Evaluation of the Male Pubertal Onset Assay to Detect Testosterone and Steroid Biosynthesis Inhibitors in CD Rats

M. S. Marty, J. W. Crissman, and E. W. Carney

Toxicology and Environmental Research and Consulting, The Dow Chemical Company, 1803 Building, Midland, Michigan 48674

Received August 21, 2000; accepted January 2, 2001

The male pubertal onset assay has been recommended by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) as an alternate Tier I screening assay to detect potential endocrine-active chemicals (EACs). Recently, this assay was evaluated by several laboratories using a variety of dosing schemes. This study used a 30-day dosing period to confirm and extend previous work on the assay’s ability to detect steroid biosynthesis inhibitors. Weanling male rats were dosed by gavage from 21 to 50 days of age with vehicle (0.5% methocel) or chemicals from the following EAC classes: an androgen (testosterone propionate [TP], 0.1 or 0.4 mg/kg/day), a broad-spectrum steroid biosynthesis inhibitor (ketoconazole [KETO], 24 mg/kg/day), a 5α-reductase inhibitor (finasteride [FIN], 20 or 80 mg/kg/day), a moderately specific aromatase inhibitor (testolactone [TL], 220 mg/kg/day), or a highly specific aromatase inhibitor (fadrozole [FAD], 0.6 or 6.0 mg/kg/day). None of these treatments altered relative thyroid weights. However, TL, KETO, and FIN were positive for endocrine activity based on decreases in one or more reproductive or accessory sex gland organ weights. Of these three inhibitors, only TL significantly increased the age at PPS, indicating that PPS was less sensitive for detecting these EACs. Based on its profile of effects, TL may have been detected as an androgen. TP and FAD were negative in this assay, even at doses that caused effects in other studies. With TP, oral administration limited assay sensitivity such that higher TP doses would be needed for detection. FAD decreased body weight gains, but did not significantly alter any other assay end points; thus, the capacity of this assay to detect aromatase inhibitors remains in question.

Key Words: endocrine disruption; endocrine modulation; EDSTAC; puberty; pubertal onset; preputial separation; testosterone; steroid inhibitors; thyroid.

Government mandates passed in 1996 (Food Quality Protection Act and the Safe Drinking Water Act) require the U.S. Environmental Protection Agency to institute a screening program to evaluate whether xenobiotics can modulate endogenous endocrine function. Toward this goal, the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) was established and proposed a battery of tests designed to detect compounds altering estrogen, androgen, or thyroid function (EDSTAC, 1998). The mammalian in vivo components of the recommended Tier I in vivo battery consist of the uterotrophic assay, the female pubertal onset assay, and the Hershberger assay, but alternative assays have been proposed. One alternative battery replaces the female pubertal onset assay and the Hershberger assay with the 14-day intact male assay.

The other alternate assay under consideration is the male pubertal onset assay. According to the EDSTAC report, 33-day-old male rats are dosed with test material daily by gavage for 20 days, and the age and weight at which these rats attain puberty is measured. Balano-preputial separation (PPS) serves as a biomarker of puberty onset. Body weights are monitored throughout the dosing period, and once dosing is complete, rats are euthanized and the weights of the testes, epididymides, levator ani-bulbocavernous muscles (LABC), ventral prostate and seminal vesicles with coagulating glands are recorded. At necropsy, a serum sample is collected for the analysis of serum hormone levels, including thyroid stimulating hormone (TSH) and thyroxine (T4). Additional hormone analyses (i.e., testosterone, estradiol, leutinizing hormone [LH], prolactin [PRL], and triiodothyronine [T3]) are optional. Testes, epididymides, and thyroid glands are examined histopathologically. Weights and histology for the liver, kidneys, adrenals, and pituitary are optional.

PPS, an androgen-dependent process, serves as an easily measured, external indicator of pubertal onset in male rats. PPS normally occurs at approximately 43.6 ± 1.0 days of age in Sprague-Dawley rats (Clark, 1999). Aside from androgens, agents operating through a variety of mechanisms may alter pubertal assay end points (reviewed in Stoker et al., 2000).

