Antiandrogenic Mechanisms of Pesticides in Human LNCaP Prostate and H295R Adrenocortical Carcinoma Cells

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ABSTRACT

Several pesticides suspected or known to have endocrine disrupting effects were screened for pro- or antiandrogenic properties by determining their effects on proliferation, prostatic-specific antigen (PSA) secretion and androgen receptor (AR) expression, and AR phosphorylation in androgen-dependent LNCaP human prostate cancer cells, as well as on the expression and catalytic activity of the enzyme CYP17 in H295R human adrenocortical carcinoma cells, an in vitro model of steroidogenesis. Effects on SRD5A1 gene expression were determined in both cell lines. Benomyl, vinclozolin, and prochloraz, but not atrazine, concentration dependently (1-30 μM) decreased dihydrotestosterone (DHT)-stimulated proliferation of LNCaP cells. All pesticides except atrazine decreased DHT-stimulated PSA secretion, AR nuclear accumulation, and AR phosphorylation on serines 81 and 213 in LNCaP cells. Benomyl and prochloraz, but not vinclozolin or atrazine, decreased levels of CYP17 gene and protein expression, as well as catalytic activity in H295R cells. In the case of prochloraz, some of these effects corresponded with cytotoxicity. H295R cells expressed AR protein and SRD5A1, but not SRD5A2 transcripts. SRD5A1 gene expression in H295R cells was increased by 10 nM DHT, whereas in LNCaP cells significant induction was observed by 0.1 nM DHT. AR protein expression in H295R cells was not increased by DHT. Vinclozolin decreased DHT-induced SRD5A1 gene expression in LNCaP, but not H295R cells, indicating a functional difference of AR between the cell lines. In conclusion, pesticides may exert antiandrogenic effects through several mechanisms that are cell type-specific, including AR antagonism and down-regulation or catalytic inhibition of androgen biosynthetic enzymes, such as CYP17 and SRD5A1.

Key words: androgen receptor; pesticides; LNCaP; H295R; CYP17; SRD5A1

Accumulating evidence implicates certain pesticides in causing endocrine disrupting effects that can be associated with reproductive and developmental dysfunction as well as with an increased risk of development of hormone-dependent cancers (Brevini et al., 2005; Schug et al., 2011). Many studies focus on estrogenic effects, but pro- or antiandrogenic mechanisms of action of endocrine disruptors have not been investigated in the same detail. A notable exception is the fungicide vinclozolin (Kavlock and Cummings, 2005), which has been reported to directly antagonize the androgen receptor (AR) (Wong et al., 1995), thus preventing its activation by endogenous androgens such as testosterone and dihydrotestosterone (DHT). The consequence of this disruption is a demasculinization of male offspring in rats that were exposed to 100 and 200 mg vinclozolin/kg body weight/day from gestational day 14 until postnatal day 3 (Gray et al., 1994) and alterations in androgen-dependent developmental processes such as prostate growth and androgen-mediated gene expression in this tissue in adult male rats (Kelce et al., 1997). A study in female offspring of rats exposed to 1 mg vinclozolin/kg body weight/day for the duration of pregnancy showed alterations in female mammary glands, including an increase in epithelial branching and loss of epithelial cell polarization at postnatal day 35, but which were less pronounced at postnatal day 50 (El Sheikh Saad et al., 2013).
Such effects were consistent with altered mammary gland development in female mice lacking AR (Yeh et al., 2003).

Pesticides may act through various potential mechanisms to cause disruption of androgenic function, which beside an interaction with the AR, as exemplified by vinclozolin, may include interferences with the function of steroidogenic enzymes or “cross-talk” pathways that influence AR signaling by altering AR phosphorylation or degradation, or its affinity for coregulators (Balk and Knudsen, 2008). The AR is a key target for pro- or antiandrogenic compounds and is involved in (sexual) development in both males and females, as well as in the progression of certain hormone-dependent cancers such as that of prostate. Key steroidogenic enzymes that may be targeted by androgen-disrupting chemicals are cytochrome P450 17 (CYP17), which conveys steroid 17α-hydroxylase and 17,20-lyase activities and is responsible for the synthesis of precursors for all androgens, and steroid 5α-reductase (SRD5A), which converts testosterone to the significantly more potent androgen DHT (Sanderson, 2006).

