

Pubertal Development in Female Wistar Rats following Exposure to Propazine and Atrazine Biotransformation By-Products, Diamino-S-Chlorotriazine and Hydroxyatrazine

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We showed previously that the chlorotriazine herbicide, atrazine (ATR), delays the onset of pubertal development in female rats. ATR and its biotransformation by-products are present in soil and groundwater. Since current maximum contaminant levels are set only for ATR, it is important to determine whether these by-products can also alter pubertal development and possibly pose a cumulative exposure hazard. We evaluated the effects of two ATR by-products, diamino-s-chlorotriazine (DACT) and hydroxyatrazine (OH-ATR), and a structurally similar chlorotriazine, propazine (PRO), on female pubertal development. Rats were gavaged from postnatal days (PNDs) 22 through PND 41 with DACT (16.7, 33.8, 67.5, 135 mg/kg), OH-ATR (22.8, 45.7, 91.5, 183 mg/kg), or PRO (13, 26.7, 53, 106.7, 213 mg/kg). The dose range for each chemical was selected as the molar equivalent of ATR (12.5, 25, 50, 100, 200 mg/kg). The females were monitored daily for vaginal opening (VO) and killed on PND 41. DACT, a by-product of ATR that occurs in the environment and is also the primary chlorinated metabolite of ATR in animal tissue, delayed VO by 3.2, 4.8, and 7.6 days compared to the controls (33.1 ± 0.4 (SE) days of age) following exposure to 33.8, 67.5, and 135 mg/kg, respectively. The no effect level (NOEL) for DACT (16.7 mg/kg) was identical to the equimolar NOEL for ATR (25 mg/kg). Although the body weight (BW) on PND 41 was reduced by the high dose of DACT (8.4% reduction), this reduction did not exceed the criteria for selecting the maximum tolerated dose (e.g., a dose that causes >10% decrease in BW at necropsy). None of the lower doses of DACT caused a significant difference in BW gain. Additionally, 33.8, 67.5, and 135 mg/kg of DACT significantly increased the BW on the day of VO. PRO (107 or 213 mg/kg) delayed VO by 4 days but did not alter the BW on PND 41. While no significant delays in pubertal development were observed in

two separate dose–response studies with doses ranging up to 183 mg/kg (OH-ATR), a minor but statistically significant delay in the onset of puberty in a pilot study using OH-ATR raises the possibility that an effect might occur following exposure to higher doses. However, it is clear from these data that OH-ATR has a much lower potency when compared with equimolar doses of DACT and PRO. Together, these data demonstrate that PRO and DACT can delay the onset of puberty in the female rat at doses equimolar to ATR and provide the scientific basis for the use of additivity in the upcoming risk assessments.

Key Words: atrazine; propazine; metabolites; female; pubertal development; reproductive toxicology.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; ATR) is a chloro-s-triazine herbicide that is used extensively throughout the United States and the world for broadleaf and grassy weed control in corn, sorghum, sugarcane, cotton, and pineapple crops and landscape vegetation. The U.S. Department of Agriculture (1990–1994) estimates that 64 to 75 million pounds of ATR are used annually within the United States. Biotransformation by-products of ATR, as well as the parent compound, are highly persistent in water and mobile in soil (Seiler *et al.*, 1992), and have been detected in surface and ground water in areas of major usage (Baker, 1998). Recent monitoring of surface and ground water has detected levels of ATR in the Midwestern river basins and in community water supplies that exceed the maximum contaminant level (MCL) of 3 $\mu\text{g/l}$ set by the U.S. Environmental Protection Agency (U.S. EPA) (Kello, 1989; Thurman *et al.*, 1991). The highest levels of ATR and biotransformation by-products are typically observed in the Midwestern states during the seasonal months of farming, when ATR is used for corn (Balu *et al.*, 1998).

The environmental fate of ATR can affect the potential for exposure of humans and wildlife to ATR and its by-products. It has been estimated that the surface runoff of ATR usually ranges from 0.5 to 3% of the applied ATR (Snedeker and Clark, 1999). While the half-lives of ATR and its by-products vary with geographic conditions, the predominate forms found

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in the soil, surface, and ground water are hydroxyatrazine (OH-ATR), (2-chloro-4-amino-6-(ethylamino)-s-triazine (DIA), 2-chloro-4-amino-6-(isopropylamino)-s-triazine (DEA), and, to a lesser extent, 2-chloro-4,6-diamino-s-triazine (DACT) (Buchanan and Hiltbold, 1973; Eldridge *et al.*, 1994; Kolpin *et al.*, 1997; Koskinen and Clay, 1997; Muir and Baker, 1978; Sorenson *et al.*, 1993; Thurman *et al.*, 1998; Winkelmann and Klaine, 1991). These biotransformation by-products are a result of microbial degradation, photodegradation, chemical hydrolysis, and plant metabolism.

The metabolism of ATR in animals and humans has been thoroughly studied, and metabolites identical to those found in the environment are also produced *in vivo*. Major urinary metabolites identified in the adult rat include OH-ATR, DIA, DEA, DACT, and ammeline (Bakke *et al.*, 1972; Bradway and Moseman, 1982). McMullin *et al.* (2003) recently reported that DACT is the major, persistent plasma metabolite in the rat following oral exposure. Finally, the metabolites identified in the urine of occupationally exposed males are DIA, DEA, and DACT (Catenacci *et al.*, 1990; Ikonen *et al.*, 1988). Thus, the potential exists for humans and wildlife to be exposed to a number of these chemicals resulting from either environmental sources and/or metabolism following exposure to ATR.

In 1994, ATR and the related s-triazine herbicides, simazine and cyanazine, were placed under a special review by the U.S. EPA based on concerns that ATR was a possible cancer risk to humans ([//www.epa.gov/oppsrrd1/reregistration/atrazine/hed_redchap_16apr02.pdf](http://www.epa.gov/oppsrrd1/reregistration/atrazine/hed_redchap_16apr02.pdf)). Subsequently, the Agency's Scientific Advisory Panel (SAP) concluded that the cancer mode of action responsible for the earlier onset of mammary tumors in the Sprague-Dawley rat following chronic exposure to ATR was not relevant to humans (www.epa.gov/scipoly/sap/2000/june27/finalatrazine.pdf). Although ATR might cause adverse effects on hypothalamic-pituitary functions in humans, the hormonal environment conducive to tumor development (i.e., elevated or prolonged exposure to estrogen and prolactin) that is found in Sprague-Dawley rats is not expected to occur in humans. However, the SAP did conclude that the findings on the neuroendocrine mode of action for ATR in the rat did raise a developmental concern for children. These later conclusions were based on recent studies that have shown that ATR can adversely affect reproductive function in the laboratory rat through a neuroendocrine mode of action (Cooper *et al.*, 2000; Das *et al.*, 2001). Cooper *et al.* (1996) reported the disruption of estrous cyclicity in adult Long Evans and Sprague-Dawley rats during a 21-day exposure to ATR (75–300 mg/kg, oral gavage). These authors concluded that the effects on estrous cyclicity were most likely mediated via alterations in the neurotransmitter and hormonal control of the gonadal function. Specifically, ATR has been reported to increase dopamine and reduce norepinephrine concentrations in the hypothalamus (Cooper *et al.*, 1998) and to diminish the estrogen-induced surge of luteinizing hormone (LH) and prolactin in ovariectomized rats following single or multiple (3 and 21 days) doses