PPS has been used as a biomarker of endocrine function for many years (Korenbrot et al., 1977), yet a standard protocol for the male pubertal assay has not been firmly established or validated. This study was designed as an initial evaluation of this assay’s ability to detect known endocrine-active compounds (EACs). The EACs used in this study encompassed different modes of action and included an androgen (testosterone propionate [TP]), a broad-spectrum steroid biosynthesis...
inhibitor (ketoconazole [KETO]), a 5α-reductase inhibitor (finasteride [FIN]) that blocks the conversion of testosterone to the more potent androgen dihydrotestosterone (DHT), and two aromatase inhibitors that block the synthesis of C18 estrogens from C19 androgens (testolactone [TL] and fadrozole [FAD]). When possible, these compounds were administered at dose levels reported to cause endocrine-mediated effects by the oral route in previous studies. With TP, no previous studies were found in which oral administration to intact juvenile male rats was performed; therefore, doses were selected by increasing the concentration of TP that stimulated endocrine changes when using an alternate dosing route. Specifically, Freitag and Döcke (1987) reported decreased testicular weights when administering 50 μg TP/kg/day subcutaneously during the pre- and peripubertal periods. With KETO, oral administration at 24 mg/kg/day to male rats for 30 days decreased sperm number and percent motile sperm and increased percent abnormal sperm (Vawda and Davies, 1986). The dose of TL (220 mg/kg/day) was greater than the dose required to inhibit aromatase in humans but was not anticipated to produce antiandrogenic effects (Vigersky et al., 1982). FIN (10 mg/kg/day) given by gavage for 14 or 20 days decreased seminal vesicle, levator ani, and ventral prostate weights in rats (Di Salle et al., 1993; Häusler et al., 1996) and significantly decreased fertility at 80 mg/kg/day (Wise et al., 1991). The dose of the aromatase inhibitor FAD was based on previous reports, wherein FAD at 6.0 mg/kg/day severely decreased the number of estrous cycles (Nunez et al. 1996), delayed puberty onset, and decreased uterine weight in juvenile female rats (Marty et al., 1999).

**MATERIALS AND METHODS**

*Animals.* Litters of CD® (Sprague-Dawley-derived) rats were received from Charles River Laboratories (CRL; Portage, MI). At CRL, pups from contemporary litters were cross-fostered into study litters to reach the required number of male pups per litter. Cross-fostered pups from the same litter were not placed in multiple study litters, in order to control for litter effects at the time of randomization into treatment groups. Once received at 14 days of age, pups were housed and maintained under laboratory conditions as previously described (Marty et al., in press).

*Chemicals.* TP and FIN were purchased from Sigma (St. Louis, MO). KETO was from ICN Biomedicals (Costa Mesa, CA), and TL tablets were purchased from Bristol-Myers-Squibb (Princeton, NJ). Novartis Pharmaceuticals Corporation (Summit, NJ) generously donated FAD for these experiments. Compound purity was estimated to be ≥ 98% by its respective chemical supplier.

*Experimental design.* In the first series of experiments, 10 litters, each containing 12 pups and 1 lactating dam, were used. Each litter comprised six male and six female pups, so that both the male and female pubertal onset assays could be conducted simultaneously (female assay results in Marty et al., 1999). In the first experiment, males were treated with vehicle, low-dose TP, low-dose FIN, KETO, and TL. In the second experiment, 11 litters containing 10 male pups per litter were used. In this experiment, rats were treated with high-dose TP, high-dose FIN, and FAD. A second manuscript with male pubertal onset assay results using different chemicals is reported separately (Marty et al., in press). Pups were weighed at weaning (21 days of age) and randomized into treatment groups such that each group had approximately equal mean body weights and variances. Furthermore, pups were blocked by litter to ensure that littersmates were not assigned to the same treatment group. Ten males were assigned to each treatment group and animals were dosed daily by gavage as previously described (Marty et al., in press). Because control males from the first series of experiments were similar to control males from the second series of experiments (mean weaning weight: 51.7 ± 2.7 vs. 52.7 ± 4.6 g; body weight at PPS: 237.1 ± 18.6 vs. 237.3 ± 21.1 g; age at PPS: 43.8 ± 1.5 vs. 45.0 ± 2.3 days of age; body weight at study termination: 259.6 ± 20.2 vs. 251.1 ± 23.1 g), results from the controls were combined prior to analysis.