Given the time-consuming and expensive aspects of in vivo experiments to determine disruptive effects of chemicals on androgenic function, a number of in vitro tools have been developed to rapidly screen for pro- or antiandrogenic effects of compounds of interest to be selected for in vivo studies (Beck et al., 2008; Kolle et al., 2010; Sohoni and Sumpter, 1998).

**MATERIALS AND METHODS**

Pesticides. All pesticides were purchased from Sigma-Aldrich (St. Louis, MO). Atrazine and benomyl were Fluka PESTANAL grade with purities of 97.4% and 99.0%, respectively. Vinclozolin and prochloraz were Riedel deHaen analytical grade with purities of 99.6% and 99.1%, respectively. Their chemical structures are shown in Table 1.

<table>
<thead>
<tr>
<th>Pesticide (CAS Number)</th>
<th>Structure</th>
<th>Classification</th>
<th>LNCaP Cell Bioassay</th>
<th>H295R Cell Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine (1912-24-9)</td>
<td><img src="atrazine.png" alt="Structure" /></td>
<td>Triazine herbicide</td>
<td>Cell proliferation; PSA secretion; AR accumulation; AR phosphorylation</td>
<td>n.e. CYP17 gene expression; n.e. CYP17 protein expression; n.e. CYP17 catalytic activity; n.e.</td>
</tr>
<tr>
<td>Benomyl (17804-35-2)</td>
<td><img src="benomyl.png" alt="Structure" /></td>
<td>Benzimidazole fungicide</td>
<td>Cell proliferation; PSA secretion; AR accumulation; AR phosphorylation</td>
<td>↓ CYP17 gene expression; ↓ CYP17 protein expression; ↓ CYP17 catalytic activity; ↓</td>
</tr>
<tr>
<td>Vinclozolin (50471-44-8)</td>
<td><img src="vinclozolin.png" alt="Structure" /></td>
<td>Dicarboximide fungicide</td>
<td>Cell proliferation; PSA secretion; AR accumulation; AR phosphorylation; SRD5A1 gene expression</td>
<td>↓ CYP17 gene expression; ↓ CYP17 protein expression; ↓ CYP17 catalytic activity; ↓</td>
</tr>
<tr>
<td>Prochloraz (67747-09-5)</td>
<td><img src="prochloraz.png" alt="Structure" /></td>
<td>Triazole fungicide</td>
<td>n.e. CYP17 gene expression; n.e. CYP17 protein expression; n.e. CYP17 catalytic activity; n.e. SRD5A1 gene expression; n.e.</td>
<td></td>
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Notes: The effects of vinclozolin on DHT-mediated induction of SRD5A1 gene expression were studied in both cell lines. Abbreviations and symbols: (n.e.) no effect. Inhibition observed at (↓) 1 μM; (↓↓) 10 μM; (↓↓↓) 30 μM.
Cell culture. LNCaP human prostate carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia) and grown in RPMI 1640 medium (Life Technologies; Cat. No. 21870-076) supplemented with 1% L-glutamine (21051-024), 1% penicillin/streptomycin (15070-063), 1% HEPES buffer (15630-080), 10% fetal bovine serum (FBS) (12483-020), and 1% sodium pyruvate (11360-070). Cells were exposed to pesticides in phenol red-free medium (13835-030), supplemented with 2% dextran-charcoal-stripped FBS (12676-011). Pesticides and DHT (A2570-000; Steraloids, Newport, Rhode Island) were dissolved in dimethyl sulfoxide (DMSO) (D4540; Sigma-Aldrich) as 1000-fold stock solutions. DMSO was used as solvent control and final DMSO concentrations in culture medium were either 0.1% or 0.2% dependent on whether the experiment required single or dual compound exposures. H295R human adrenocortical carcinoma cells (ATCC) were grown in Dulbecco’s Modified Eagle’s medium with phenol red, complemented with 1% L-glutamine, 1% penicillin/streptomycin, 1% insulin-transferrin/sodium selenite/bovine serum albumin/linoleic acid (ITS - 1) (Sigma-Aldrich), and 2.5% Nu-serum (BD Biosciences). H295R cells were seeded in Dulbecco medium without supplement, when exposed with hormones treatments.