of ATR (Cooper *et al.*, 2000). The observation that intravenous injections of gonadotropin-releasing hormone (GnRH) restored the estrogen-induced secretion of LH in these animals provided additional evidence for a central nervous system (CNS)-pituitary mode of action. Similarly, significant delays in pubertal development observed in both female (50–200 mg/kg [Laws *et al.*, 2000] and 30–100 mg/kg [Ashby *et al.*, 2002]) and male rats (12.5–200 mg/kg [Stoker *et al.*, 2000]) following exposure to ATR are possibly due to alterations in the hormonal signaling within the hypothalamic-pituitary-ovarian axis. Recent studies by Stoker *et al.* (2002) have shown that at least three chlorinated by-products also delay pubertal development in male rats at doses similar to that of ATR. Together, these data suggest that there is a need to understand the potential for cumulative toxic effects of the chlorotriazines and their biotransformation by-products.

ATR is currently undergoing a re-registration process by the U.S. EPA (www.epa.gov/oppsrrd1/reregistration/atrazine/) and is one of a group of s-triazine pesticides for which the agency is conducting a reassessment of tolerances. In carrying out the tolerance reassessment provisions of the 1996 Food Quality Protection Act (FQPA), the U.S. EPA recently determined that the triazine pesticides—ATR, PRO, and simazine—and the by-products—DACT, DIA, and DEA—share a common mechanism of toxicity due to their ability to suppress the LH ovulatory surge and produce consequent effects on reproductive function and reproductive development. Thus, for purposes of a cumulative risk assessment (and as part of the tolerance reassessment process for triazine pesticides), these compounds will be considered as a “Common Mechanism Group” (<http://www.epa.gov/oppsrrd1/cumulative/triazines/triazinescommonmech.pdf>).

To date, there have been few studies that have compared the toxicological effects following equimolar doses of these chemicals. Such studies are needed to provide the necessary data for the cumulative risk assessment of this group of s-triazine compounds. In addition, comparative studies to determine structure-activity relationships between the chlorinated and nonchlorinated by-products are also needed. Thus, the studies reported here were conducted to compare the effects of two by-products of ATR, DACT and OH-ATR (Fig. 1), on female pubertal development and thyroid function in young Wistar rats. A structurally similar s-triazine, propazine (2-chloro-4,6-bis (isopropylamino)-s-triazine; PRO) was also tested to compare its potency with ATR. The Protocol for the Assessment of Pubertal Development and Thyroid Function in the Female Rat (Goldman *et al.*, 2000) was used for these studies, which was identical to that used in our previous studies with ATR (Laws *et al.*, 2000). Since this protocol is currently undergoing an extensive validation process by the U.S. EPA for possible use in a Tier I screening battery of the agency's Endocrine Disruptor Screening Program, all endpoints included in the protocol were evaluated to provide additional performance criteria data for the protocol (www.epa.gov/scipoly/oscpendo/). Thus,

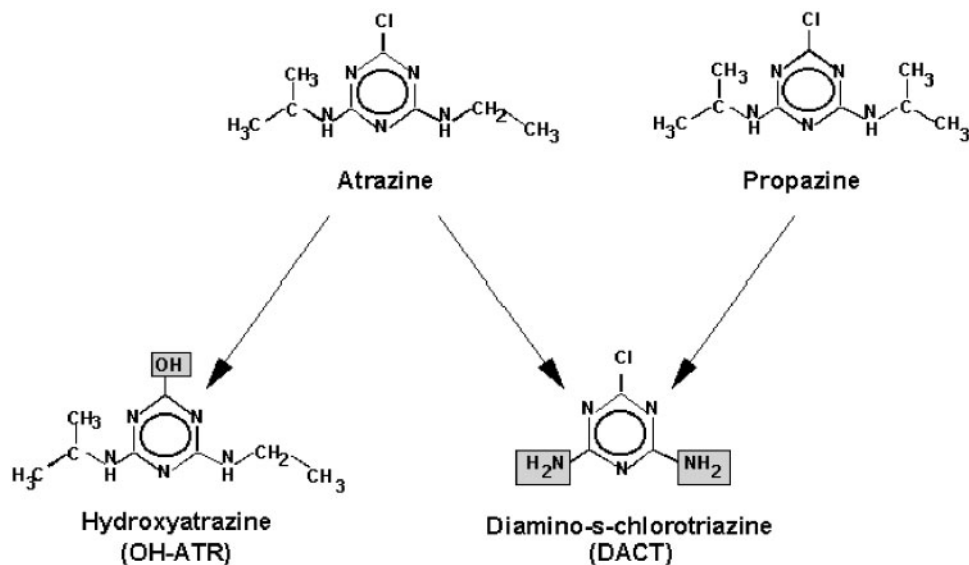


FIG. 1. Chemical structures of atrazine, propazine (PRO), and metabolites, diamino-s-chlorotriazine (DACT) and hydroxyatrazine (OH-ATR).

thyroid hormones were evaluated in these studies even though no previous data suggested that the chlorotriazines would affect thyroid function. To facilitate the comparison of the potency of all of the test chemicals, the dose range for each was equimolar to the doses used for ATR in our previous studies. Thus, it should be noted that the doses used are higher than that which would be expected from environmental exposures. Since DACT is a common chlorinated environmental by-product as well as a human and animal metabolite of ATR and PRO, this study tested the hypothesis that DACT is the active compound for both herbicides. The use of a nonchlorinated by-product, OH-ATR, provides information as to whether the chlorine moiety is a necessary structural component for reproductive toxicity. Finally, this study provides data to evaluate whether environmental exposure to multiple chlorotriazines and their biotransformation by-products could possibly have an additive effect on pubertal development.

