-immunostaining in the Leydig cells and in the peritubular myoid cells was compared by visual inspection of the control and the intact prochloraz-treated males. In the Sertoli cells the staining-reaction alters with the cycle of the seminiferous epithelium. Therefore the staining intensity of the AR in the Sertoli cells was compared in stage III–IV tubules, in stage VII tubules, and in stage XII–XIII tubules in 40 tubules per tissue section.

Statistical analyses. *In vitro* data was analyzed by ANOVA and when the corresponding F test for differences among groups was significant, pair-wise comparisons between test and control group were made with Dunnett's test. Significance was judged at $p < 0.05$. IC_{50} values were determined using a four-parameter logistic function (SigmaPlot ver. 7.0, Statistical Solutions)

For *in vivo* data all calculations and statistical analysis were generated in SAS version 8 (SAS Institute Inc, Cary, NC). For comparison between the group treated with testosterone (control) and the groups treated with testosterone and flutamide or testosterone and prochloraz, a one-way ANOVA (General linear models procedure: Proc Glm) was used. Likewise the comparison between the untreated castrated group and the untreated intact males was analyzed using Proc Glm. For analysis of organ weights, the body weight was used as a covariate. When the overall ANOVA was significant, Dunnett's test ($p < 0.05$) was conducted for pair-wise comparison between the control group and the treatment groups. Nonprocessed and ln transformed data were checked for normal distribution and homogeneity of variance. If the data did not fulfill these conditions, data was subjected to Wilcoxon's test followed by Kruskall-Wallis test for pair-wise comparison, if statistically significant.

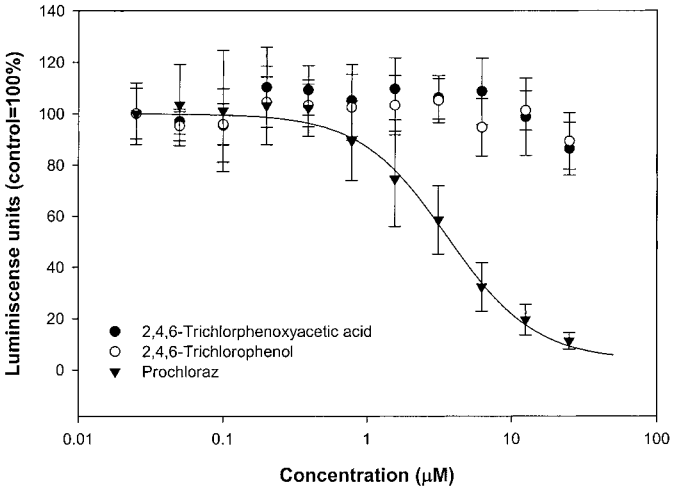

FIG. 2. Prochloraz-induced androgen receptor inhibition in the presence of 0.01 nM R1881 determined in an AR reporter gene assay in CHO cells. Data represent the mean ± SD of $n = 5$ performed in quadruplicate. The major and a minor metabolite of prochloraz, 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol, caused no statistically significant inhibition of the R1881-induced response ($n = 2$).

RESULTS

Antiandrogenic effects of prochloraz were tested *in vitro* by a transactivation assay involving transient cotransfection of CHO cells with the AR expression vector and the MMTV-LUC reporter plasmid. Prochloraz inhibited the response induced by 0.01 nM R1881 that was statistically significant at concentrations of 1.6 μM and up to 25 μM. The IC_{50} was determined from the nonlinear regression line to be 3.6 μM (Fig. 2). The major and a minor metabolite of prochloraz, 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol, caused no statistically significant inhibition of the R1881-induced response.

Potential cytotoxic effects of prochloraz on the transactivating process were assessed by transfecting the cells with the truncated and constitutively active human pSV-AR13 vector. Prochloraz was found to decrease the transcriptional activity at the highest concentration of 50 μM, whereas no effects were observed at concentrations at and below 25 μM (data not shown).

