In Vitro and in Vivo Effects of 17β-Trenbolone: A Feedlot Effluent Contaminant

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Concern has arisen regarding the presence and persistence of trenbolone in the environment. Trenbolone acetate is an anabolic steroid used to promote growth in beef cattle. It is hydrolyzed to the active compound, 17β-trenbolone (TB), which is also one of the metabolites excreted by cattle. Reproductive alterations have been reported in fish living in waters receiving cattle feedlot effluent, and in vitro androgenic activity displayed by feedlot effluent samples has been related to these effects. In the current study, the androgenic potency of TB was examined both in vitro and in short-term in vivo assays. TB was a high affinity ligand for the androgen receptor (AR), with an IC50 of about 4 nM in rat ventral prostate cytosol and about 33 nM in cells transfected with the human AR when competed with 1 nM [3H]R1881. TB induced AR-dependent gene expression in MDA-kb2 cells with a potency equal to or greater than dihydrotestosterone. In immunocytochemistry experiments with the human AR, concentrations as low as 1 pM significantly induced androgen-dependent translocation of the AR into the cell nucleus. TB also displayed antiguocorticoid activity in vitro, inhibiting dexamethasone-induced transcriptional activity, and reduced adrenal gland size in vivo. In the Hershberger assay (in vivo), TB was as potent as testosterone propionate -reductase but less effective at increasing weight of tissues with this enzyme. Such tissue specificity was anticipated because other C-19 norsteroidal androgens display a similar profile in this assay. Subcutaneous TB treatment was about 50- to 100-fold more effective in stimulating growth of androgen-dependent tissues than was oral treatment. In our in utero screening assay, maternal TB administration increased AGD and attenuated the display of nipples in female offspring in a dose-related manner, similar to the published effects of testosterone propionate. Previous studies have documented that these types of malformations in newborn and infant rats are not only permanent effects but are also highly correlated with serious reproductive malformations as adults. In summary, TB is a potent environmental androgen both in vitro and in vivo and, in contrast to other reports, can induce developmental abnormalities in the fetus.

Key Words: 17β-trenbolone; environmental androgen; feedlot contaminant; in vivo, in vitro, in utero screen.

Most of the chemicals identified as endocrine disruptors display weak hormonal activity as an unintended side effect. In contrast, there are a variety of other chemicals that have been developed to intentionally alter the endocrine system. These synthetic hormones are used for medical purposes or as growth promoters in farm animals. There is growing concern that these synthetic hormones are making their way into surface water and even ground water via animal and human wastes. Public concern initially focused on the synthetic components of oral contraceptives and their discharge from sewage treatment facilities after their incomplete removal during the treatment process (Ternes et al., 1999). These chemicals have high physiological activity at low concentrations and sewage treatment effluents have been shown to induce vitellogenin production, an estrogen-inducible protein normally expressed only in females, in male oviparous fish (Purdom et al., 1994). Thus there is evidence that wildlife is impacted by effluents containing synthetic steroids, while the potential impact on human populations remains uncertain. Concerns are not limited to sewage treatment effluents, however. Recently, both in vitro androgenic activity in feedlot effluent samples (Gray et al., 2001; Jegou et al., 2001) and reproductive alterations in fish living downstream from animal feedlot operations (Jegou et al., 2001) and in water dosed with low levels of 17β-trenbolone in the lab (Ankley et al., in preparation) have been reported.

The anabolic steroid, trenbolone acetate (TBA; 17β-hydroxy-estra-4,9,11-trien-3-one-17-acetate), is a growth promoter used in cattle in the U.S. and Canada either alone or in combination with an estrogenic compound. After absorption, TBA is hydrolyzed to the active androgen, 17β-trenbolone (TB; 17β-hydroxy-estra-4,9,11-trien-3-one). A portion of the active androgen, TB, is excreted by the cattle along with its metabolites, primarily, 17α-trenbolone and triendione (Pottier...
et al., 1981). TB, along with its metabolites, has been identified in liquid and solid waste from cattle, and studies conducted on stored liquid cattle waste indicated half-lives of 267 and 257 days for the 17α-isomer (α-TB) and 17β-isomer (TB), respectively (Schiffer et al., 2001).