The present study differed from the protocol proposed by EDSTAC in several ways. First, there is uncertainty as to whether a 20-day dosing period is sufficient (Ashby and Lefevre, 2000); therefore, animals were dosed for 30 days beginning at 21 days of age. A 30-day dosing period had been recommended previously (Kelce and Wilson, 1997). Additional changes included a) the weight of the LABC was not recorded due to the redundancy of this end point, because the weights of several other tissues (e.g., epididymides, prostate, seminal vesicles) are androgen dependent. Furthermore, the LABC is somewhat laborious to dissect during a large-scale screening assay; b) whole-prostate weights were measured rather than ventral prostate weights, to reduce variability introduced during the dissection process; c) after fixation, thyroid weights were recorded in order to monitor thyroid size; and d) histological evaluations were done on the thyroid, but not the testes and epididymides. The optional measurements of serum testosterone, DHT, and liver weights were added to this study. In our initial work with this assay, we focused on puberty onset, the reproductive organs, and the thyroid; hence, accessory sex organ and liver weights were not collected for compounds run in the first experiment, which included low-dose TP, KETO, low-dose FIN, and TL. Ultimately, this omission did not alter the outcome of the study, because accessory sex gland weights were collected at higher dose levels of TP and FIN, and effects of KETO and TL were detected without inclusion of these end points.

*Observations.* All animals were examined at least once per day throughout the study for clinical signs of toxicity. Body weights were recorded daily and used to adjust dose volume. Body weights were compared statistically at 21, 27, 34, 41, and 50 days of age, and body weight gains were analyzed during the intervals of 21–27, 21–34, 21–41, and 21–50 days of age.

Males were examined daily for PPS beginning at 35 days of age. On the day that PPS was achieved, age and body weights of the affected animals were recorded. If an animal had not achieved PPS by 50 days of age, that animal was arbitrarily assigned a value of 51 days of age. This artificial value was applied to one animal in each of the KETO and low-dose TP groups and to two animals in the high-dose FIN group.

*Pathology.* Approximately 24 h after the final dose, animals were weighed, anesthetized with methoxyflurane, and given a limited gross necropsy. The testes, epididymides, seminal vesicles, prostate, and liver were removed and weighed. Seminal vesicle weights included seminal fluid. Thyroid glands were removed, fixed in neutral, phosphate-buffered 10% formalin, weighed, and examined histologically. Terminal serum samples were collected for analysis of testosterone and DHT. Hormone assays were conducted by Analytix, Inc. (Gaithersburg, MD).

*Statistical analysis.* Age at PPS, body weight at PPS, body weights, serum hormone data, and absolute and relative organ weights were evaluated by Bartlett’s test for equality of variances (Winer, 1971). Due to heterogeneity of variances, some data transformations were required prior to analyses. Data that were log transformed included thyroid weights for TP-, KETO- and TL-treated animals, relative thyroid weights for KETO- and TL-treated groups, relative seminal vesicle weights for TP- and FIN-treated animals, and serum testosterone and/or DHT levels in the TP, KETO, and FAD treatment groups. Heterogeneity of variance required that inverse weights for relative testicular, epididymal, and thyroid weights were used for TP data. After Bartlett’s test, age at PPS, body weight at PPS, serum hormone data, body weights, and absolute organ weights were evaluated by an analysis of covariance (ANCOVA) with body weight at weaning as the covariate, as was recommended in the EDSTAC report (EDSTAC, 1998). Relative organ weights were evaluated by analysis of variance (ANOVA). In accordance with the recommendations in the EDSTAC
final report (1998), significant differences \( p < 0.05 \) were examined using least square means (LSM) to compare the vehicle to treatment groups during post hoc comparisons. In most cases, analyses were grouped based on test compound such that each analysis contained results from control animals compared with results from animals treated with one test compound. The level of statistical significance for all analyses was set \( a \) priori at \( \alpha = 0.05 \).