MATERIALS AND METHODS

Animals. Wistar rats (14-day timed pregnant) were obtained from Charles Rivers Laboratories (Raleigh, NC) and were maintained under controlled temperature (20–24°C), humidity (40–50%), and light (14-h light/10-h dark) conditions with Purina Laboratory Rat Chow (5001) and water available *ad libitum*. The pregnant dams were allowed to deliver their pups naturally; 3 days postpartum (postnatal day [PND] 3; PND 0 = the morning of birth) all litters were culled to 10 pups. The females were weaned on PND 21, ranked by body weight and litter, and placed into treatment groups such that the mean body weights \pm SE for all groups were similar. In addition, littermates were equally distributed between the treatment groups.

Dosing solutions and procedures. DACT (purity 96.8%) and OH-ATR (purity 97.1%) were gifts from Syngenta Crop Protection, Inc. (Greensboro, NC). PRO (purity 99.8%) was a gift from Griffin LLC (Valdosta, GA). All of the chemicals were administered by oral gavage in a suspension of 1% methyl cellulose/distilled water (M-7140, Lot No. 64H0619, Sigma Chemical Co., St. Louis, MO) in a volume of 5.0-ml dosing solution/kg body weight. To facilitate the comparison of the potency of each test chemical with that of ATR,

the dose ranges for each test chemical were the molar equivalent of ATR (atrazine equimolar dose; AED; see Table 1) and were selected based on results reported in a previous study of ATR on female pubertal development (Laws *et al.*, 2000). The doses used for the study were as follows: DACT (16.7, 33.8, 67.5, 135 mg/kg); OH-ATR (22.8, 45.7, 91.5, 183 mg/kg); and PRO (13, 26.7, 53, 106.7, 213 mg/kg). The control animals received the 1% methylcellulose.

Experimental design. The overview of the study protocol is shown in Figure 2. The female rats were dosed daily by oral gavage, beginning on PND 22 and continuing through PNDs 41–42. The body weights were recorded daily, and the dose administered each day was adjusted for body weight. Throughout the dosing period, the animals were monitored daily for vaginal opening. The age at complete vaginal opening was recorded. For those animals where vaginal opening failed to occur prior to necropsy, the age of vaginal opening was recorded as the day after necropsy to determine a mean for each treatment group. Beginning on the day of vaginal opening, daily vaginal smears were collected and observed under a low-power light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells to monitor the estrous cycle until necropsy. The vaginal smears were classified as diestrus (presence of leukocytes), proestrus (nucleated epithelial cells), or estrus (cornified epithelial cells) as characterized by Everett (1989).

TABLE 1
Actual Doses of Chemicals as Compared to the Molar
Equivalent of Atrazine

Moles	ATR FW 215.7 ^a	DACT FW 145.5	OH-ATR FW 197.2	PRO FW 230.1
0.058	12.5	—	—	13
0.116	25	16.7 ^b	22.8	26.7
0.232	50	33.8	45.7	53
0.464	100	67.5	91.5	106.7
0.930	200	135	183	213

Note. ATR, atrazine; DACT, diamino-s-chlorotriazine; OH-ATR, hydroxyatrazine; PRO, propazine.

^aFW (formula weight in g).

^bAED (atrazine equimolar dose, mg/kg).

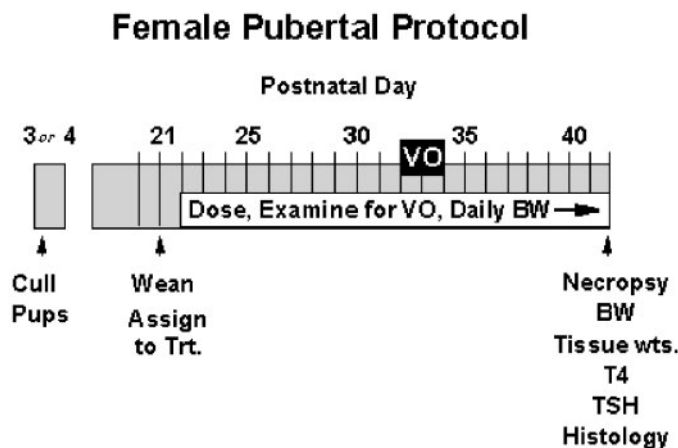


FIG. 2. Overview of the study protocol. Female rats were weaned on post-natal day (PND) 21, ranked by body weight (BW) and litter, and randomly assigned to treatment groups such that the mean BW and variance were approximately equal for all groups. During the treatment period (PND 22–41), the females were evaluated daily for vaginal opening (VO). Beginning at VO, estrous cyclicity was evaluated daily. Animals were necropsied on PND 41–42 ($n = 15/\text{treatment group}$) and serum and tissues collected.

Extended estrus was defined as exhibiting cornified cells with no leukocytes for three or more days and extended diestrus as the presence of leukocytes for four or more days (Cooper and Goldman, 1999). The animals were killed by decapitation 2 h after the last dose on PNDs 41–42. Tissue weights for each animal were recorded for liver, kidney, adrenals, ovaries, uterus, and pituitary. The serum was frozen at -80°C for triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone (TSH) assays. Each chemical was evaluated in at least two separate experiments. The range of the dose response used for DACT and PRO was lowered in the second experiments to determine the lowest observable effect level (LOEL) for each chemical. Two dose response experiments were conducted using OH-ATR to confirm the no effect level (NOEL). To document the reproducibility between experiments, the age of vaginal opening is reported for all experiments. As recommended in the protocol, the data for tissue and body weights are reported as treatment means ($n = 15$). The study for DACT was conducted in two blocks with 7–8 animals per treatment group. The data were combined after a statistical analysis demonstrated that there was no significant block effect or block–treatment interaction.

Histology. Immediately after necropsy the thyroid, uterus, and ovaries were placed in formalin for 24 h. The tissues were rinsed and stored in 70% alcohol until embedded in paraffin, sectioned ($4\text{--}6\ \mu\text{m}$), and stained with hematoxylin and eosin. For the thyroid, each slide to be evaluated contained a transverse section of the thyroid gland (bracketing the trachea). The histological evaluations for pathologic abnormalities and potential treatment-related effects were conducted by Veritas Laboratories, Inc. (Burlington, NC).

Radioimmunoassays. Serum TSH was measured by radioimmunoassay using material supplied by the National Hormone and Pituitary Agency (e.g., iodination preparation I-9, reference preparation RP-3, and antisera S-6). The iodination material was radiolabeled with ^{125}I (Dupont/New England Nuclear) by a modification of the chloramine-T method (Greenwood *et al.*, 1963). The labeled TSH was separated from the unreacted iodide by gel filtration chromatography as described in Laws *et al.* (2000). Total triiodothyronine (T_3) and thyrotropin (T_4) were measured using coat-a-count radioimmunoassay kits obtained from Diagnostic Products Corp. (Los Angeles, CA). The detection limits for T_3 and T_4 were 0.2 and 10 ng/ml, respectively.

