Cumulative and Antagonistic Effects of a Mixture of the Antiandrogens Vinclozolin and Iprodione in the Pubertal Male Rat

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Vinclozolin and iprodione are dicarboximide fungicides that display antiandrogenic effects in the male rat, which suggests that a mixture would lead to cumulative effects on androgen-sensitive end points. Iprodione is a steroid synthesis inhibitor, but androgen receptor antagonist activity, which is displayed by vinclozolin, has not been fully evaluated. Here, we demonstrate that iprodione binds to the human androgen receptor (IC50 = 86.0 μM), reduces androgen-dependent gene expression, and reduces androgen-sensitive tissue weights in castrated male rats (Hershberger assay). Since vinclozolin and iprodione affect common targets in the pubertal male rat, we tested the hypothesis that a mixture would have cumulative antiandrogenic effects. An iprodione dose, that does not significantly affect androgen-dependent morphological end points, was combined with vinclozolin doses (2 × 5 factorial design). Sprague-Dawley rats were dosed by gavage with vinclozolin at 0, 10, 30, 60, and 100 mg/kg/day with and without 50 mg iprodione/kg/day from postnatal day (PND) 23 to 55–57 (n = 8 per group). The age at puberty (prepubertal separation [PPS]), organ weights, serum hormones, and ex vivo testis steroid hormone production were measured. Vinclozolin delayed PPS, reduced androgen-sensitive organ weights, and increased serum testosterone. The addition of iprodione enhanced the vinclozolin inhibition of PPS (PND 47.5 vs.49.1; two-way ANOVA: iprodione main effect p = 0.0002). The dose response for several reproductive and nonreproductive organ weights was affected in a cumulative manner. In contrast, iprodione antagonized the vinclozolin-induced increase in serum testosterone. These results demonstrate that these fungicides interact on common targets in a tissue-specific manner when coadministered to the pubertal male rat.

Key Words: iprodione; vinclozolin; mixture; puberty; testosterone; androgen receptor; endocrine disruption.

The cumulative toxic effect of chemical mixtures is of concern to regulating agencies such as the United States Environmental Protection Agency (EPA). Several studies demonstrate that mixtures of antiandrogenic chemicals have a cumulative effect on the developing male rat reproductive system (Hass et al., 2007; Hotchkiss et al., 2004; Howdeshell et al., 2007, 2008). Although antiandrogen chemicals may act via different mechanisms (e.g., androgen receptor [AR] antagonist vs. steroid synthesis inhibitor), ultimately, they disrupt the androgen-signaling pathway, which can lead to a cumulative effect in androgen-sensitive tissues (National Research Council, 2008; Rider et al., 2008). In the present study, two antiandro
genic dicarboximide fungicides were evaluated for cumulative antiandrogenic effects in the pubertal male rat. Since these dicarboximide fungicides can be active against the same fungal diseases, they can be used alternately on the same crop in some countries (International Union of Pure and Applied Chemistry/1991), and residues of more than one active ingredient therefore may be present in the harvested crops and in the fields. Vinclozolin and iprodione have antiandrogenic activity in vivo. Vinclozolin adversely affects male rat reproductive development after in utero or pubertal exposure via antagonism of the AR (Gray et al., 1994, 1999; Kelce et al., 1994; Ostby et al., 1999). Iprodione lowers circulating testosterone levels, inhibits testicular testosterone production, and delays pubertal developmental in the male rat (Blystone et al., 2007b). These two chemicals were not previously considered for inclusion in a cumulative risk assessment because it was determined that there was insufficient evidence that they shared a narrowly defined mechanism of action (i.e., AR antagonism) (EPA, 2003). However, the AR activity of iprodione has not been fully evaluated and both chemicals target male reproductive development. Since recent studies demonstrate cumulative
effects by mixtures of antiandrogenic compounds that target the same end points via different mechanisms of action (Hotchkiss et al., 2004; Rider et al., 2008), it is likely that a vinclozolin and iprodione mixture would have a cumulative effect on male rat development.