**RESULTS**

**Observations, Body Weights, and Body Weight Gains**

There were no significant treatment-related clinical observations in any of the treatment groups. One animal from the low-dose FAD group was removed from study due to ocular enlargement unrelated to treatment.

Mean body weight gain data are shown in Figures 1A and 1B. Males treated with TL and both doses of FAD exhibited reduced body weight gains at one or more of the time intervals evaluated. Animals treated with TL had body weight gains that were 6, 8, and 12% less than control animals during the intervals from 21–34, 21–41, and 21–50 days of age, respectively. Low-dose FAD–treated animals had significantly decreased body weight gains over 21–41 days of age (8% lower than controls), and animals treated with both doses of FAD had 14% decreases in body weight gains over the entire dosing period. KETO significantly decreased body weight gains during the 21–41 day interval (7% less than controls), but body weight gains for these animals were not significantly different over the entire dosing period (21–50 days of age). Terminal body weights were decreased significantly in TL-treated and low- and high-dose FAD–treated animals by 7.3, 10.7, and 8.8%, respectively (Fig. 2; control terminal body weight was 255.4 ± 21.6 g). Neither body weight gains nor terminal body weights differed significantly with TP or FIN treatments.

**Preputial Separation**

The mean ages and body weights for PPS in the various treatment groups are illustrated in Figures 3A and 3B. Control animals achieved PPS at 44.4 ± 2.0 days of age and at a mean body weight of 237.2 ± 19.4 g.

In an evaluation of assay sensitivity to androgenic com-
pounds, neither dose of TP significantly altered the age or body weight at PPS. However, the broad-spectrum steroid biosynthesis inhibitor KETO (24 mg/kg/day) delayed puberty onset to 46.1 ± 2.5 days of age, although this delay was not statistically identified (\( p = 0.051 \)). FIN, a 5α-reductase inhibitor, significantly accelerated PPS at 20 mg/kg/day (42.9 ± 1.4, \( p = 0.041 \)), but delayed this end point at 80 mg/kg/day (45.6 ± 3.0; \( p = 0.074 \)). Mixed results were seen with the aromatase inhibitors, TL (220 mg/kg/day) and FAD (0.6 or 6.0 mg/kg/day). TL significantly delayed the age at PPS (47.0 ± 1.6; \( p = 0.001 \)), whereas FAD did not alter the age at puberty onset, but animals achieved PPS at a lower body weight (215.6 ± 33.5 at 0.6 mg/kg/day, \( p = 0.023 \); 220.1 ± 16.2 at 6.0 mg/kg/day, \( p = 0.070 \)). The mean body weights at puberty onset did not differ in KETO-, FIN-, or TL-treated animals.

**Liver Weights**

Absolute and relative liver weights, optional end points in the male pubertal assay, are illustrated in Figure 4. Low-dose

![FIG. 4. Mean liver weights (X ± SD) were collected for each treatment group to examine the potential for increased liver metabolism and enhanced hormone clearance. Low-dose FAD significantly decreased absolute liver weight, but relative liver weight was not changed with this treatment. FIN (80) significantly increased relative liver weight, suggesting that enzyme induction has occurred with this treatment. Liver weights were not collected for animals dosed with low-dose TP, low-dose FIN, KETO, or TL. Asterisks (*) mark significantly different liver weights \( p < 0.05; n \geq 9 \) animals per treatment group.](image)
FAD significantly decreased absolute liver weights compared with liver weights in control animals (Fig. 4A; control liver weight was 8.842 ± 1.003 g); however, this decrease appeared to be secondary to decreased body weights (Fig. 4B). Relative liver weights were significantly increased by 7.6% in animals treated with high-dose FIN. Note that liver weights were not recorded in our initial experiments with the male pubertal onset assay; therefore, there are no values for liver weights in animals treated with low-dose TP, low-dose FIN, KETO, or TL.