Statistical analyses. The data from each of the dose–response experiments for PRO and OH-ATR ($n = 15$) were analyzed separately by analysis of variance (ANOVA) using the General Linear Model (GLM) procedure (Sta-

tistical Analysis System (SAS), SAS Institute, Inc., Cary, NC). The data from the two dose–response block studies for DACT ($n = 7\text{--}8$) were initially analyzed separately by ANOVA. The data were then combined to yield a sample number of 15/treatment group and analyzed by ANOVA for block, treatment, and block–treatment interaction effects. In cases where a significant treatment effect ($p < 0.05$) was observed, the dose–response data were further evaluated by the Dunnett multiple comparison (control compared with each treatment group). The treatment means for each endpoint were tested for homogeneity of variance using the Bartlett test (*GraphPad InStat*, GraphPad Software, San Diego, CA), and, where heterogeneity was evident, the Welch *t*-test or Kruskal-Wallis Nonparametric test with Dunn's multiple comparison test were used. Organ weights were analyzed by analysis of covariance (ANCOVA) using the body weight at necropsy as a covariate. Means and adjusted means relative to necropsy body weight were calculated for organ weights for which a significant effect of body weight was observed. Adjusted means were compared with the control using a pairwise *t*-test with the Bonferroni correction. All data are reported as mean \pm SE (n).

RESULTS

Body Weight

The effects of each test chemical on growth during the exposure period are shown in Figure 3 (A, B, and C). The mean body weights \pm SE were similar for all treatment groups on the first day of dosing (PND 22). Exposure to DACT (16, 33.8, or 67.5 mg/kg) did not cause any significant changes in body weight during the dosing period. However, animals receiving the highest dose of DACT (135 mg/kg) did not gain any weight during the first 3 days of treatment (Fig. 3A). Although these animals began to gain weight by PND 25, their mean body weight was 18.5% lower than the control group. At necropsy, the body weight in these animals remained significant lower (10.4%) than the control. The body weights of animals receiving the highest dose of PRO (213 mg/kg; Fig. 3B) also lagged behind that of the controls. However, the magnitude of the reduction in body weight was not as great as that observed with DACT and was not significantly different from the control. At PND 25 and necropsy, the mean body weight of this PRO group was 8.7% and 4.9% lower than the control. OH-ATR (Fig. 3C) did not alter body weight gain at any point during the exposure period.

Age and Body Weight at Vaginal Opening

The age and body weight at vaginal opening for each test chemical are shown in Table 2. DACT (33.8, 67.5, and 135 mg/kg) significantly delayed the age at vaginal opening by 3.2, 4.8, and 7.6 days, respectively. Four of 15 animals in the highest DACT treatment did not attain vaginal opening by necropsy at PNDs 41–42. Body weight at the age of vaginal opening was also significantly increased in the three highest dose groups of DACT. To determine a possible effect of a lower body weight at the average age of onset of puberty in the Wistar rat (e.g., 33 days of age), the body weight at PND 33 was compared for each treatment group. A significant reduction in body weight was observed at PND 33 in the group receiving the highest dose of DACT (135 mg/kg) as compared

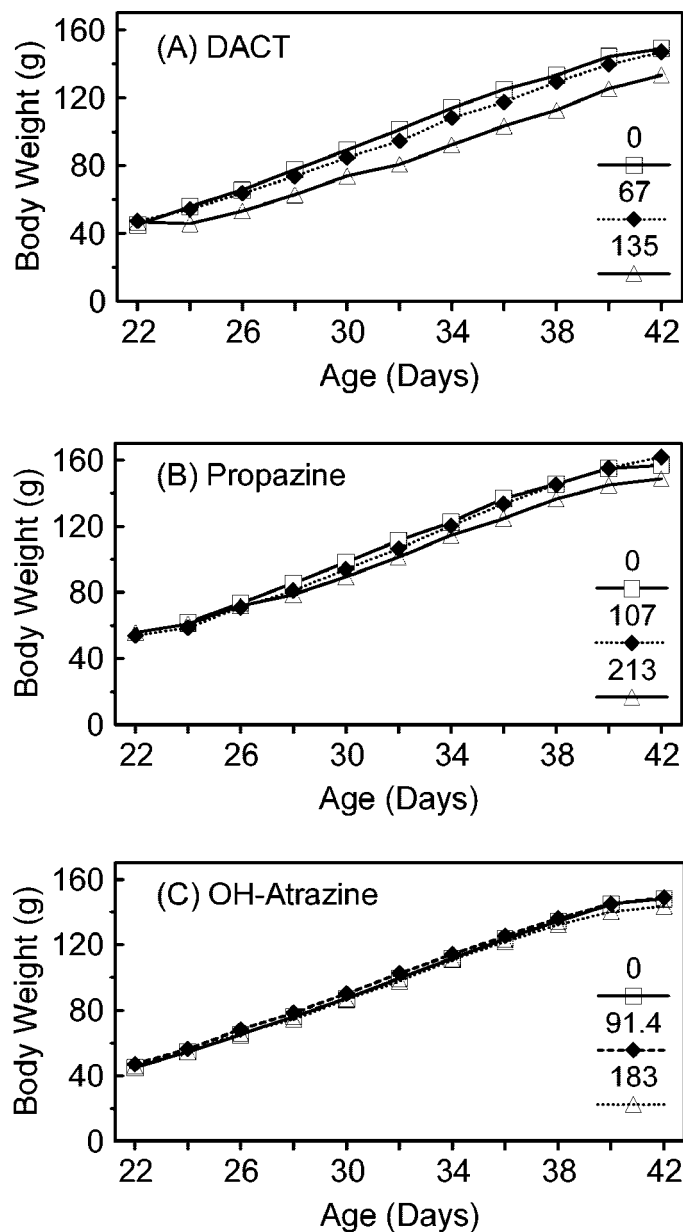


FIG. 3. Effects of PRO, DACT, and OH-ATR on growth during exposure. Mean body weights (g) are shown for controls and test chemicals from PND 22–42. (A) The highest dose of DACT (135 mg/kg; 200 AED) caused a significant reduction in body weight at PND 42. (B) PRO did not significantly alter body weight at any dose. (C) OH-ATR did not alter body weight during the treatment period.

with the controls. However, the body weight at PND 33 was not significantly lower for the 33.8- and 67.5-mg/kg groups, where there was also a significant delay in the onset of puberty. PRO (106.7 and 213 mg/kg) also significantly delayed the age of vaginal opening by 3.6–4 days. Again, having to obtain a threshold body weight did not appear to be the reason for the delay in puberty, since a significant increase in body weight at vaginal opening was observed in the females receiving the

highest dose of PRO. Doses of OH-ATR (up to 200 mg/kg) did not cause a significant delay in the age of vaginal opening.