This study first tested the hypothesis that iprodione is an AR antagonist using a competitive AR-binding assay and an AR-mediated transcription assay. In addition, the Hershberger assay was used to evaluate AR antagonism in castrated males that received daily testosterone injections. Understanding iprodione’s AR activity is important for assessing its contribution within a mixture of other antiandrogenic compounds and provides mechanistic data for cumulative risk assessment. Next, the hypothesis was tested that a vinclozolin and iprodione mixture would have cumulative effects in the male rat during pubertal development. Similar to male sexual differentiation, puberty requires androgen action for successful development, which may be inhibited by antiandrogenic compounds (e.g., vinclozolin, p,p’-DDE, DE-71, and prochloraz; Blystone et al., 2007a; Kelce et al., 1995; Monosson et al., 1999; Stoker et al., 2005). In the present study, an iprodione dose, that did not significantly delay preputial separation or reduce androgen-sensitive organ weights (Blystone et al., 2007b), was combined with several doses of vinclozolin in order to determine iprodione’s effect on the vinclozolin dose-response curve. We hypothesized that administration of iprodione with vinclozolin would produce statistically significant shifts in the dose-response curves as compared to administration of vinclozolin alone, leading to the following:

- A longer delay in preputial separation with the mixture compared to vinclozolin alone.
- A greater decrease in androgen-sensitive organ weights with the mixture compared to vinclozolin alone.
- A decrease in serum and ex vivo testicular testosterone production levels by the mixture compared to vinclozolin alone (vinclozolin exposure increases serum testosterone).
- A greater increase in liver and adrenal organ weights in the mixture response compared to vinclozolin alone.

The last point is based upon evidence that these fungicides increase liver and adrenal weights in the male pubertal rat through unknown mechanisms (Blystone et al., 2007b; Monosson et al., 1999). By testing these hypotheses, we hope to demonstrate whether iprodione has AR-binding activity and that a mixture of iprodione and vinclozolin can have a cumulative effect on reproductive and nonreproductive end points. The present study is the first use of the pubertal male rat assay to test the hypothesis that antiandrogenic chemicals display cumulative effects in a mixture, which we used to evaluate two fungicides that may be applied on the same crops on a rotational basis or in component mixtures.

**MATERIALS AND METHODS**

**Chemicals.** Iprodione (CAS# 36734-19-7, 97% purity determined by vendor; Sigma Aldrich, St. Louis, MO; Lot# MO 07526JO) and vinclozolin (CAS# 50471-44-8, 99.5% purity as determined by vendor; Riedel-de Haen, Seelze, Germany; Lot# 2296X) were used in the in vitro and in vivo studies. Corn oil served as the in vivo vehicle (CAS# 8001-30-7; Sigma Aldrich). 3H-labeled methyltrienolone, i.e., R1881 (specific activity 83.5 Ci/mMol, >97% purity determined by vendor).
TABLE 1
Hershberger Assay Results. Mean (± SEM) Organ Weights and Serum Hormone Levels of Castrated Rats Exposed to Iprodione and TP for 10–11 Daysa

<table>
<thead>
<tr>
<th>End points</th>
<th>Iprodione (mg/kg/day)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nec. Wt.(g)</td>
<td>305.1 ± 3.7</td>
<td>312.2 ± 4.1</td>
<td>305.7 ± 2.4</td>
<td>299.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>80.4 ± 3.1</td>
<td>88.9 ± 5.9</td>
<td>82.0 ± 3.6</td>
<td>75.2 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Liver (g)</td>
<td>13.67 ± 0.51</td>
<td>14.33 ± 0.19</td>
<td>14.55 ± 0.50*</td>
<td>15.03 ± 0.20***</td>
<td></td>
</tr>
<tr>
<td>Paired kidneys (g)</td>
<td>2.21 ± 0.06</td>
<td>2.27 ± 0.06</td>
<td>2.36 ± 0.05</td>
<td>2.20 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Paired adrenals (mg)</td>
<td>44.8 ± 3.0</td>
<td>44.6 ± 3.8</td>
<td>48.0 ± 3.2</td>
<td>64.6 ± 5.1***</td>
<td></td>
</tr>
<tr>
<td>Glans penis (mg)</td>
<td>83.0 ± 5.1</td>
<td>83.7 ± 5.6</td>
<td>80.0 ± 2.8</td>
<td>80.0 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>142.4 ± 10.1</td>
<td>135.0 ± 13.1</td>
<td>127.6 ± 8.1</td>
<td>99.0 ± 3.3**</td>
<td></td>
</tr>
<tr>
<td>Seminal vesicle (mg)</td>
<td>554.6 ± 46.5</td>
<td>464.4 ± 27.8</td>
<td>467.0 ± 34.6</td>
<td>426.8 ± 46.2</td>
<td></td>
</tr>
<tr>
<td>LABC (mg)</td>
<td>594.1 ± 25.2</td>
<td>535.6 ± 17.5*</td>
<td>529.8 ± 23.6*</td>
<td>470.7 ± 17.8***</td>
<td></td>
</tr>
<tr>
<td>Cowper’s gland (mg)</td>
<td>36.1 ± 4.5</td>
<td>38.1 ± 3.5</td>
<td>37.3 ± 2.6</td>
<td>29.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>1.60 ± 0.17</td>
<td>1.53 ± 0.17</td>
<td>2.00 ± 0.29</td>
<td>1.96 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>15.56 ± 6.38</td>
<td>11.28 ± 3.30</td>
<td>10.37 ± 1.20</td>
<td>8.39 ± 2.57</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Organ Weights and Serum Hormone Levels of Castrated Rats Exposed to Iprodione and TP for 10–11 Days