Reproductive Organ Weights

Control animals in this study had mean absolute and relative testicular weights of 2.603 ± 0.231 g and 1.024 ± 0.108 g/100 g body weight, respectively (Fig. 5). Only the aromatase inhibitor TL caused a significant (6.6%) decrease in absolute testicular weight ($p = 0.046$).

Mean paired epididymal weights in control animals were 0.396 ± 0.035 g and 0.156 ± 0.020 g/100 g body weight (Figs. 5C and 5D). KETO and FIN (both doses), compounds which inhibit androgen synthesis, and the aromatase inhibitor TL significantly decreased absolute epididymal weights by 17.4, 13.4, 14.9, and 21.2%. These compounds also reduced relative epididymal weights. The more specific aromatase inhibitor FAD, and the androgen TP, were without effect on this parameter.

Accessory Sex Gland Weights

Monosson et al. (1999) and Asbhy and Lefevre (2000) recently examined the relationship between accessory sex gland weight, body weight, and age in peripubertal male rats. These investigators consider absolute accessory sex gland weights in juvenile animals to be most relevant. In this study, both absolute and relative accessory sex gland weights are presented for completeness (Figs. 6A–6D).
Of the steroid biosynthesis inhibitors tested, only FIN (80 mg/kg/day) significantly reduced prostate weight by 30.4% and seminal vesicle weight by 64.0% compared with control values (controls: 0.598 ± 0.071 g for prostate and 0.472 ± 0.099 g for seminal vesicles; p < 0.0001 in both cases). None of the other treatments significantly altered accessory sex gland weights. Note that in our initial experiment with the male pubertal onset assay, accessory sex gland weights were not recorded for low-dose TP, KETO, low-dose FIN, or TL groups.

Serum Testosterone and DHT Levels

To examine the effectiveness of the steroid biosynthesis inhibitors, levels of serum testosterone and DHT were analyzed in samples collected 24 h after administration of the final dose of test material (Fig. 7). Despite transforming data for statistical analyses, the interanimal variability inherent in these hormonal end points limited their utility. For example, KETO reduced mean serum testosterone by 43% and FIN reduced mean serum DHT by 45% compared with their respective controls (control testosterone = 2.12 ± 1.54 ng/ml; control DHT = 0.62 ± 0.47 ng/ml); however, these changes were not statistically identified. Furthermore, FIN increased mean serum testosterone by 30%, although this change was not significant (p = 0.208).

Despite this variability, serum hormone data revealed two significant differences. First, there was a significant reduction (64%) in mean serum testosterone with 0.1 mg TP/kg/day, but high-dose TP did not affect serum testosterone levels. Second, the aromatase inhibitor TL significantly reduced serum testosterone by 50% (p = 0.032), whereas the more specific aromatase inhibitor FAD was without effect (p = 0.757).
Both FAD doses affected absolute thyroid weight by $ \geq 12.2\%$ (Fig. 8A; control thyroid weight was $18.8 \pm 2.9$ mg). However, this difference was lost when relative thyroid weights were examined (Fig. 8B). Thyroid glands from study animals also were evaluated histologically, but no treatment-related effects were noted.

**DISCUSSION**

These experiments were undertaken to evaluate the sensitivity and specificity of the male pubertal assay to detect a variety of known endocrine-active agents. Male rats were treated with test compound from 21 through 50 days of age, examined for pubertal onset, and organ weights (testes, epididymides, prostate, seminal vesicles, thyroid, and liver) were measured at 51 days of age. Serum testosterone and DHT were measured. Results from this study were compared with results obtained with other proposed Tier I assays (Tables 1 and 2).