To evaluate the variability of the age of vaginal opening as an endpoint of pubertal development, data from each replicate study are reported (Table 3). The age of vaginal opening for the controls in all of the replicate studies ranged from 32.2 to 33.6 days of age, with the coefficient of variation (CV) ranging from 2.6 to 7.3%. The replicate studies for DACT were conducted with 7–8 animals per dose group rather than the standard sample size of 15 (Goldman *et al.*, 2000). When the data for each replicate study were analyzed separately, a significant treatment effect on the age at vaginal opening was observed in each study. Additionally, the mean values for each treatment group were equal between the two replicate studies. Similar results were observed in the two studies of PRO, where significant effects on the age of vaginal opening were detected following exposure to 106 mg/kg of PRO regardless of a sample size of 7 or 15. As expected, the variance associated with the age at vaginal opening increased slightly for the higher dose groups for DACT and PRO. In these groups, the CV ranged from 4.5 to 10.7%. Data from two dose response studies of OH-ATR are shown in Table 3. Although the age of vaginal opening was significantly delayed in a pilot study (control 33.7 ± 0.44 (15), OH-ATR 35.9 ± 0.88 (15) days of age) by 183 mg/kg OH-ATR, these two subsequent dose–response studies failed to produce a significant effect at the same dose.

Tissue Weight

Tissue weights at necropsy following exposure to DACT, PRO, and OH-ATR are shown in Tables 4–6. In the cases where a significant reduction in body weight during treatment was observed, tissue weights were analyzed with and without the body weight at necropsy as a covariate. Significant reductions in kidney, pituitary, and adrenal weights were observed following exposure to the highest dose of DACT (Table 4). Using ANCOVA, the adjusted mean for pituitary weight remained significantly lower than the controls. Uterine (with and without fluid) and ovarian weights were also significantly reduced in the highest treatment group of DACT, where 4 of 15 females failed to show vaginal opening at necropsy. Since uterine weight varies during the estrous cycle, the number of females in each stage of the estrous cycle at necropsy is reported for each treatment group. However, uterine weights were not analyzed separately according to the endocrine status of each female. In contrast to DACT, no significant differences in tissue weights were observed in any of the OH-ATR animals (Table 5). Only pituitary weights in the two highest groups of PRO (106.7 and 213 mg/kg) were significantly different from the controls (Table 6). Since no significant differences were observed in the necropsy body weights following exposure to OH-ATR or PRO, adjusted means for the tissue weights following ANCOVA are not reported.

TABLE 2
Comparison of Body Weight Gain and Pubertal Development in Female Wistar Rats

Chemical	Dose (mg/kg)	Dose (AED) ^a	Body weight PND 22 (g)	Body weight PND 33 (g)	Body weight at VO (g)	Age at VO (days)	No. of days VO delayed
DACT ^c	0	0	45.1 ± 1.3 ^b	107 ± 2.0	108 ± 3.2	33.1 ± 0.39	—
	16.7	25	47.5 ± 1.7	106 ± 2.8	114 ± 4.1	34.5 ± 0.68	1.4
	33.8	50	45.4 ± 0.6	103 ± 0.9	122 ± 3.9 ^f	36.3 ± 0.70 ^f	3.2
	67.5	100	47.3 ± 0.9	102 ± 1.7	128 ± 3.4 ^f	37.9 ± 0.73 ^f	4.8
	135	200	46.8 ± 3.6	87 ± 2.4 ^f	127 ± 3.1 ^f	40.7 ± 0.66 ^f	7.6
OH-ATR ^d	0	0	45.1 ± 1.2	106 ± 2.4	106 ± 3.1	33.0 ± 0.62	—
	22.8	25	46.9 ± 1.2	111 ± 2.1	107 ± 2.7	32.7 ± 0.62	—
	45.7	50	47.2 ± 1.3	109 ± 2.1	112 ± 3.2	33.7 ± 0.51	0.7
	91.5	100	47.1 ± 1.3	107 ± 2.4	112 ± 3.4	33.7 ± 0.53	0.7
	183	200	46.1 ± 0.5	105 ± 1.2	111 ± 4.1	34.3 ± 0.77	1.3
PRO ^e	0	0	51.3 ± 1.2	111 ± 1.9	113 ± 2.7	32.2 ± 0.22	—
	13	12.5	51.3 ± 1.2	110 ± 1.6	117 ± 3.7	32.9 ± 0.55	0.7
	26.7	25	51.0 ± 1.3	110 ± 2.3	117 ± 3.1	33.0 ± 0.39	0.8
	53	50	50.8 ± 1.5	109 ± 1.9	116 ± 3.2	33.2 ± 0.37	1.0
	106.7	100	50.6 ± 1.3	106 ± 1.9	132 ± 3.0 ^f	35.8 ± 0.57 ^{g,h}	3.6
	213	200	55.8 ± 1.0	101 ± 1.7 (14)	126 ± 3.1 ^f (14)	36.2 ± 0.38 ^{g,h} (14)	4.0

Note. ATR, atrazine; DACT, diamino-s-chlorotriazine; OH-ATR, hydroxyatrazine; PRO, propazine.

^aDoses (mg/kg) used for each chemical were equimolar to the doses of atrazine (AED).

^bMean ± SE (*n* = 15 unless noted).

^cDACT: Combined data from DACT Studies 1 and 2.

^dOH-ATR: Data from OH-ATR Study 1.

^ePRO: Data for propazine (213 mg/kg) from Propazine Study 1, and all other dose groups from Study 2.

^fSignificantly treatment effect by ANOVA (GLM) and different from control by Dunnett's multiple comparison test, *p* < 0.05.

^gHeterogeneity of variance (Bartlett's test, *p* < 0.05).

^hSignificantly different from control by Kruskal-Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test, *p* < 0.05.

Thyroid Hormones

Serum thyroid hormone concentrations are shown in Table 7. No significant dose effects were observed for serum T3 or TSH following exposure to any of the test

chemicals. Although T4 was significantly increased by 67.5 mg/kg DACT, this change was not dose responsive and no other changes in T4 were observed for any of the test chemicals.