*aOrgan weights analyzed using necropsy weight (Nec. Wt.) as a covariate.

*p < 0.05, **p < 0.01, ***p < 0.001, shaded areas differ significantly from control values.

TABLE 2
Average (± SEM) of Body Weights and Weight Gain After Vinclozolin or Vinclozolin + Iprodione Exposure in the Pubertal Male Rat

<table>
<thead>
<tr>
<th>End pointa</th>
<th>Vinclozolin dose (mg/kg/day)</th>
<th>Vinclozolin dose + iprodione dose (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Initial body weight</td>
<td>46.2 ± 1.6</td>
<td>46.8 ± 1.6</td>
</tr>
<tr>
<td>Weight gain</td>
<td>263.7 ± 10.8</td>
<td>268.1 ± 8.9</td>
</tr>
<tr>
<td>Necropsy body weight</td>
<td>316.7 ± 11.3</td>
<td>322.2 ± 9.4</td>
</tr>
</tbody>
</table>

Table 2: Body Weights and Weight Gain After Vinclozolin or Vinclozolin + Iprodione Exposure

*aVinclozolin and vinclozolin + iprodione treatments did not significantly affect body weights or weight gain.
DHT + 0.1 μM hydroxyflutamide (an AR antagonist). The luciferase activity was measured the following day using a BMG Labtech luminometer (Offenburg, Germany), and induction was calculated as fold increase over background (media alone wells). Cytotoxicity was evaluated by the MTT assay that measures mitochondrial function of the cells through the conversion of the yellow tetrazolium dye to blue formazan crystals (Berridge and Tan, 1993; Mosmann, 1983).

In vivo AR antagonism (Hersberger assay). Immature Sprague-Dawley rats were castrated on postnatal day (PND) 42 at Charles River Laboratories (Raleigh, NC) and shipped to our facility. Animals were provided with Purina Rat Chow 5001 and water ad libitum. Environmental conditions were 23°C, 37–41% humidity, and a 12L:12D light cycle (lights on at 0600 h). Prior to dosing, animals were weight ranked and assigned to dose groups to minimize weight differences among treatment groups. The animal use protocol for these studies was approved by the National Health and Environmental Effects Research Laboratory’s Institutional Animal Care and Use Committee.

Iprodione was mixed in corn oil and administered by gavage for a final volume of 2.5 ml/kg weight. At 100 and 200 mg/kg/day, the iprodione and corn oil formed a suspension, which was constantly stirred throughout dosing. Starting on PND 49 each rat received the iprodione dose (0, 50, 100, or 200 mg/kg/day) and then 100 μg testosterone propionate (TP) (Sigma Aldrich) by sc injection in 0.1 ml corn oil (n = 6 per treatment). After 10–11 days of treatment, animals were necropsied for organ weights (glans penis, ventral prostate, seminal vesicle, Cowper’s gland, levator ani + bulbocavernous muscle [LABC], liver, adrenals, and kidneys) and blood was collected via cardiac puncture to avoid contamination of blood with sc TP depots. Serum levels of testosterone and late-maturing hormone (LH) levels were quantified by radioimmunoassay (RIA), as described below.