One factor of critical importance in the pubertal male screening assay is the effect of body weight on assay endpoints. In this study, TL significantly delayed age at PPS, yet animals had approximately equal body weights to control animals at the time PPS was achieved. This scenario poses the question as to whether the delay in pubertal onset was mediated by changes in rate of growth. It seems unlikely that the effect of TL on pubertal onset is solely mediated by changes in rate of growth, because TL also affected reproductive and accessory sex gland organ weights and serum testosterone levels. However, in instances where body weight gains prior to puberty are altered, the effect of body weight should be considered in view of other assay end points.

To test the ability of the male pubertal assay to detect androgens, males were dosed with 0.1 or 0.4 mg TP/kg/day. TP failed to significantly alter body weight gains, the age or body weight at PPS, or androgen-dependent organ weights. The lack of effect on puberty onset agrees with work by Freitag and Döcke (1987), who reported that puberty was not altered in rats treated from 30 days of age through PPS with subcutaneous injections of 0.1 mg TP/kg/day. Serum testosterone was decreased significantly at low-dose TP. This effect on serum testosterone appears to be either transient or artifactual due to the lack of a dose response and the presence of a slight, nonsignificant increase in testicular weight, which is inconsistent with reduced testosterone production. Furthermore, de-
creases in serum testosterone and DHT were not accompanied by reduced epididymal or accessory sex gland weights, although these organs are androgen dependent (Cook et al., 1993). Taken together, these data suggest that the male pubertal onset assay was unable to detect TP at doses up to 0.4 mg/kg/day. Because androgens have been linked with alterations in several end points measured in the male pubertal assay, it seems likely that higher doses of TP would have been detected.

Results for testosterone using an alternate Tier I assay, the 14-day intact male assay, are presented in Table 1. In this assay, intraperitoneal injection of testosterone at dose levels of ≥ 0.5 mg/kg/day produced significant organ weight and hormonal effects. These dose levels are higher than those employed in the present study and utilize a route of exposure likely to result in greater activity. Using the broad-based steroid biosynthesis inhibitor KETO at 24 mg/kg/day, the age at PPS was increased slightly (not statistically identified) by 1.7 days. KETO had no effect on testicular weight, but significantly reduced both absolute and relative epididymal weights. This result confirms a previous finding, where epididymal weight was decreased at 49–50 days of age following a 14-day treatment with 15 mg KETO/kg/day (Table 2). Interestingly, these investigators did not find significant changes in epididymal weights with 25 mg KETO/kg/day at 35–36 or 54–55 days of age when it was administered for 14 or 20 days, respectively. This may suggest that 49–51 days of age is a highly sensitive time point in which to measure changes in epididymal weight.

KETO caused a 43% reduction in serum testosterone and a 21% decrease in serum DHT. These androgens are known to contribute to the maintenance of epididymal weight (Awoniyi et al., 1993; George et al., 1989). It seems likely that a greater change in serum hormone concentrations would have been detected if the animals had been necropsied at a time point closer to the administration of the last dose of KETO instead of 24 h later (Wang et al., 1992). Because of the rapid recovery from KETO treatment, many studies administer multiple doses of KETO per day to maintain suppression of testosterone levels (English et al., 1986; Irsy and Koranyi, 1990; Trachtenberg, 1984). In the final version of the EDSTAC male pubertal assay (EDSTAC, 1998), animals are sacrificed on the same day that the final dose of test material is administered, which presumably would result in more uniform steroid hormone suppression. The weight of evidence for KETO endocrine activity would have been improved with accessory sex gland weight

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Testes</th>
<th>Epididymides</th>
<th>Accessory sex gland</th>
<th>Prostate</th>
<th>Seminal vesicles</th>
<th>Testosterone</th>
<th>DHT</th>
<th>Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>—</td>
<td>—</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>—</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Finasteride</td>
<td>—</td>
<td>—</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>—</td>
<td>—</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Note. Alterations in other hormones (prolactin, gonadotropins, thyroid stimulating hormone, and thyroid hormones) not included in this table. See original references.*

*Data from O'Connor et al., 2000.
*Data from Cook et al., 1997.
*Data from O'Connor et al., 1998.