Cell proliferation and viability. LNCaP cells were seeded in 96-well plates (5 × 10^3 cells/well) in phenol red- and steroid-free medium and acclimatized for 24 h to arrest cell proliferation. Cells were then exposed to fresh medium containing 1, 3, 10, or 30μM pesticides in presence or absence of 0.1 nM DHT; cells were re-exposed to identical treatments in fresh medium 24 h later and after another 72 h the number of viable cells was determined using a WST-1 cell viability kit (Roche, Mississauga, Ontario, Canada). Cells were incubated with WST-1 substrate for 2 h, after which the formation of formazan was quantified by measuring its absorbance at a wavelength of 440 nm and a reference wavelength of 630 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, California). H295R cell viability was determined in 24-well plates (5 × 10^4 cells/well) using a WST-1 assay after a 24-h exposure to 1, 3, 10, 30, or 100 μM of each pesticide.

PSA secretion. LNCaP cells (0.3 × 10^5 cells/well) in 12-well plates (Corning) were exposed to pesticides with or without 0.3 nM DHT for 24 h, after which medium was collected and centrifuged. PSA levels were determined using a commercial enzyme-linked immunosorbance assay kit (No. EL10050; Anogen, Mississauga, Ontario, Canada). Media from cells treated without DHT were diluted 10 times and from those treated with DHT 20 times prior to assay. Cellular protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, Illinois). This colorimetric test is based on the reduction of Cu²⁺ to Cu⁺ by proteins in alkaline medium. Proteins were quantified by measuring the absorbance of Cu⁺ at a wavelength of 562 nm using a SpectraMax M5 spectrophotometer. PSA secretion levels were expressed in ng per 100 ng cellular protein.

AR protein expression and phosphorylation. LNCaP cells (5 × 10⁵ cells/well) in 6-well plates (Corning) were exposed to the pesticides in the presence or absence of 10 nM DHT for 24 h, after which cytoplasmic and nuclear proteins were extracted using a NE-PER extraction kit (Pierce Biotechnology). Nuclear proteins (25 μg) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by migration for 1 h through a 10% SDS-PAGE gel at 150 V, after which proteins were transferred over a 10-min period to a polyvinylidene fluoride membrane using a Trans-Blot Turbo system (Bio-Rad, Mississauga, Ontario, Canada). Membranes were blocked with Tris-buffered saline containing 5% milk powder for 1 h and then incubated overnight in the same blocking buffer containing primary antibody. H295R cells (5.5 × 10⁵ cells/well) were treated with various concentrations of DHT, and with 10 μM vinclozolin in the presence or absence of 10 nM DHT, after which AR protein levels were determined in total lysates, as described for LNCaP cells above. Phosphorylated AR levels were determined in total lysates of LNCaP or H295R cells exposed for 1, 6, or 24 h to pesticides (H295R cells were exposed to vinclozolin only) with or without DHT. When using phospho-antibodies, the blocking buffer contained bovine serum albumin instead of milk. Primary antibodies against AR were AR-21 (Cat. No. FCAB5415PE, Millipore, Billerica, Massachusetts), p-AR-Ser81 (07-1375, Millipore), and p-AR-Ser210/213 (ab45089, Abcam, Cambridge, UK). Secondary antibodies were horse radish peroxidase-conjugated antirabbit (31210, Pierce Biotechnology) for AR and p-AR-Ser81 and HRP-conjugated antimouse (31172, Pierce Biotechnology) for p-AR-Ser213. Protein bands were made visible by immunofluorescence with Clarity Western ECL substrate (170-5061) and a ChemiDoc MP Image (Bio-Rad). Densities of the protein bands were analyzed by Image Lab Software (Bio-Rad).