Pubertal mixture study. Immature Sprague-Dawley rats were delivered from Charles River Laboratories and housed in the EPA’s Reproductive Toxicology Division Animal Facility on PND 22. Animals were provided with Purina Rat Chow 5001 and water ad libitum. Environmental conditions were 23°C, 37–45% humidity, and a 12L:12D light cycle (lights on at 0600 h). Prior to dosing, animals were weight ranked and assigned to dose groups to minimize differences in means and variance among treatment groups.

Vinclozolin and iprodione were mixed in corn oil and administering by gavage for a final volume of either 2.5 or 5.0 ml/kg body weight. Rats were dosed each morning with a dose volume of 5.0 ml/kg body weight from PND 23–30 due to the small size of the animals and then a dose volume of 2.5 ml/kg for the remainder of the study. At 60 and 100 mg/kg/day vinclozolin (with and without iprodione), the chemical(s) formed a suspension in the corn oil, which was constantly stirred throughout dosing.

Rats were dosed daily by gavage with vinclozolin (0, 10, 30, 60, and 100 mg/kg/day) or with these doses of vinclozolin plus 50 mg/kg/day iprodione from PND 23 to 55–57 (n = 8 per treatment, 80 total animals). The vinclozolin doses were selected to provide a dose response for the androgen-sensitive end points (Monosson et al., 1999). The 50 mg/kg/day dose of iprodione was selected on the basis that it would decrease serum testosterone but did not statistically affect androgen-sensitive end points (Blystone et al., 2007b). The experiment was conducted in two blocks in order to manage the study, each block contained four animals per dose. The progression of preputial separation (PPS) was inspected daily from PND 37 to necropsy at PND 55–57. The prepucce was gently retracted far enough to note the presence of either a constriction at the base of the glans penis or connective tissue that prevented the full retraction of the prepuce. Complete PPS was distinguished from incomplete PPS in which portions of the prepuce remained attached to the glans.

On the final day of dosing, animals were necropsied within 1.25–3.50 h of their final dose. Organ weights (glans penis, ventral prostate, seminal vesicle, Cowper’s gland, LABC, testes, epididymides, liver, kidneys, and adrenals) were recorded at necropsy, and trunk blood was collected following decapitation for serum measurements of testosterone, progesterone, and LH. In addition to serum measurements, ex vivo hormone production by the right testis of each animal was assessed in stimulated (100 IU/ml) human chorionic gonadotropin (hCG; Sigma Aldrich) or unstimulated conditions (−hCG) (described previously; Blystone et al., 2007a).

Hormones. Testosterone (limit of detection [LD] 0.04 ng/ml) and progesterone (LD 0.02 ng/ml) were measured using Diagnostic Products Corporation’s Coat-A-Count kit (Los Angeles, CA). Serum LH (LD 0.115 ng/ml) was measured by RIA as previously described (Goldman et al., 1986) using materials supplied by the National Hormone and Pituitary Agency: iodination preparation 1-6, reference preparation RP-3, and antiserum S-11. Iodination material was radiolabeled with 125I (Dupont/New England Nuclear, Boston, MA) by a modification of the chloramine-T method (Greenwood et al., 1963).

Statistical analysis. All in vivo data were analyzed using the PROC GLM procedure from SAS (SAS v8; Cary, NC). To demonstrate the iprodione effect (shift) on the vinclozolin dose response in the pubertal male rat, data were analyzed by two-way ANOVA. The two-way ANOVA tested for two main factors, vinclozolin and iprodione, and an interaction term vinclozolin×iprodione for PPS, weights, and hormone levels. Block effect or interaction between block and other terms in the model were also examined since the pubertal study was conducted in two blocks. If the block effect was nonsignificant, then it was removed from the model. PPS and weight at PPS data were analyzed using the initial weight as a covariate. Organ weights at necropsy were analyzed using body weight at necropsy as a covariate. Hormone data were log10 transformed for analysis to normalize variance. If the
hormone level of an individual sample was below the standard curve, the lowest value on the standard curve was used for the statistical analysis. Significant effects ($p < 0.05$) were further analyzed using least squares (LS)-means to determine significance between the control and the treatment groups. AR binding data were analyzed using Prism 5.0 (Graphpad Software, Inc., San Diego, CA).