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Age at PPS</th>
<th>Testes weight</th>
<th>Epididymal weight</th>
<th>Seminal vesicles</th>
<th>Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>NE</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Finasteride</td>
<td>NE</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CPA</td>
<td>NE</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>NE</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

*Note. Note that exposure times in these studies varied from the present study, in which males were dosed from 21 to 50 days of age. NE, not evaluated in the studies by Ashby and Lefevre.*

*Data from Ashby and Lefevre, 2000.
*Denotes similar results with the present study. TL results are compared with the antiandrogen CPA (cyproterone acetate) and FAD results are compared with the aromatase inhibitor anastrozole.
*These parameters were not measured in the present study for KETO or TL.
*Data from Ashby and Lefevre, 1997.
data (Table 2), which were not collected for this compound in the present study. In the 14-day intact male assay, KETO was detected due to hormonal changes at 25 mg/kg/day, and at 125 mg/kg/day decreases in androgen-sensitive organs weights were seen (Table 1).

The endocrine activity of FIN, a 5α-reductase inhibitor that blocks the conversion of testosterone to DHT, was readily detected with the male pubertal assay. Animals given high-dose FIN (80 mg/kg/day) had a slight increase in age and body weight at puberty, a response profile that may be indicative of a true endocrine-active compound. Unlike other male pubertal studies (Table 2), FIN had no effect on testicular weights; however, epididymal, prostate, and seminal vesicle weights were significantly decreased. These results were predicted, because prostate and seminal vesicle growth are under DHT control (Wise et al., 1991). Furthermore, these findings are consistent with previous reports (Tables 1 and 2). Interestingly, mean age at PPS was increased in high-dose FIN-treated animals (not significant), yet males achieved PPS at a significantly younger age than control animals with low-dose FIN treatment. The reason for this response is not known. The rationale for accelerated PPS is further complicated by the serum hormone data, whereby high-dose FIN increased serum testosterone and low-dose FIN decreased serum testosterone relative to control animals. One hypothesis is that the relative proportion of testosterone to DHT influenced the achievement of PPS, a possibility given that the male reproductive system is shifting from DHT to testosterone as the dominant androgen during this period (Ojeda and Urbanski, 1994). Alternatively, the body weights of low-dose FIN-treated animals were the same as the control animals at the time they reached puberty, suggesting that these FIN-treated animals had slightly faster growth rates. Indeed, although not statistically identified, the body weight gains and final body weights of these animals were slightly higher than weights in control animals. Lastly, the lack of a readily apparent explanation for the acceleration of PPS by low-dose FIN allows the possibility that this result is artifactual. Despite this unusual finding, the variety of effects with FIN treatment readily suggests endocrine activity (Table 2).

The capacity of the male pubertal assay to detect aromatase inhibitors is somewhat uncertain, particularly in light of the limited role of estrogens in male pubertal onset. In this study, the two aromatase inhibitors (TL and FAD) produced different results. Although both compounds significantly decreased body weight gains, a finding reported previously for aromatase inhibitors (Nunez et al., 1996; Vanderschueren et al., 1997), TL also delayed PPS, decreased absolute testicular and epididymal weights, and reduced serum testosterone levels. FAD did not significantly alter reproductive or accessory sex gland weights or serum hormone concentrations. The overall response differences between TL, a less-specific aromatase inhibitor, and FAD, a highly specific aromatase inhibitor, suggest that TL is not operating solely, if at all, through aromatase inhibition. A previous report by Vigersky et al. (1982) demonstrated that a high dose of TL (75 mg/day) competed with DHT for androgen-receptor binding and produced antian- drogenic effects in male rats. Although the dose level of TL used in the present study was less than that used by Vigersky and colleagues, the results of this study on puberty onset and reproductive organ weights are consistent with the effects reported for other aromatase inhibitors (Ashby and Lefevere, 1997, 2000; Dhar et al., 1983; Dhar and Setty, 1990a,b; Gray et al., 1999; Monosson et al., 1999). Although the decreased reproductive organ weights by TL concur with the antiandrogen hypothesis (see results for CPA in Table 2), serum testosterone levels were decreased in TL-treated animals, a finding which is contrary to other studies with aromatogens in which elevations in serum testosterone were observed (Dhar and Setty, 1990b; O’Connor et al., 1998). This difference may be related to the time interval (24 h) between antiandrogen dosing and serum sample collection for testosterone analysis.