In vivo, body weights, paired kidney weights, and pituitary weights were unaffected by the prochloraz or flutamide treatment (Tables 1 and 2), whereas liver weights were increased in both intact (Table 1) and castrated testosterone-treated animals exposed orally to prochloraz (Tables 1 and 2). The increase in liver weights was statistically significant at doses of 100, 200, and 250 mg/kg. Flutamide at a dose of 20 mg/kg sc did not affect the liver weight (Table 2), but Experiment 1 involving oral administration showed that flutamide also increased liver weights using this exposure route (Table 1).

Castrated vehicle-exposed rats compared to intact males of the same age had significantly reduced weights of all the reproductive organs and increased levels of LH and FSH and lowered testosterone (Figs. 3 and 5, Table 3). The serum levels of TSH and T_4 were unaffected by castration. On the gene expression level both PBP C3 and ODC mRNA were reduced by castration (Fig. 6).

In the intact prochloraz-treated males (250 mg/kg) the absolute and relative weights of the seminal vesicles were significantly decreased by approximately 30%, whereas the other reproductive organs (testis, ventral prostate, musc. levator ani, and bulbourethral gland) were unaffected (Table 1). Prochloraz administration to castrated testosterone-treated males reduced significantly both absolute and relative weights of the follow-

TABLE 1
Final Body Weights and Organ Weights Obtained in the Initial Experiment 1

	Intact young adult rats		Castrated immature rats		
	Control	Proc. (250 mg/kg)	T	T + flut. (75 mg/kg)	T + proc. (250 mg/kg)
Body weight (g)	309 ± 11	301 ± 10	208 ± 11	197 ± 17	201 ± 11
Liver weight (g)	11.6 ± 1.0	15.7 ± 1.4*	8.9 ± 0.5	11.0 ± 1.1**	11.7 ± 0.6**
Paired kidney weight (g)	1.90 ± 0.15	1.95 ± 0.21	1.53 ± 0.13	1.44 ± 0.06	1.55 ± 0.16
Ventral prostate (g)	0.34 ± 0.05	0.29 ± 0.07	0.098 ± 0.014	0.012 ± 0.006**	0.053 ± 0.012**
Vesicula seminalis (g)	0.87 ± 0.11	0.58 ± 0.11*	0.175 ± 0.017	0.035 ± 0.009**	0.110 ± 0.022**
Musc.levator ani/bulbocavernosus (g)	0.64 ± 0.06	0.57 ± 0.04	0.260 ± 0.030	0.109 ± 0.014**	0.179 ± 0.032**
Gl. bulbourethralis (mg)	63.0 ± 8.7	55.9 ± 16.9	14.1 ± 1.0	2.2 ± 0.6**	9.2 ± 1.5**
Left testis (g)	1.71 ± 0.10	1.60 ± 0.10	—	—	—
Right testis (g)	1.68 ± 0.09	1.66 ± 0.14	—	—	—

Note. Intact young adult Wistar rats were treated with or without 250 mg/kg prochloraz and castrated Wistar rats were treated with testosterone propionate (T) alone or together with flutamide (75 mg/kg orally) or prochloraz (250 mg/kg orally). Data represent the mean ± SD of six animals per group.

*Statistically significant difference ($p < 0.05$) from the intact vehicle-treated animals.

**Statistically significant difference ($p < 0.05$) from the testosterone-treated castrated animals.

TABLE 2
Final Body Weights and Organ Weights Obtained in Experiment 2

	Control				T + proc.		
	Intact	Castrated	T	T + flut. (20 mg/lg)	50 (mg/kg)	100 (mg/kg)	200 (mg/kg)
Body weight (g)	194 ± 18	183 ± 9	192 ± 16	187 ± 13	185 ± 14	185 ± 9	184 ± 10
Liver weight (g)	8.1 ± 0.8	7.2 ± 0.6**	7.6 ± 0.8	7.6 ± 0.8	8.1 ± 0.6	8.6 ± 0.6*	10.0 ± 0.7*
Paired kidney weight (g)	1.5 ± 0.2	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Pituitary weight (mg)	6.7 ± 1.2	8.1 ± 0.6**	8.2 ± 1.1	8.4 ± 1.1	7.8 ± 1.1	7.4 ± 0.5	7.3 ± 1.2