CYP17 and SRD5A gene expression. Gene expression was quantified by real-time reverse transcriptase-polymerase chain reaction (RT-qPCR). Effects on CYP17 gene expression were studied in H295R cells only, because LNCaP cells were found not to express CYP17. H295R cells (5.5 × 10⁵ cells/well) were added to 6-well plates in 4 ml of culture medium and left to adhere for 24 h. Cells were then exposed to fresh medium containing 30 μM of the pesticides atrazine, benomyl, or vinclozolin, or 10 μM prochloraz. Forskolin (F6886; Sigma-Aldrich) at a final concentration of 10 μM was used as a positive control for CYP17 gene induction to verify the responsiveness of the H295R cells. To determine effects on SRD5A1/2 expression, H295R and LNCaP cells (5.5 × 10⁵ cells/well) that had acclimatized in steroid-free medium were exposed to various concentrations of DHT to determine the induction response of SRD5A1 in each cell type; SRD5A2 was not expressed in either cell line. H295R and LNCaP cells were then exposed to vinclozolin (10 μM) in the presence or absence of an optimally inducing concentration of DHT. For all gene expression experiments, wells without cells were used as negative controls. Total RNA was extracted using a High Pure RNA Isolation kit (Cat. No. 11828665001, Roche). RNA was quantified by measuring its absorbance at a wavelength of 260 and 280 nm using a SpectraMax M5 spectrophotometer. After reverse transcription using a Thermocycler T3000 (Biometra, Goettingen, Germany), 1 μg of the resultant cDNA was amplified by PCR using a Thermal Cycler C1000 (Bio-Rad) and primers that selectively recognized either CYP17 (fw: 5’-GCC-TTC-CTG-CTG-CAC-AAT-CTT-3’; rev: 5’-AAA-CTC-ACC-GAT-GCT-GGA-GTC-AAC-3’; amplicon: 207 bp), SRD5A1 (fw: 5’-GGC-AGG-AGG-AAA-GCC-TAT-GC-3’; rev: 5’-CAG-GGC-ATA-GCC-ACA-CCA-CT-3’; amplicon: 316 bp), or SRD5A2 (fw: 5’-ATT-GCG-CCA-GTC-CAG-GAA-G-3’; rev: 5’-TGG-AAT-AAG-GGC-TTT-CCG-AGA-T-3’; amplicon: 256 bp) (Soderstrom et al., 2001). Beta-actin and glyceraldehyde-3-phosphate dehydrogenase were verified to be suitable reference genes and used to normalize gene expression.

CYP17 protein expression. H295R cells (5.5 × 10⁵ cells/well) were added to 6-well plates in 4 ml of medium and acclimatized for...
Cells were exposed to fresh medium containing 30 μM atrazine, benomyl, or vinclozolin, or 10 μM prochloraz. Forskolin (10 μM) was used as positive control and cells exposed to DMSO acted as a solvent control. Proteins were extracted using a radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) with 1% anti-protease and 1% anti-phosphatase. Total protein (50 μg) underwent SDS-PAGE and immunoblotting using the same protocol as for AR, except with CYP17-selective antirabbit antibodies at a 1:100 dilution (Cat. No. 14447-1-AP; Acris, San Diego, California) and HRP-conjugated secondary antirabbit antibodies 1:5000 dilution (Cat. No. 31210, Pierce Biotechnology).

CYP17 catalytic activity. H295R cells (5 × 10^4 cells/well) were added to 24-well plates in 1 ml medium and left to adhere for 24 h. Cells were then exposed to pesticides in fresh medium for another 24 h, after which the medium was replaced with fresh medium without supplements, containing 100 nM of the CYP17 substrate pregnenolone and 5 μM of the 3β-hydroxysteroid dehydrogenase (3β-HSD) inhibitor trilostane. After a 90-min incubation, the reaction medium was removed and dehydroepiandrosterone (DHEA) content was quantified using an ELISA kit (EIA3415; DRG Intl., New Jersey). This assay design allowed for the integrated measurement of both the 17α-hydroxylase and 17,20-lyase activity of CYP17 without the rapid loss of either substrate or product via 3β-HSD-mediated reactions, which are known to be strong in H295R cells (Canton et al., 2006).