Our inability to detect the specific aromatase inhibitor fadrozole with the male pubertal assay differs from previous work with anastrozole (Table 2). Similar to FAD, anastrozole is considered to be highly specific for aromatase inhibition (Dukes et al., 1996). Although the reason for this discrepancy is not readily apparent, the study by Ashby and Lefevere used a different strain of rats and an alternate dosing duration (14 days), with animals sacrificed 24 h after the final dose at 36–37 or 49–50 days of age. As shown in Table 1, aromatase inhibitors have different effects in adult rats, decreasing relative seminal vesicle and accessory sex gland weights.

To examine the requirement to control for weaning weight during statistical analyses, data from these experiments also were analyzed by analysis of variance (ANOVA) with Dunnett’s test and compared with the results using ANCOVA and LSM analyses. Although most analyses yielded similar results whether ANOVA or ANCOVA was used, four differences were detected with the ANCOVA analysis that were not detected using ANOVA and Dunnett’s test. Specifically, ANOVA failed to identify a) significant decreases in terminal body weight (p = 0.12) in TL-treated animals and b) decreased absolute testes weight (p = 0.11) with TL treatment. Furthermore, Dunnett’s test failed to detect c) statistically significant differences in age at PPS for low-dose FIN–treated animals and d) decreased absolute thyroid weights for both doses of FAD, although the overall ANOVA analyses were significant in each case (p = 0.03). A TL-induced decrease in testicular weight was consistent with other effects produced by TL treatment, which were detected by ANOVA analyses. Although an acceleration in PPS with low-dose FIN was not identified with Dunnett’s test, this analysis identified significantly decreased epididymal weight with low-dose FIN. The effect of FAD on absolute thyroid weight was not detected by Dunnett’s test, but this decrease was secondary to decreased body weights. Neither ANOVA nor ANCOVA detected differences in relative thyroid weight with FAD treatment. These statistical discrepancies related to the use of Dunnett’s test, which controls type
I error with multiple comparisons ($\alpha = 0.05$), may reflect the more conservative nature of this type of analysis or the marginal nature of these findings. Overall, the use of either ANOVA or ANCOVA yielded similar results with regard to detecting EACs.

Overall, the male pubertal assay is capable of detecting chemicals that operate through a variety of mechanisms. KETO, TL, and FIN were deemed positive for endocrine activity in the current male pubertal assay study; however, TP and FAD were considered negative. The negative designation for TP assumes that altered serum testosterone alone was insufficient to trigger a Tier II test in the absence of other effects. This interpretation seems reasonable, given the variability in serum hormone data and the idea that hormone data represent a single time point, whereas organ weights represent a culmination of events over time. Hypothetically, higher doses of TP would be detectable with this assay, but the ability of this assay to identify aromatase inhibitors remains questionable.

With regard to assay specifics, the present study suggests that PPS was not the most sensitive end point for detection of these endocrine-active compounds. Changes in the age at PPS were observed only at concentrations that altered reproductive/ accessory sex gland organ weights. In most cases, organ weight changes observed in this study deviated from control values by $< 22\%$, with the exception of marked effects on accessory sex gland weights by FIN. This may reflect the adaptability of the male endocrine system to maintain homeostasis. Measurement of steroid hormones had limited value in this study due to interanimal variability. A greater number of animals per treatment group would be needed in future studies to improve the utility of these data. Additional work is needed to establish criteria for a positive response in the male pubertal onset assay, especially in light of the apical nature of its end points. Finally, it is worth noting that the compounds used in the present study were strong endocrine-active agents. The capacity of the male pubertal onset assay to detect weakly active environmental agents, including aromatase inhibitors, requires further investigation.

REFERENCES