Note. Results from intact Wistar rats and castrated Wistar rats treated with testosterone propionate alone or together with flutamide (20 mg/kg sc), or prochloraz 50, 100, and 200 mg/kg orally. T, testosterone propionate; flut., flutamide; proc, prochloraz. Data represent the mean ± SD of six animals per group.

*Statistically significant difference ($p < 0.05$) from the testosterone-treated castrated animals.

**Statistically significant difference ($p < 0.05$) from the intact vehicle-treated animals.

ing tissues: ventral prostate (54%), seminal vesicles (38%), musc. levator ani/bulbocavernosus (25%), and the bulbourethral gland, also called Cowpers gland (48%, Fig. 3). The numbers in the parentheses refer to reductions of absolute weights caused by 200 mg/kg prochloraz. These weight reductions were evident even at the lowest test dose of 50 mg/kg. As expected flutamide also caused pronounced weight reductions of these organs. In general, the weight reductions observed for the castrated rats in the second experiment were comparable to those obtained in Experiment 1. Furthermore it is evident that the young adult intact rats are less sensitive for detection of the antiandrogenic effects of prochloraz compared to the castrated rats in the Hershberger assay.

A photo of representative seminal vesicles from the intact and castrated animals is shown in Figure 4 illustrating the size reduction of the organ caused by prochloraz treatment.

These prochloraz-induced effects on organ weights were accompanied by an increase in LH that was statistically significant for intact and castrated testosterone-treated animals given 250 mg/kg prochloraz or flutamide (Fig 5A). The increase caused by prochloraz in castrated and intact animals was 440% and 75%, respectively. Flutamide gave rise to a significant increase by 830% of the LH level in the castrated testosterone-treated animals.

In the second animal experiment, the LH increase was statistically significant for the castrated flutamide-treated animals (980% increase) but not for the prochloraz-treated animals, although a tendency for an increase with 200 mg/kg prochloraz was seen (Fig. 5B).

Comparison of the intact animals to the castrated testosterone-treated animals showed that both testosterone and FSH levels were significantly higher in the testosterone-treated animals than at normal physiological conditions (Table 3), whereas LH levels were close to physiological levels (Fig. 5). The approximately five- to six-fold higher testosterone level in castrated testosterone-treated animals was not reflected in the weights of the reproductive organs that were either similar or less than physiological organ weights (Fig. 3). However, the animals have been castrated 14 days prior to dosing start and all testosterone-induced growth processes have slowed down, and it is conceivable that a lag time exists for the organs to regain maximum growth rates.

FSH serum levels were unaffected after prochloraz administration whereas a statistically significant increase was seen in flutamide-exposed animals (Table 3). Neither prochloraz nor flutamide affected the serum testosterone levels in testosterone-treated castrated animals. Thyroid hormone analyses were performed in order to evaluate any prochloraz-induced effects on

TABLE 3
Hormone Levels of Intact Wistar Rats and Castrated Wistar Rats Treated with Testosterone Propionate Alone or Together with Flutamide

	Control				T + proc.		
	Intact	Castrated	T	T + flut. (20 mg/kg)	50 (mg/kg)	100 (mg/kg)	200 (mg/kg)
FSH (ng/ml)	9.6 ± 1.0	40.6 ± 1.7*	37.4 ± 4.1	56.5 ± 2.7**	34.2 ± 3.6	36.5 ± 4.7	41.8 ± 4.8
Testosterone (nM)	4.4 ± 2.6	0.05 ± 0.03*	25.7 ± 7.3	31.4 ± 8.3	23.9 ± 7.8	19.1 ± 8.5	22.6 ± 7.5
T_4 (nM)	95.7 ± 6.4	107.6 ± 9.6	136.8 ± 14.0	118.8 ± 11.8	112.0 ± 7.7	81.2 ± 12.5**	68.9 ± 10.0**
TSH (ng/ml)	13.4 ± 0.9	15.7 ± 1.8	12.3 ± 1.1	13.8 ± 0.7	8.5 ± 0.7**	10.6 ± 0.6	7.8 ± 0.7**

Note. Data represent the mean ± SEM of five to six animals per group. T, testosterone propionate; flut., flutamide; proc, prochloraz.