TABLE 3
Comparison of the Age (Days) at Vaginal Opening between Chemicals and Study

Chemical	Experiment	ATR equimolar dose (AED; mg/kg)*					
		0	12.5	25	50	100	200
DACT	1	33.6 ± 0.50 ^{a,b} (8)	— ^c	—	36.5 ± 0.46 (8)	38.0 ± 1.28 ^d (8)	40.6 ± 1.08 ^e (8)
	2	32.6 ± 0.57 (7)	—	34.3 ± 0.50 (15)	36.0 ± 1.46 (7)	37.8 ± 0.67 ^g (7)	40.7 ± 0.78 ^g (7)
OH-ATR	1	33.0 ± 0.62 (15)	—	32.7 ± 0.62 (15)	33.6 ± 0.51 (15)	33.7 ± 0.53 (15)	34.3 ± 0.77 (15)
	2	32.3 ± 0.37 (15)	—	—	—	32.2 ± 0.48 (15)	32.2 ± 0.56 (15)
PRO	1	32.4 ± 0.37 (14)	—	—	34.3 ± 0.62 ^f (8)	36.3 ± 1.04 ^g (7)	36.2 ± 0.38 ^g (14)
	2	32.2 ± 0.22 ^b (15)	32.9 ± 0.55 (15)	33.0 ± 0.39 (15)	33.2 ± 0.37 (15)	35.8 ± 0.57 ^c (15)	—

Note. ATR, atrazine; DACT, diamino-s-chlorotriazine; OH-ATR, hydroxyatrazine; PRO, propazine.

^aMean ± SE (*n*).

^bHeterogeneity of variance (Bartlett).

^cNo data collected for dose level.

^dSignificantly different from control by Kruskal-Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test (*p* < 0.05).

^eSignificantly different from control by Kruskal-Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test (*p* < 0.01).

^fSignificantly different from control by ANOVA and Dunnett's multiple comparison test (*p* < 0.05).

^gSignificantly different from control by ANOVA and Dunnett's multiple comparison test (*p* < 0.01).

*See Table 1 to determine actual dose of test chemical used in study.

TABLE 4
Body Weight and Organ Weights at Necropsy following Exposure to DACT

Parameter	DACT mg/kg (AED) ^a				
	0	16.7 (25) ^a	33.8 (50) ^a	67.5 (100) ^a	135 (200) ^a
Body weight (g)	154 ± 3.5 ^b	150 ± 3.6	153 ± 2.5	153 ± 2.5	141 ± 2.7 ^c
Liver (g)	6.99 ± 0.19	6.87 ± 0.20	6.94 ± 0.14	6.95 ± 0.21	6.65 ± 0.18
ANCOVA mean (g)	6.82 ± 0.13	6.91 ± 0.13	6.78 ± 0.13	6.84 ± 0.13	7.18 ± 0.14
Kidney (g)	1.47 ± 0.03	1.42 ± 0.03	1.43 ± 0.03	1.45 ± 0.05	1.29 ± 0.04 ^c
ANCOVA mean (g)	1.44 ± 0.03	1.43 ± 0.03	1.41 ± 0.03	1.44 ± 0.03	1.37 ± 0.03
Pituitary (mg)	8.52 ± 0.18	8.18 ± 0.37	8.04 ± 0.31	7.63 ± 0.19	5.96 ± 0.30 ^d
ANCOVA mean (g)	8.41 ± 0.28	8.21 ± 0.29	7.94 ± 0.28	7.56 ± 0.28 ^e	6.36 ± 0.32 ^e
Adrenals (mg)	49.1 ± 1.35	44.7 ± 2.86	45.5 ± 1.55	47.9 ± 1.95	42.4 ± 1.40 ^c (13)
ANCOVA mean (g)	48.4 ± 1.88	44.8 ± 1.87	44.9 ± 1.88	47.5 ± 1.88	44.4 ± 2.00 (13)
Endocrine Status ^f					
Diestrus	8	6	8	7	6
Proestrus/estrus	7	9	7	8	5
Not cycling	0	0	0	0	4
Ovary (mg)	61.3 ± 2.42	57.1 ± 2.63	54.4 ± 2.41	56.9 ± 2.53	34.9 ± 3.18 ^d
Uterus + fluid (mg)	309.1 ± 49.9	419.7 ± 67.3	288.4 ± 35.1	309.6 ± 40.0	177.4 ± 25.5 ^d
Uterus – fluid (mg)	249.4 ± 22.5	306.9 ± 28.5	252.9 ± 18.3	246.8 ± 16.9	163.6 ± 22.9 ^d

Note. DACT, diamino-s-chlorotriazine.

^aAED: Used dose of DACT equimolar to ATR (mg/kg; see Table 1).

^bMean ± SE (*n* = 15, unless noted); combined data from DACT Studies 1 and 2.

^cSignificantly treatment effect by ANOVA (GLM) and significantly different from control by Dunnett multiple comparison test (*p* < 0.5).

^dSignificantly treatment effect by ANOVA (GLM) and significantly different from control by Dunnett multiple comparison test (*p* < 0.1).

^eSignificant treatment effect by ANCOVA and significantly different from control by pairwise *t*-test and Bonferroni correction (*p* < 0.05).

^fNumber of females in each stage of the estrous cycle at necropsy as characterized by vaginal epithelial cytology.

Histology

No pathologic abnormalities or treatment-related changes were observed in the uterine or ovarian tissue from any of the controls. An absence of corpora lutea was noted in the four animals in the

highest DACT treatment group that did not attain vaginal opening prior to necropsy. No treatment-related microscopic changes were observed in any animals in the PRO or OH-ATR groups. Additionally, no pathologic abnormalities were detected in the thyroids

TABLE 5
Body Weight and Organ Weights at Necropsy following Exposure to OH-ATR

Parameter	OH-ATR, mg/kg (AED) ^a				
	0	22.8 (25) ^a	45.7 (50) ^a	91.5 (100) ^a	183 (200) ^a
Body weight (g)	148 ± 3.3 ^b	152 ± 3.3	153 ± 3.2	149 ± 2.8	140 ± 4.3
Liver (g)	6.58 ± 0.29	7.22 ± 0.19	6.96 ± 0.21	7.21 ± 0.17	6.81 ± 0.17
Kidney (g)	1.44 ± 0.04	1.53 ± 0.05	1.48 ± 0.04	1.48 ± 0.04	1.48 ± 0.04
Pituitary (mg)	8.75 ± 0.41	8.68 ± 0.42	8.43 ± 0.34	8.54 ± 0.43	8.23 ± 0.42
Adrenals (mg)	47.0 ± 1.97	46.7 ± 1.97	46.2 ± 1.63	46.3 ± 1.61	44.8 ± 1.11
Endocrine status ^c					
Diestrus	9	11	9	9	10
Proestrus/estrus	7	4	6	6	5
Not cycling	0	0	0	0	0
Ovary (mg)	61.3 ± 3.25	63.6 ± 3.78	62.5 ± 4.31	57.3 ± 3.37	60.6 ± 2.73
Uterus + fluid (mg)	259.8 ± 29.6	305.2 ± 28.9	284.3 ± 27.3	343.8 ± 43.8	288.9 ± 46.0
Uterus – fluid (mg)	235.2 ± 15.9	284.4 ± 22.6	247.6 ± 13.5	275.8 ± 21.0	235.1 ± 23.9

Note. OH-ATR, hydroxyatrazine.