RESULTS

In Vitro AR Binding

A competitive binding assay was used to evaluate the ability of iprodione to displace $[^3]$H R1881 from the hAR. In the hAR COS whole cell-binding assay, iprodione bound to AR with an $IC_{50}$ of 86.0 $\mu$M compared to the vinclozolin’s $IC_{50}$ of 1.4 $\mu$M (Fig. 1). Crystals were apparent at the 200 $\mu$M concentration of iprodione, suggesting that the chemical was insoluble at this concentration.

In Vitro AR Transcriptional Activation

In order to determine if iprodione affects AR activity, MDA-kb2 cells were exposed to iprodione or iprodione + 0.1nM DHT. Iprodione alone did not increase luminescence, and there was a significant increase in cytotoxicity at 300 $\mu$M as measured in the MTT assay (Fig. 2A). In the iprodione + 0.1nM DHT treatments, there was a decrease in DHT fold induction as iprodione concentrations increased ($IC_{50}$ = 245.9 $\mu$M), suggesting that iprodione may act as an AR antagonist (Fig. 2B). Crystals were observed within the media at 300 $\mu$M, suggesting iprodione was insoluble at this concentration.

In Vivo AR Antagonism

Immature castrated male rats were dosed with iprodione and TP to assess in vivo AR antagonism. Iprodione did not affect weight at necropsy ($p = 0.3393$) or weight gain ($p = 0.1806$) suggesting no overt systemic toxicity (Table 1). Since there was no effect on body weight, organ weights were analyzed using body weight at necropsy as a covariate. Iprodione significantly decreased the androgen-dependent ventral prostate ($p = 0.0322$) at 200 mg/kg/day and LABC ($p = 0.0068$) at all doses (Table 1). Although the seminal vesicle ($p = 0.1981$) or Cowper’s gland ($p = 0.2377$) weights were not statistically reduced, there was a significant ($p = 0.0479$) decreasing trend for the seminal vesicle weights but not Cowper’s gland weights ($p = 0.1082$). Adrenal ($p = 0.0049$) and liver ($p = 0.0003$) weights were significantly increased at 200 mg/kg/day and at 100 and 200 mg/kg/day, respectively. There was no effect by iprodione on the kidneys ($p = 0.1844$) (Table 1). Serum testosterone levels were not significantly different ($p = 0.2993$), which suggests that iprodione does not reduce circulating testosterone by increasing its metabolism. Serum LH levels were unaffected by treatment ($p = 0.8031$), and although LH appears to decrease with treatment, a trend analysis was not statistically significant ($p = 0.4390$) (Table 1).
Ventral prostate (mg) 217.9 ± 0.10
Testes (g) 2.80 ± 0.07
Cowper’s gland (mg) 64.4 ± 0.09
Kidneys (g) 2.68 ± 0.07

There was a significant block effect on the initial unaffected by vinclozolin or the vinclozolin mixture (Fig. 4 and Table 3). Testes and kidney weights were vesicle, epididymides, LABC, and Cowper’s glands weights (Fig. 4 and Table 3). Of these reproductive organs, iprodione decreased the weight of several reproductive end points: glans penis, ventral prostate, organ weights. Vinclozolin significantly reduced the weight of treatment, it was used as a covariate in the two-way analysis of treatment did not induce systemic toxicity (Table 2). The age at which the animal reached complete preputial separation and the corresponding body weights were significantly affected by vinclozolin dose response by iprodione was indicated by the design that was analyzed by two-way ANOVA. A shift in the dose-response curve was tested through a 2 factor study

Serum progesterone (ng/mL) 0.339 ± 0.044
Ex vivo progesterone (ng/g testis) 10.2 ± 2.1
Ex vivo progesterone (+hCG) (ng/g testis) 19.0 ± 2.5

Ex vivo progesterone (+hCG) (ng/g testis) 21.5 ± 1.2

p = 0.0519). One block of animals was initially larger than the other block, but significant body weight differences did not exist at the end of the study. The adrenal glands were not affected by vinclozolin treatment by two-way ANOVA, although there was a significant difference between the control and 100 mg/kg/day vinclozolin dose by LS-means (p = 0.0395). Iprodione did increase the adrenal weights, and there was not a significant interaction between the treatments (p = 0.1390) (Fig. 5). Vinclozolin increased liver weights and there was a greater increase in liver weights by the addition of iprodione (Fig. 5) but neither chemical affected kidney weights (Table 3).