*Statistically significant difference ($p < 0.05$) from the intact vehicle-treated animals.

**Statistically significant difference ($p < 0.05$) from the testosterone-treated castrated animals.

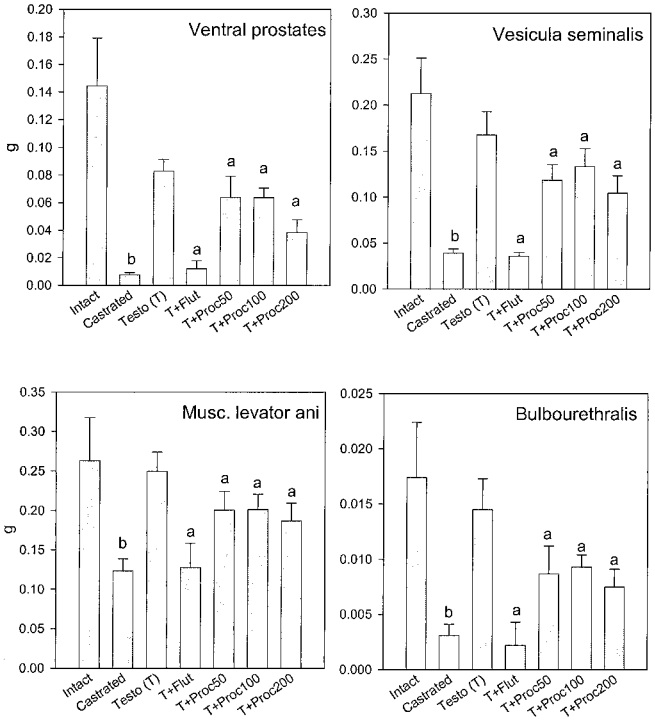

FIG. 3. Absolute weights of ventral prostates, seminal vesicles, musc. levator ani/bulbocavernosus, and bulbourethral glands from castrated rats treated for 7 days with testosterone propionate (0.5 mg/kg sc) with or without flutamide (20 mg/kg sc) and prochloraz at doses of 50, 100, and 200 mg/kg given orally. Mean ± SD ($n = 6$). [a]Statistically significant difference from castrated testosterone-treated animals ($p < 0.05$). [b]Statistically significant difference from intact nontreated animals ($p < 0.05$).

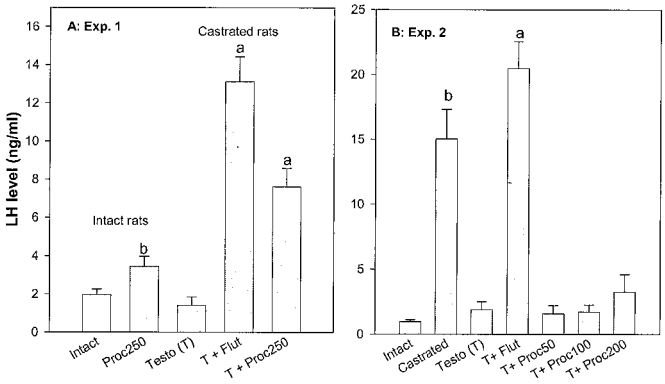

FIG. 5. (A) Serum levels of luteinizing hormone in intact rats treated for 7 days with prochloraz and in castrated rats treated for 7 days with testosterone propionate (0.5 mg/kg sc) with or without flutamide (20 mg/kg sc) or 250 mg/kg prochloraz given orally. Mean ± SEM ($n = 6$). (B) Serum levels of luteinizing hormone in intact rats and in castrated rats treated for 7 days with testosterone propionate with or without flutamide or prochloraz 50, 100, and 200 mg/kg. Mean ± SEM ($n = 6$). [a]Statistically significant difference from castrated testosterone-treated animals ($p < 0.05$). [b]Statistically significant difference from intact nontreated animals ($p < 0.05$).