Statistical analysis. Results were presented as means with standard errors. Statistically significant differences (*P < 0.05) from control were determined by Student’s t test or by one-way analysis of variance (ANOVA) and Dunnett’s post hoc test to correct for multiple comparisons to control. All statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software, San Diego, California).
RESULTS

Antiandrogenic Effects in LNCaP Cells

None of the pesticides affected basal LNCaP cell proliferation under steroid-deprived conditions at concentrations between 1 and 30 μM (Fig. 1A). An incubation of LNCaP cells with 0.1 nM DHT increased cell proliferation 5- to 6-fold above control (Fig. 1B) that corresponded to a 75% effective concentration (EC75) for this response, which was consistent with our previous studies (Rivest et al., 2011). Passage number had a strong effect on androgen-stimulated cell proliferation, with passage numbers above 18 resulting in strongly diminished stimulation, as reported previously (Sanderson et al., 2013). DHT-stimulated LNCaP cell proliferation was reduced concentration dependently and statistically significantly by benomyl (30 μM), vinclozolin (≥10 μM), and prochloraz (≥10 μM), but not by atrazine (Fig. 1B). At 30 μM, benomyl, vinclozolin, and prochloraz reduced DHT-stimulated cell proliferation by 55%, 60%, and 88%, respectively.

FIG. 3. Nuclear accumulation of androgen receptor (AR) in LNCaP cells exposed for 24 h to atrazine (A), benomyl (B), vinclozolin (C), or prochloraz (D) in the absence or presence of 50 nM DHT. Beta-actin was used as reference protein. Relative protein levels were expressed as a percentage of DMSO control. Hash (#), A statistically significant difference between DHT and DMSO control; Student’s t test; P < 0.05. Asterisks (*), A statistically significant differences between pesticide treatment and DHT control; one-way ANOVA and Dunnett’s post hoc test; P < 0.05. Experiments were performed in triplicate; per experiment, each concentration was tested in duplicate (as shown here in representative gels).
CYP17 Expression and Catalytic Activity in H295R Cells

As an alternate mechanism of pro- or antiandrogenicity, the effects of the pesticides on CYP17 expression and catalytic activity were evaluated in H295R cells; we did not detect CYP17 expression or activity in LNCaP cells (not shown). To differentiate inhibitory effects from possible cytotoxicity, the viability of H295R cells was determined after a 24 h exposure to each pesticide (1–100 μM). Prochloraz was cytotoxic at concentrations greater than 10 μM; vinclozolin was cytotoxic at 100 μM (Fig. 5). Exposure to benomyl (30 μM) or prochloraz (10 μM) decreased CYP17 gene expression in H295R cells (Fig. 6A). These two pesticides also decreased CYP17 protein expression (Fig. 6B). Pregnenolone-stimulated DHEA production, our measure of CYP17 activity, was decreased in H295R cells exposed to benomyl (30 μM) and prochloraz (1 and 10 μM) (Fig. 6C). Neither atrazine nor vinclozolin had an effect on CYP17 gene and protein expression or its catalytic activity.

SRD5A Expression in H295R and LNCaP Cells

Another possible steroidogenic mechanism of pro- or antiandrogenicity is a chemical-induced change in the expression of SRD5A genes. We found similar levels of SRD5A1 gene expression in H295R and LNCaP cells; neither cell line expressed SRD5A2 (not shown). In both cell lines, SRD5A1 gene expression was inducible by DHT, although statistically significant increases occurred at a 10-fold lower concentration in LNCaP than H295R cells (Fig. 7A). DHT-mediated induction of SRD5A1 expression was decreased statistically significantly by 10 μM vinclozolin in LNCaP but not H295R cells (Fig. 7B). Similarly, neither atrazine nor vinclozolin had an effect on SRD5A1 expression in H295R cells (not shown).

AR Expression in H295R Cells

The similar induction of SRD5A1 expression by DHT in LNCaP and H295R cells suggests that H295R cells contain functional AR. We found that AR protein was expressed at relatively high basal levels in H295R cells, but that its expression was not increased by a 24-h exposure to 10 nM DHT. Neither did a 24-h exposure to 1 or 10 μM vinclozolin in the presence or absence of DHT have an effect on AR expression relative to control (Fig. 8). In addition, we did not observe increased phosphorylation of AR on Ser81 in response to DHT and failed to detect any phosphorylation of AR on Ser213 (not shown).