Serum hormones and ex vivo hormone production by the testes were examined to assess the effects of vinclozolin and the mixture of vinclozolin and iprodione on these end points. Serum testosterone increased from vinclozolin exposure but iprodione significantly decreased this effect with the effect of iprodione being most prominent when combined with vinclozolin at 60 mg/kg/day. The effects of the mixture on testosterone production ex vivo showed the same trends as the serum levels, but the effects were less robust (Fig. 6). Ex vivo testosterone production with and without hCG stimulation was increased by vinclozolin treatment (p < 0.0001; Fig. 6). There were significant interactions between iprodione and vinclozolin treatments for testosterone in the unstimulated and the stimulated (p = 0.016 and p = 0.027, respectively) ex vivo
conditions with the greatest suppression of testosterone production occurring when iprodione was combined with 60 mg vinclozolin/kg/day (p < 0.01 in both cases) (Fig. 6). However, serum progesterone was not affected by vinclozolin or the vinclozolin + iprodione mixture (Table 3). Serum LH was increased by vinclozolin (p < 0.0001) and iprodione did not affect this response (p = 0.8033) (Fig. 6). Testosterone and progesterone production by the ex vivo testis under stimulated or nonstimulated conditions was increased by vinclozolin. There was a significant block effect on progesterone levels in the unstimulated and hCG-stimulated conditions. The effects of the mixture on progesterone production ex vivo were similar to the effects on ex vivo testosterone production (Table 3). Ex vivo progesterone production with and without hCG stimulation was increased by vinclozolin treatment (p < 0.0001; Table 3). There were interactions between iprodione and vinclozolin treatments on progesterone production ex vivo with and without hCG (p = 0.07 and p = 0.25, respectively) with the greatest suppression occurring when iprodione was combined with 60 mg vinclozolin/kg/day (t-tests were p < 0.01 in both cases). The main effect of iprodione on ex vivo progesterone production was significant in the absence of hCG (p = 0.045).

**DISCUSSION**

These data clearly support the hypotheses that the vinclozolin and iprodione mixture has a cumulative effect on several androgen-sensitive and nonreproductive end points in the pubertal male rat. This is the first study to use the pubertal male assay to test the effects of a chemical mixture, and the results indicate that this assay is sensitive to detect cumulative effects of antiandrogens. The pubertal model allows for assessment of antiandrogenic activity after a relatively short exposure period and these results predict that exposure to a similar mixture in utero would lead to malformations in the androgen-sensitive tissue of male offspring. The combination of vinclozolin’s AR antagonism with iprodione’s AR antagonism and ability to reduce circulating levels of testosterone likely have a cumulative effect of reducing testosterone binding to the AR, resulting in the cumulative delay in PPS and reduced reproductive organ weights. Previous reports demonstrate that in utero exposures to antiandrogen chemical mixtures, which contain components with various mechanisms of action, have also produced cumulative effects on the male reproductive development (Hass et al., 2007; Hotchkiss et al., 2004; Howdeshell et al., 2007, 2008).

The in vitro and Hershberger experiments demonstrated that iprodione is an AR antagonist (Figs. 1 and 2 and Table 1), which is a common mechanism displayed by the other dicarboximide fungicides vinclozolin and procymidone. The AR antagonism of vinclozolin and procymidone has been demonstrated in vitro and in vivo (Hosokawa et al., 1993; Kelce et al. 1994, 1997; Ostby et al., 1999). The metabolites of vinclozolin were demonstrated to be the active AR antagonists. The Kᵢ for vinclozolin was calculated to be > 700µM, while the M1 and M2 metabolites Kᵢ’s were 92 and 9.7µM, respectively (Kelce et al., 1994). It is unknown if procymidone metabolites contribute to the AR antagonism in vivo (Freyberger and Ahr, 2004; Hosokawa et al., 1993; Ostby et al., 1999). Further investigation will be needed to determine if iprodione or a metabolite of iprodione is the AR antagonist, but preliminary results suggest that a metabolite may be responsible. These iprodione results and the reported reduced testosterone levels by iprodione in the pubertal male (Blystone et al., 2007b) suggest that iprodione is a mixed mechanism antiandrogen. These mechanisms would likely act jointly in vivo since the reduction of available testosterone (ligand) along with iprodione competition for the AR would result in less activation of the AR. A comparison of effects on the
androgen-sensitive organ weights in the Hershberger assay demonstrates that iprodione is a weaker AR antagonist in vivo compared to vinclozolin (Gray et al., unpublished data) (Fig. 7). This comparison complements the present in vitro findings that vinclozolin is a more potent AR antagonist than iprodione.