thyroid hormone homeostasis. Prochloraz at doses of 100 and 200 mg/kg induced a significant decrease of serum T_4 that was reduced to 50% of control levels with the highest dose. A reduction in the serum level of the pituitary hormone TSH was also evident after prochloraz treatment, indicating that at least part of the decrease in T_4 level was secondary to effects on TSH secretion.

The relative expression of the genes PBP C3 and ODC was analyzed in ventral prostates by real-time RT-PCR (Fig. 6). Highly significant decreased levels of ODC and PBP C3 mRNA were seen in castrated testosterone-treated animals given flutamide and all dose levels of prochloraz. The effect of prochloraz at the highest dose was a 99% reduction of PBP C3 mRNA and a 92% reduction of ODC mRNA. No effects on expression of the investigated genes in intact prochloraz-treated animals could be found (data not shown).

The histopathological investigation of haematoxylin and eosin stained testis indicated that no changes in the prochloraz-treated animals had taken place. In addition, AR immunohis-

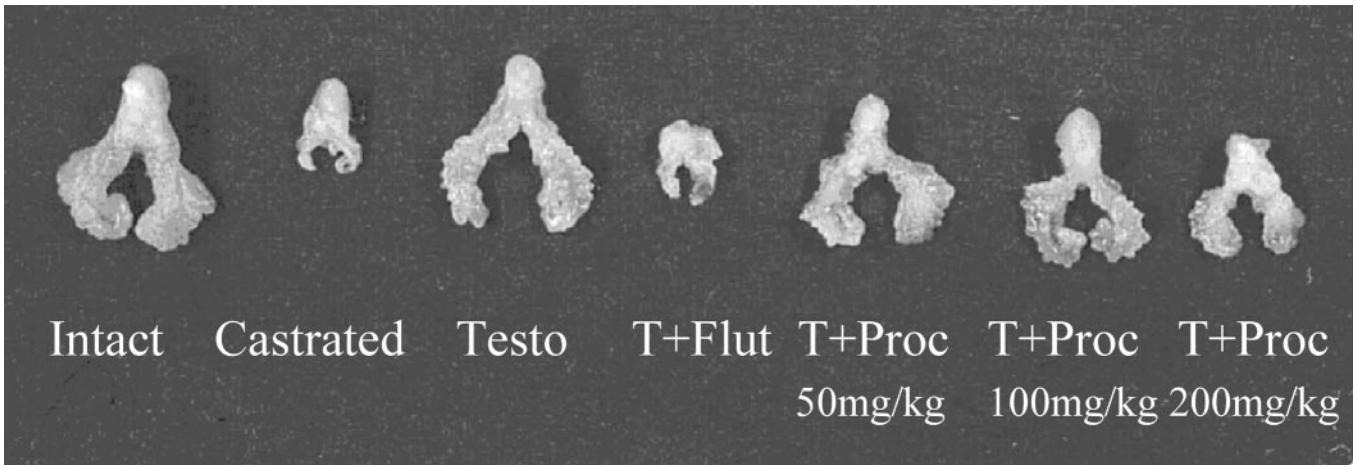

FIG. 4. Photo of seminal vesicles dissected from intact rats and castrated rats treated with testosterone propionate with or without flutamide and prochloraz at doses of 50, 100, and 200 mg/kg.