DISCUSSION

Antiandrogenic Effects in LNCaP Cells

Our results using LNCaP cells confirm that certain pesticides, in particular vinclozolin, exert antiandrogenic effects, such as
reduced DHT-stimulated LNCaP cell proliferation (Fig. 1), PSA secretion (Fig. 2), and nuclear AR accumulation (Fig. 3). Prochloraz and benomyl appeared to act similar to vinclozolin, although benomyl was less potent. The observed antiandrogenic effects of vinclozolin are consistent with other studies of its endocrine disrupting properties, which have been attributed specifically to an interference with AR ligand binding, resulting in disrupted male development and demasculinization (Gray et al., 1994, 1999; Kavlock and Cummings, 2005; Kubota et al., 2003). Prochloraz has also been reported to have antiandrogenic effects in vivo and in vitro (Vinggaard et al., 2002, 2005), but benomyl has not been associated with any antiandrogenic activity, neither in vitro nor in vivo (Scippo et al., 2004; Yamada et al., 2005). However, we find that benomyl, at least at 30 μM, reduces DHT-stimulated LNCaP cell proliferation (Fig. 1) and PSA secretion (Fig. 2), as well as decreasing nuclear AR accumulation (Fig. 3) and AR phosphorylation on serine positions 81 and 213 (Fig. 4), indicating it is antiandrogenic at high concentrations. This appears to be corroborated by a study showing that at a high dose of 100 mg/kg body weight for 10 days, benomyl reduced the number of seminal vesicles and weight of the levator ani-bulbocavernosus muscles in castrated rats supplemented with testosterone (Hershberger assay) (Yamada et al., 2005).

Atrazine did not exert any antiandrogenic effects in LNCaP cells. This is also consistent with the known behavior of atrazine, which has clear endocrine disrupting properties in vivo, but that are estrogenic and feminizing in nature (Belloni et al., 2011; Hayes et al., 2002; Roy et al., 2009; Wilhelms et al., 2006) and not related to an interaction with the AR signaling pathway (Vinggaard et al., 2008).

The antiandrogenic potencies of the pesticides appeared to be dependent on the nature of the DHT-stimulated response in question. This may in part have to do with the amount of DHT.
required to exert a specific androgenic effect in LNCaP cells (under our conditions). Far less DHT was required to induce LNCaP cell proliferation or PSA secretion, than to observe significant nuclear AR accumulation. It is probable that far greater receptor occupancy is required for AR protein stabilization, which is the main mechanism of androgen-mediated increases in cellular and nuclear AR levels (Zhou et al., 1995), than for other more sensitive AR signaling responses. Among the anti-androgenic responses elicited by the pesticides in LNCaP cells, the DHT-stimulated secretion of PSA appears to be the most sensitive endpoint as it was decreased by each of the three anti-androgenic pesticides at a concentration as low as 1 μM.

**AR Phosphorylation in LNCaP Cells**

We are the first to report that certain pesticides can inhibit the androgen-induced phosphorylation of AR at sites known to be associated with increased transactivational activity (Gioeli et al., 2002; Gioeli and Paschal, 2012; Koryakina et al., 2014). The DHT-induced phosphorylation of AR on positions serine 81 and 213, which reside in the N-terminal domain of the receptor, is reported to convey transactivational activity, possibly by enhancing the interaction of this domain with AR coactivating factor, AF-1 (Gioeli, 2005). It is thought that the binding of DHT to the AR induces a conformational change that increases exposure of the phosphorylation site(s) to certain kinases. Cyclin-dependent kinases CDK1 (Chen et al., 2006), CDK9 (Gordon et al., 2010), and CDK5 (Hsu et al., 2011) have been reported to phosphorylate AR on serine 81, which is the highest stochiometric phosphorylation site stimulated by DHT. Serine 81 phosphorylation results in reduced ubiquitination of AR, and subsequent protein stabilization and rapidly increased intracellular levels of AR in the absence of increased AR gene expression (Chen et al., 2006; Hsu et al., 2011). The Ser213 position is phosphorylated by Akt (protein kinase B), which has an established role in sustained proliferation of prostate cancer cells when overexpressed due to a loss of phosphatase and tensin homolog (PTEN) activity, which normally keeps levels of the PI3K product phosphatidylinositol-3,4,5-triphosphate low enough to suppress PI3K/Akt-mediated proliferative signaling (Mulholland et al., 2005).