The mechanism of action by which the three antiandrogenic dicarboximide fungicides iprodione, vinclozolin, and procymidone affect adrenal gland weight is unknown. Iprodione alone increased adrenal gland weights in the Hershberger assay and in the pubertal male rat (Blystone et al., 2007b), and there are similar results for procymidone and vinclozolin (Kennel et al., 2004; Matsuura et al., 2005; Stoker et al., 2005). Together, this suggests that all three dicarboximides in a mixture would produce a cumulative effect on the adrenal gland, as with the androgen-sensitive end points. In the present study, vinclozolin alone had no effect on adrenal weights in the pubertal male rat by the two-way ANOVA, but there was significant difference when the control and 100 mg/kg/day vinclozolin dose are analyzed, suggesting a high overall variability in adrenal weights. Previous reports indicate an effect on adrenal weights at 100 or 200 mg/kg/day in the castrated male rat (Lambright et al., 2000; Yamasaki et al., 2003) or 100 mg/kg/day in the pubertal male rat (unpublished data). The adrenal weights increased in a dose-dependent manner in the iprodione and vinclozolin mixture, and a cumulative effect may have been detected if there were more animals per group. Iprodione and vinclozolin separately increase liver weights in the male rat (Blystone et al., 2007b; Monosson et al., 1999), which may involve cytochrome P450 induction (Sapone et al., 2003). The shift of the vinclozolin liver weight response by the addition of iprodione, at an iprodione dose that does not affect liver weight, suggests that the mixture of the two has a cumulative effect on the liver.

The response of serum testosterone to the dicarboximide mixture (Fig. 6) was consistent with the mechanism of action for these two chemicals (Blystone et al., 2007b; Monosson et al., 1999). As an AR antagonist, vinclozolin increased serum LH in the pubertal male, presumably by blocking the negative feedback loop of the hypothalamic-pituitary regulation. Iprodione exposure did not affect serum LH in the pubertal male, while it decreased serum testosterone levels. The reason why LH is unaffected by iprodione treatment when testosterone levels are reduced is not known, but LH appears to be a consistently unaffected by iprodione treatment (Blystone et al., 2007b). The serum data presented here demonstrate that the testosterone increase induced by vinclozolin’s LH stimulation is negated to a certain degree by iprodione’s inhibition of testis testosterone production with the effect being greater when combined with 60 mg vinclozolin/kg/day. The effects of iprodione plus vinclozolin on ex vivo testosterone production showed the same trends as the effects on serum testosterone levels, but the ex vivo effects were less robust (Fig. 6). This is consistent with the previous report that 50 mg iprodione dose did not significantly lower media testosterone levels from the ex vivo testis incubation (Blystone et al., 2007b), which indicates that it is not as sensitive as serum testosterone measurements after iprodione treatment. It is not clear why the
ex vivo testis incubation was not as sensitive as the serum testosterone measurements for iprodione, but this may be due to the dilution of iprodione during the incubation period.

In summary, this study demonstrates that iprodione is an AR antagonist in vitro and in vivo, a mechanism of action shared with the other dicarboximide fungicides vinclozolin and procymidone. Coadministration of iprodione and vinclozolin has a cumulative effect on androgen-sensitive end points in the pubertal male rat exceeding the response expected had they acted independently. Furthermore, iprodione and vinclozolin had a cumulative effect on nonreproductive organs that are known targets of these chemicals. While it has generally been held that vinclozolin and iprodione did not share a common mechanism of toxicity (EPA, 2003), these data do not support this assumption and indicate that iprodione is an AR antagonist, albeit less potent in vitro and in vivo than vinclozolin. The mixture of iprodione, a mixed mechanism antiandrogen, and vinclozolin, an AR antagonist, act jointly and have cumulative antiandrogenic effects in the pubertal male rat.

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**FIG. 7.** Comparison of iprodione (black square, current study, solid line) and vinclozolin (white square, Gray et al., unpublished data, dashed line) Hershberger results. Values are means ± SE (n = 6 per group for iprodione and 4 per group for the vinclozolin studies).
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REFERENCES