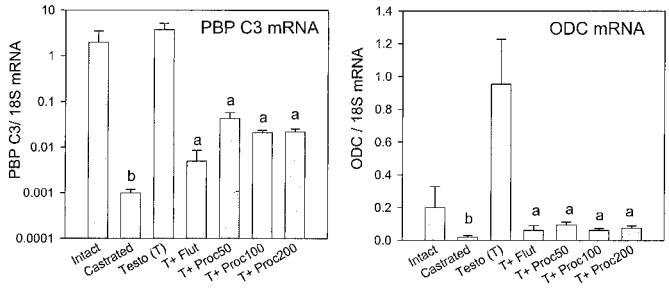

FIG. 6. Expression of PBP C3 and ODC mRNAs taken relative to expression of the house-keeping gene 18S rRNA in ventral prostates from castrated animals treated for 7 days with testosterone propionate (0.5 mg/kg sc) with or without flutamide (20 mg/kg sc) or prochloraz (50, 100 and 200 mg/kg orally). Mean ± SEM ($n = 6$). Note the logarithmic scale on the y-axis in the figure for PBP C3. [a]Indicates statistically significant difference from castrated testosterone-treated animals ($p < 0.05$). [b]Indicates statistically significant difference from intact nontreated animals ($p < 0.05$).

tochemical investigation of testis from prochloraz-treated intact animals showed that the AR distribution was unchanged (data not shown). In accordance with this a preliminary investigation of the AR mRNA in prostate determined by real-time RT-PCR showed that AR mRNA was also unaffected by prochloraz in this tissue (data not shown).

DISCUSSION

The Hershberger assay responds to antiandrogenic compounds acting through different mechanisms of action, so it is important to confirm any purported AR-activity with other methods. The *in vitro* transcriptional assay showed that prochloraz acted as an AR antagonist having an IC_{50} of 4 μM. For comparison the IC_{50} of vinclozolin and *p,p′*-DDE in our AR assay is approximately 10-fold lower, being around 0.4 μM and 0.3 μM, respectively. In the Hersberger assay performed in our lab, vinclozolin administered at doses of 50, 100, and 200 mg/kg reduced prostate weight by 50, 63, and 79%, respectively, (manuscript in preparation), while prochloraz reduced prostate weights by 21, 21, and 51% at the same dose levels. The corresponding numbers for weight reductions of seminal vesicles were 45, 65, and 82% for vinclozolin and 26, 17, and 34% for prochloraz. Thus, the *in vivo* potency of prochloraz compared to vinclozolin is within the range from 26–65% when looking at organ weight changes. However, *p,p′*-DDE at a dose level of 50 mg/kg did not significantly reduce prostate weight (unpublished data). Thus, concerning effects on weight of reproductive organs, vinclozolin is more potent and *p,p′*-DDE is less potent than prochloraz. Compared to relations between potencies observed *in vitro and in vivo* for vinclozolin and *p,p′*-DDE, the *in vivo* response induced by prochloraz was higher than predicted from the *in vitro* assay. However, several toxicokinetic factors, e.g., the gastrointestinal uptake, the tissue distribution, the cellular uptake, and the rate of hepatic metabolism affect the response *in vivo* but are not evaluated *in vitro*. In addition, prochloraz is capable of disturbing hormone function *in vitro* via several mechanisms, i.e., interaction with the AR and possibly the ER and by affecting enzymes involved in synthesis and degradation of steroids. Overall the IC_{50} values for the *in vitro* effects of prochloraz on aromatase, AR, and ER antagonism is 0.3, 3.6, and approximately 25 μM, respectively (Andersen *et al.*, 2002; Vinggaard *et al.*, 2000) indicating that the compound affects aromatase at lower concentrations than those needed to block the AR and that the effect on ER is very weak. The effect detected *in vivo* is an integrated response of all the reactions affected by the chemical while the *in vitro* response in the AR transactivation assay mainly is a result of direct action of the chemical with the AR, although some metabolism may take place in the CHO cells. An additional explanation could be that the *in vivo* antiandrogenic effect was caused by one or more metabolites of prochloraz formed more extensively *in vivo* than *in vitro*. In general, prochloraz is rapidly metabolized and excreted after oral exposure ($T_{1/2}$ is approximately 24 h in male rats) with urinary excretion being the predominant route in most animals tested (Laignelet *et al.*, 1992; Needham and Challis, 1991). The principal metabolites in urine are 2,4,6-trichlorophenoxy acetic acid and 2,4,6-trichlorophenoxy-ethanol, whereas 2,4,6-trichlorophenol is formed as a minor metabolite (Laignelet *et al.*, 1992; Needham and Challis, 1991). However, neither 2,4,6-trichlorophenoxy-acetic acid nor 2,4,6-trichlorophenol gave rise to a significant AR inhibition *in vitro*. 2,4,6-Trichlorophenoxyethanol was not analyzed as no supplier was found, and thus this metabolite could be responsible for at least part of the effect. Also in trout, prochloraz-induced effects on hepatic biotransformation enzymes were observed at lower concentrations *in vivo* than predicted from *in vitro* experiments (Sturm *et al.*, 2001).