^aAED: Used dose of OH-ATR equimolar to ATR at the doses indicated (mg/kg; see Table 1).

^bMean ± SE (*n* = 15). Data from OH-ATR (Study 1).

^cNumber of females in each stage of the estrous cycle at necropsy as characterized by vaginal epithelial cytology.

TABLE 6
Body Weight and Organ Weights at Necropsy following Exposure to PRO

Parameter	PRO, mg/kg (AED) ^a					
	0	13 (12.5) ^a	26.7 (25) ^a	53 (50) ^a	106.7 (100) ^a	213 (200) ^a
Body weight (g)	159 ± 2.1 ^b	161.4 ± 1.9	159 ± 3.0	157 ± 2.6	160 ± 2.4	152 ± 2.9 (14)
Liver (g)	7.06 ± 0.16	7.14 ± 0.16	6.97 ± 0.17	7.19 ± 0.22	7.51 ± 0.20	6.88 ± 0.24
Kidney (g)	1.48 ± 0.02	1.50 ± 0.03	1.47 ± 0.03	1.43 ± 0.03	1.46 ± 0.04	1.37 ± 0.04
Pituitary (mg)	8.89 ± 0.21	8.56 ± 0.11	8.49 ± 0.24	8.52 ± 0.22	8.09 ± 0.25 ^c	7.11 ± 0.24 ^c
Adrenals (mg)	45.7 ± 1.46	45.5 ± 1.57	43.4 ± 1.46	45.1 ± 1.94	42.1 ± 1.93	42.5 ± 1.69
Endocrine status ^d						
Diestrus	5	8	5	10	8	8
Proestrus/estrus	10	7	10	5	7	6
Not cycling	0	0	0	0	0	0
Ovary (mg)	64.3 ± 2.29	60.5 ± 1.83	61.8 ± 2.37	66.0 ± 2.49	59.4 ± 2.70	58.4 ±
Uterus + fluid (mg)	402.8 ± 60.5	286.2 ± 18.2	330.9 ± 39.3	242.6 ± 14.5 ^c	279.0 ± 25.3	273.0 ± 41.2 (14)
Uterus – fluid (mg)	304.1 ± 21.5	262.5 ± 11.0	273.1 ± 16.8	246.7 ± 17.5	258.0 ± 18.6	233.7 ± 18.8

Note. PRO, propazine.

^aMean ± SE (*n* = 15). Data for PRO, 213 mg/kg, from Study 1, and all other data from PRO Study 2.

^bAED: Used dose of PRO equimolar to ATR (mg/kg; see Table 1).

^cSignificantly treatment effect by ANOVA (GLM) and significantly different from control by Dunnett multiple comparison test (*p* < 0.5).

^dNumber of females in each stage of the estrous cycle at necropsy as characterized by vaginal epithelial cytology.

of controls, PRO-, or OH-ATR-treated animals. There was a slight increased incidence of dystrophic thyroid follicles (5, 3, and 4 of 15 animals/treatment group in the animals dosed with 33.7, 67.5, and 135 mg/kg of DACT, respectively).

DISCUSSION

The purpose of this study was to compare the effects of two biotransformation by-products of ATR, DACT, and OH-ATR,

and a structurally similar s-triazine herbicide, PRO, on pubertal development and thyroid function in young Wistar rats. These data show that DACT, a by-product common to both ATR and PRO, can delay the onset of puberty at doses equimolar to the LOEL observed for ATR (Table 8). These results are in agreement with the observations reported by Stoker *et al.* (2002) of delayed pubertal onset in male Wistar rats following exposure to DACT and two other chlorinated by-products of ATR.

TABLE 7
Serum Thyroid Hormone Concentrations at Necropsy

Chemical	Dose (mg/kg) ^a	Dose (AED) ^b	T ₄ (ng/ml)	T ₃ (ng/ml)	TSH (ng/ml)
DACT	0	0	50.0 ± 3.2 ^c	1.06 ± 0.05	0.858 ± 0.093
	16.7	25	52.9 ± 3.6 (14)	1.19 ± 0.06 (14)	0.960 ± 0.088
	33.8	50	59.7 ± 3.9	1.31 ± 0.07	0.857 ± 0.090
	67.5	100	63.9 ± 3.7 ^d	1.23 ± 0.06	0.641 ± 0.081 (14)
	135	200	54.8 ± 3.0	1.18 ± 0.08	0.744 ± 0.085
OH-ATR	0	0	36.6 ± 1.67 (14)	1.35 ± 0.08	1.09 ± 0.108 (14)
	22.8	25	45.3 ± 2.40	1.30 ± 0.06	1.34 ± 0.137
	45.7	50	44.8 ± 2.49	1.27 ± 0.06	1.50 ± 0.106
	91.5	100	39.1 ± 2.44	1.23 ± 0.11	1.10 ± 0.113
	183	200	33.8 ± 2.48	1.17 ± 0.08	1.17 ± 0.100
PRO	0	0	36.7 ± 2.6	1.54 ± 0.08	1.17 ± 0.100
	13	12.5	37.7 ± 2.0	1.75 ± 0.11	1.34 ± 0.116
	26.7	25	40.3 ± 2.4	1.60 ± 0.09	1.43 ± 0.137
	53	50	42.9 ± 2.7	1.43 ± 0.10	1.40 ± 0.084
	106.7	100	40.6 ± 2.4	1.45 ± 0.01	1.28 ± 0.101 (14)

Note. DACT, diamino-s-chlorotriazine; OH-ATR, hydroxyatrazine; PRO, propazine.

^aActual dose (mg/kg) of each test chemical used.

^bDoses used for each chemical were equimolar to doses for ATR (mg/kg, AED)

^cMean ± SE (*n* = 15 unless noted). DACT: Combined data from DACT Studies 1 and 2; OH-ATR: data from Study 1; PRO: data from Study 2.