Benomyl, vinclozolin, and prochloraz reduced levels of phosphorylated p-AR-Ser81 and p-AR-213 (Fig. 4), indicating that these pesticides are effectively reducing the transactivational capacity of the AR. This is likely caused by preventing DHT from interacting with the ligand-binding domain of the AR, given both AR phosphorylation sites are reduced to a similar extent. However, other possible mechanisms could play a role, such as inhibition by the pesticides of various kinase activities or the stimulation of specific phosphatases, such as PPA2, which is known to dephosphorylate AR at position Ser81 (Yang et al., 2007). In fact, these mechanisms are likely interdependent, as pesticide-mediated blockade of androgen binding to the AR would reduce exposure of the phospho-sites to kinases as well as enhance the recruitment of phosphatases, which would not normally be able to target androgen-bound AR (Yang et al., 2007).

**Steroidogenic Effects in H295R Cells**

We demonstrated that benomyl decreased CYP17 gene expression as well as protein levels and catalytic activity in H295R cells (Fig. 6). The genetic down-regulation of this enzyme by benomyl has not been reported previously. CYP17 expression in human adrenal cortex and in H295R cells is, in part, regulated by the cyclic AMP/protein kinase A signaling pathway, and the CYP17 promoter is under a complex control by various transcription factors.
factors and coregulators, such as steroidogenic factor-1 (SF-1), GATA-6, and Sp1 among others (Sewer and Jagarlapudi, 2009). Any number of these functions could be disrupted by benomyl and the mechanisms of benomyl-mediated down-regulation of CYP17 deserve further study.

Prochloraz was a strong inhibitor of CYP17 gene and protein expression (Figs. 6A and 6B), although this occurred at a concentration (10μM) that was partially toxic to the cells. However, prochloraz inhibited the catalytic activity of CYP17 by 73% at a nontoxic concentration of 1 μM (Fig. 6C), which is consistent with previous reports (Ohilsson et al., 2009).

To our knowledge, we report for the first time that H295R cells express SRD5A1 and AR. SRD5A1 is an AR-responsive gene, which is induced by potent androgens, as we found previously in LNCaP cells (Gasmì and Sanderson, 2010). Here, we show that SRD5A1 gene expression is induced in both LNCaP and H295R cells (Fig. 7A), suggesting that AR may control similar genes in both cell types. However, the functions of AR in H295R cells are unknown and the receptor does not respond to androgens with increased intracellular protein levels (Fig. 8). Nor did the AR antagonist vinclozolin reduce the induction of SRD5A1 in H295R cells, whereas it did so in LNCaP cells (Fig. 7B). The apparently antagonistic vinclozolin reduce the induction of SRD5A1, which is induced by potent androgens, as we found previously in LNCaP cells (Gasmì and Sanderson, 2010). Here, we show that SRD5A1 gene expression is induced in both LNCaP and H295R cells (Fig. 7A), suggesting that AR may control similar genes in both cell types. However, the functions of AR in H295R cells are unknown and the receptor does not respond to androgens with increased intracellular protein levels (Fig. 8). Nor did the AR antagonist vinclozolin reduce the induction of SRD5A1 in H295R cells, whereas it did so in LNCaP cells (Fig. 7B). The apparently high constitutive level of p-AR-Ser81 and lack of p-AR-Ser213 suggest different kinases play a role in AR phosphorylation and subsequent AR-signaling in H295R cells. The relevance of AR expression in H295R cells given the role of this cell line as a screening tool for chemical interferences with steroidogenesis is, thus far, unknown.

In conclusion, endocrine disrupting pesticides may act via multiple mechanism to exert antiandrogenic effects as summarized in Table 1. Benomyl appears to be an effective down-regulator of CYP17 gene expression, vinclozolin acts principally as an AR antagonist, and prochloraz exerts combined mechanisms of antiandrogenic action. Atrazine was consistently inactive in our in vitro models of pro- or antiandrogenicity. The effects of endocrine disrupting chemicals on kinase pathways that may phosphorylate AR, thus either activating or deactivating it androgen independently, as well as the role of AR in H295R cells, warrant further investigation.

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