Imidazole fungicides are well-known selective inhibitors of steroid aromatase (CYP19) activity (Mason *et al.*, 1987). Testolactone is an example of an aromatase inhibitor that has been tested in intact and castrated testosterone-treated rats (Vigersky *et al.*, 1982). This compound inhibited the weights of ventral prostate and seminal vesicle both in the intact and castrated animals, and a similar inhibition was seen when the castrated animals were administered a mixture of testosterone and estradiol. Furthermore, testolactone binds to the AR *in vitro*. Thus, it was concluded that testolactone acted as an antiandrogen by blocking AR and that the effect was not caused by aromatase inhibition, as the effects were also evident after estradiol or dihydrotestosterone exposure. As estradiol has been found to synergize with testosterone to increase rat seminal vesicle weight (Jackson *et al.*, 1977) and to increase androgen binding to the prostate in dogs (Moore *et al.*, 1979), it cannot be excluded that the aromatase inhibiting property of prochloraz is part of the explanation for the observed effects in this study, even though the compound itself is able to block the AR activation *in vitro*.

As mentioned, prochloraz is able to inhibit and induce several cytochrome P450 enzymes giving rise to increased

liver weights at the 100 and 200 mg/kg dose level. As the level of T_4 was decreased at doses of 100 and 200 mg/kg, it was speculated if an increased metabolism of T_4 was induced by prochloraz secondary to the liver enzyme induction. This may still be part of the explanation, however, the level of TSH was also significantly decreased by prochloraz at doses of 50 and 200 mg/kg**,** indicating that the T_4 reduction may occur secondary to decreased TSH secretion from the pituitary. TSH secretion is regulated by both the negative feedback effects of the thyroid hormones and by stimuli mediated by the CNS and the secretion of thyroid-releasing hormone, somatostatin, and possibly dopamine. The mechanism for the prochloraz-induced inhibition of TSH secretion is unknown, but may be caused by a CNS effect. A similar reduction of the T_4 level has been reported for *p,p′*-DDE at doses of 100–300 mg/kg (O'Connor *et al.*, 1999) in both CD and Long-Evans rats. In contrast T_3 level were unchanged, whereas TSH levels were increased in Long-Evans rats, but not in CD rats. Results from our lab in female rats showed that prochloraz reduced both the T_3 and T_4 level (unpublished data). Thus, the reductions in T_4 levels caused by prochloraz and *p,p′*-DDE seem to be the result of different mechanisms of action.