^dSignificantly treatment effect by ANOVA (GLM) and different from control by Dunnett's multiple comparison test, *p* < 0.05.

TABLE 8
Comparison of the LOELs for Delayed Onset of Puberty for
ATR, DACT, OH-ATR, and PRO

Parameter	AED ^a (mg/kg)	ATR	DACT	OH-ATR	PRO
Age at VO	0	32.5 ± 0.5 ^b	33.1 ± 0.4 ^b	33.0 ± 0.6 ^b	32.2 ± 0.2 ^b
Days VO delayed	25	1.8	1.4	0	0.8
	50	3.4 ^c	3.2 ^c	0.7	1.0
	100	4.5 ^c	4.8 ^c	0.7	3.6 ^c
	200	6.8 ^c	7.6 ^c	1.3	4.0 ^c

Note. ATR, atrazine; DACT, diamino-s-chlorotriazine; OH-ATR, hydroxyatrazine; PRO, propazine.

^aActual doses used for DACT (16.7, 33.8, 67.5, 135 mg/kg), OH-ATR (22.8, 45.7, 91.5, 183 mg/kg), and PRO (26.7, 53, 106.7, 213 mg/kg) were equimolar to ATR (25, 50, 100, 200 mg/kg).

^bAge (days) at vaginal opening (VO) for control used to compare with each test chemical (mean ± SE [*n* = 15]).

^cSignificant delay in age at VO when means were compared by ANOVA and Dunnett's multiple comparison (e.g., each test chemical compared with respective control, *p* < 0.05).

McMullin *et al.* (2003) have reported the rapid conversion of ¹⁴C-atrazine to metabolites in rats after oral dosing, with DACT being the predominate metabolite. Thus, these data as well as our observation that PRO also delayed pubertal onset, support the hypothesis that DACT is the active metabolite for both parent compounds. Another important observation from these studies was the reduced potency of the dechlorinated by-product, OH-ATR. While no significant delays in pubertal development were observed in two separate dose-response studies with doses ranging up to 183 mg/kg (200 mg/kg, AED), a minor but statistically significant delay in a pilot study using OH-ATR raises the possibility that an effect might occur following exposure to higher doses. However, it is clear from these data that OH-ATR has a much lower potency when compared with equimolar doses of DACT and PRO (Table 8). Thus, these data suggest that the chlorine moiety may be important for activity.

The observation that DACT is as potent as ATR in delaying reproductive development in the laboratory rat suggests that the chlorotriazines and their biotransformation by-products could pose a cumulative hazard. Currently, the maximal contaminant level (MCL) is set for ATR only, not for its environmental by-products or other chlorotriazines. Because ATR and its transformation by-products are persistent for extended periods in the environment (Rodriguez and Harkin, 1997), there is a potential for this herbicide and its metabolites, as well as other chlorotriazines, to produce additive effects. The data presented here will support efforts to assess the potential cumulative hazard of these chlorotriazines and chlorinated by-products based on a common mode of toxicity (e.g., the ability to suppress the surge of LH and produce effects on reproductive development). While serum LH was not evaluated in these

studies, the delay in vaginal opening as an indicator of pubertal onset in the female rat is consistent with this mode of action.

The protocol used in this study is currently undergoing validation by the U.S. EPA for possible inclusion in a Tier I screening battery for the detection of environmental chemicals that can disrupt the homeostasis of estrogen, androgen, and thyroid hormones (Goldman *et al.*, 2000; www.epa.gov/scipoly/oscpendo/). The data from this study demonstrate the ability of the protocol to also detect environmental chemicals that disrupt the endocrine system via another mechanism of action. Earlier reports have shown that ATR does not bind to the estrogen receptor directly, nor does it possess any estrogenic activity *in vivo* (Conner *et al.*, 1996; Eldridge *et al.*, 1994; Tennant *et al.*, 1994). However, it has been clearly demonstrated that ATR can reduce serum LH and prolactin secretion (Cooper *et al.*, 1996; Simpkins *et al.*, 1998) and that these actions are most likely mediated via an effect on the regulation of pituitary hormone synthesis at the level of the CNS (Cooper *et al.*, 2000). Thus, the use of this protocol as a screen to detect endocrine disruptors provides a broader scope for identifying chemicals with modes of action that are not necessarily associated with steroidogenesis or steroid receptors.

The age at vaginal opening was the most sensitive endpoint in this study, and it was perhaps the best indicator of the effects of DACT and PRO on pubertal development. The onset of puberty in the female encompasses a period of transition during which there are changes in the signaling within the hypothalamic-pituitary-ovarian axis (Goldman *et al.*, 2000). Vaginal opening (or vaginal patency) and the occurrence of the first ovulation are dependent on estrogen synthesis and the development of the ability of the female brain to respond to the positive feedback of estrogen (Ojeda and Urbanski, 1994). The fact that the age of vaginal opening is an apical endpoint is an advantage when using this protocol because it allows for the evaluation of multiple sites of action within the hypothalamic-pituitary-ovarian axis. Additionally, since the observation is noninvasive, the option to slightly modify the study design prior to necropsy remains available, and the study could be extended to evaluate longer periods of cyclicity or fertility. Within the confines of the pubertal protocol used in the study reported here, doses were selected that were slightly below the maximal tolerated dose (MTD, a dose that causes more than a 10% reduction in body weight), which minimized an effect of lower body weight on the age at vaginal opening. While in this study a 10.4% reduction in body weight was observed for the highest dose of DACT, delayed vaginal opening occurred in the animals treated with the next two lower dose groups for which no reductions in body weights were observed. This observation is in agreement with our earlier report (Laws *et al.*, 2000) for pair-fed controls for which vaginal opening was unaffected by an 11.6% reduction in body weight after being fed the same daily food intake as consumed by their ATR-treated counterparts.

In summary, this study shows that oral exposure to DACT and PRO from PNDs 22–41 delays the age of vaginal opening in Wistar rats in a dose-dependent manner. The LOEL for the delay in vaginal opening for DACT was equimolar to that reported for ATR. The LOEL for PRO was 2-fold higher than that for ATR (e.g., 100 vs. 50 mg/kg, AED). In contrast, the dechlorinated by-product, OH-ATR, appeared to be much less potent and did not alter the onset of puberty at doses equimolar to 200 mg/kg ATR in two dose–response studies. Importantly, none of the test chemicals had any dose responsive effect on serum thyroid hormone concentrations or histology. Together, these data demonstrate that PRO and DACT can delay reproductive development in the laboratory rat and that the persistent nature of the chlorotriazines in the environment raises the potential for additive reproductive effects in humans and wildlife.

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