The serum LH level was significantly increased by 250 mg/kg prochloraz in our first *in vivo* experiment, whereas in the second experiment 200 mg/kg prochloraz did not significantly increase LH, although a tendency towards an increase was seen. These results point to the conclusion that the central antiandrogenic effect of prochloraz is observed only at high doses above 200 mg/kg. For comparison vinclozolin given for 5 days exerts LH increases (around 430%) at a dose of 200 mg/kg (with a testosterone dose that was two-fold the physiological level; Kelce *et al.*, 1997), which may be compared to the increase in LH caused by 250 mg/kg prochloraz of 440%. However, *p,p′*-DDE was reported not to affect LH levels (Kelce *et al.*, 1997). Another antiandrogenic compound, procymidone, was given to intact rats for 14 days at doses of 2000 and 6000 ppm in the diet, corresponding to doses of approximately 100 and 300 mg/kg, respectively. The LH level was found to increase significantly at the high, but not at the low dose (Hosokawa *et al.*, 1993). Thus, the effect of prochloraz on LH levels is comparable to effects observed for vinclozolin and procymidone.

Many genes contain an androgen-responsive-element in the promotor region and their expression is directly influenced by the amount of androgen or antiandrogen available. Genes without the androgen-responsive-element in the promoter area can also be influenced by the presence of androgens via activation of transcription factors and/or cofactors, circulating hormones, etc. (Verhoeven and Swinnen, 1999). We analyzed mRNA levels of two genes (PBP-C3, ODC) that contain an androgen-responsive element in their promoter region.

PBP constitutes approximately 50% of the secreted protein from the normal rat prostate and is thereby the most abundant androgen-regulated protein in this tissue (Heyns, 1990; Pelletier *et al.*, 1988). The androgen regulation of PBP C3 is caused by an androgen-responsive element in the first intron (Heyns *et al.*, 1978; Page and Parker, 1982; Vercaeren *et al.*, 1996). Besides androgens, other compounds such as growth hormone or prolactin have been shown to have influence on the expression of PBP C3 in the rat ventral prostate (Reiter *et al.*, 1995a,b). However, the changes observed in our experiments are suggested to be mainly a result of androgen regulation.

ODC is necessary for cell growth and differentiation as an important enzyme in the synthesis of polyamines in which ODC catalyzes the conversion of ornithine to putrescine. Polyamines are found at high concentrations in prostate and can influence growth and development of prostate cancer (Betts *et al.*, 1997). The ARE element up-stream of the promoter is regulated by androgens and is up-regulated five- to ten-fold by androgens in rat kidney, prostate, and accessory sex organs (Betts *et al.*, 1997). ODC mRNA is not a specific marker for antiandrogenic activity and for instance ODC expression has also been found being induced by estrogens in rat uteri (Branham *et al.*, 1988; Russell and Taylor, 1971). Thus, the pronounced reduction of ODC mRNA levels is anticipated being an effect secondary to the impaired growth of prostate tissue.

Prochloraz caused a pronounced effect on PBP C3 and ODC mRNA that was quantitatively similar to the effect observed with flutamide. Overall, the results described in this article show that gene expression analysis can be a valuable supplement to organ weights and hormone analysis in Hershberger assays.

In an early article (Needham and Challis, 1991, p. 1481) it was mentioned that a dose of 100 mg/kg prochloraz was the high dose used for registration studies and that it "represented a dose which would not produce any deleterious toxic effects in rats." Our study showed clear effects of 50 mg/kg prochloraz on reproductive organs and mRNA levels.

Concerning the decreased organ weights in the reproductive system prochloraz behaved exactly like flutamide. However, in contrast to flutamide, prochloraz did not affect FSH levels and it decreased TSH and T_4. Our study also demonstrate that the potency for hormone disrupting properties detected *in vitro* not necessarily predict the potency of the compound *in vivo*.

In conclusion prochloraz has antiandrogenic activity *in vitro* and in rats *in vivo*. The effects are present both in intact and castrated rats. It is suggested that the toxic responses induced by prochloraz are mediated via antagonizing peripheral and central AR as well as via some unknown mechanisms of action. Some of the effect might be caused by aromatase inhibition. However, there is a great need for future studies in order to elucidate whether prochloraz administered *in utero* to pregnant animals will give rise to antiandrogenic effects in the offspring and to determine if the compound has any effects on fetal steroidogenesis.

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