Although TBA has been used in cattle feedlot operations for several decades, the affinity of 17β- and 17α-trenbolone for the human androgen receptor (AR) was only recently reported (Bauer et al., 2000). It has also been reported that TB exhibits additional endocrine activities that distinguish it from androgens like testosterone and dihydrotestosterone (DHT) by displaying potent antiluteinizing activity in vivo (Danhaive and Rousseau, 1988), which may explain its effects on adrenal gland morphology and function (Silence and Rodway, 1990; Thomas and Rodway, 1983). This steroid also has high affinity for the bovine progestin receptor (Bauer et al., 2000; Meyer and Rapp, 1985). While the in vitro effects of TBA have been extensively examined in teratology studies, multigenerational studies, and the Hershberger assay, much of this data is unpublished, having been conducted in industry laboratories, and only brief summaries are available on the internet from the World Health Organization (WHO) Joint FAO/WHO Expert Committee on Food Additives (www.inchem.org/documents/jecfa/jecmono/v25je08.htm). In this regard, TBA has been reported to be “nonteratogenic” because it failed to produce malformations in several teratology and multigenerational studies.

The purpose of this study was to examine the potency of TB in both in vitro and in vivo screening assays for androgenic activity. Initially, the ability of TB to bind to the AR was confirmed and its ability to alter AR-dependent gene transcription in the MDA-kb2 cell line was examined. This cell line contains endogenous AR and has been stably transfected with the human androgen receptor (AR) expression vector pCMVhAR as described by Wong et al. (1995). COS cells were plated at 200,000 cells/well in 12-well plates and transfected with 1 µg of pCMVhAR using diethylaminoethyl dextran. Twenty-four h later, cells were exposed to 1 nM [3H]R1881 in the presence and absence of unlabeled TB at concentrations ranging from 0.1 nM to 10 µM (two wells per concentration) and incubated for 2 h at 37°C. Nonspecific binding was determined by adding a 100-fold molar excess of unlabeled R1881. After incubation, cells were washed 3 times with 50 mM Tris buffer (centrifuged at 4°C for 30 min) and 1 µl of 60% hydroxyapatite (HAP) slurry in 50 mM Tris buffer was added to each well. Samples were washed 3 times with 50 mM Tris buffer to ensure complete removal of unbound ligand. Receptor-bound ligand was recovered using 2 ml ethanol. Counts were determined using liquid scintillation counting.

**COS whole-cell hAR binding assay.** This assay was used to evaluate the ability of TB to compete with 1 nM [3H]R1881 for binding to the rat androgen receptor (hAR). COS cells (monkey kidney cells, ATCC) were transiently transfected with the hAR expression vector pCMVhAR as described by Wong et al. (1995). COS cells were plated at 200,000 cells/well in 12-well plates and transfected with 1 µg of pCMVhAR using diethylaminoethyl dextran. Twenty-four h later, cells were exposed to 1 nM [3H]R1881 in the presence and absence of unlabeled TB at concentrations ranging from 0.1 nM to 10 µM (two wells per concentration) and incubated for 2 h at 37°C. Nonspecific binding was determined by adding a 100-fold molar excess of unlabeled R1881. After incubation, cells were washed with phosphate-buffered saline and lysed in 200 µl ZAP (0.13 M ethyldimethylhexadecylammonium bromide with 3% glacial acetic acid). Radioactivity of the lysate was determined by liquid scintillation counting. Reported data are the mean of four replicate assays.

**AR-dependent gene transcriptional activation assay in MDA-kb2 stable cell line.** The ability of TB to activate AR-mediated gene transcription was evaluated using MDA-kb2 cells stably transformed with the pMMTV.neo.luc reporter plasmid (Bauer et al., 1998). The purpose of this study was to examine the potency of TB in a short-term in vitro screening assay we have developed as a screen for chemicals with AR agonist or antagonist activities. In this assay, chemicals with androgenic activity administered to the pregnant dam from gestational day (GD) 14 to 19 increase anogenital distance (AGD) of female offspring at birth and inhibit the display of nipples in infant female rats. If these effects are noted, all offspring from the screening study are retained for further investigation. The effects of TB were compared to comparable doses of TP administered in vivo in a similar study in our laboratories (Wolf et al., 2002).

**MATERIALS AND METHODS**

**In Vitro**

**Rat ventral prostate cytosol AR binding assay.** The ability of TB to compete with 1 nM [3H]R1881 for binding to the rat androgen receptor was evaluated using a cytosolic extract prepared from rats. Ventral prostate tissue was obtained from 90-day-old Sprague-Dawley rats at 10-fold dilution using ice-cold low-salt TEDG buffer (10 mM Tris, pH 7.4, 10% glycerol, 0.1 nM sodium molybdate, 1.5 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM dinitrothiophen) at 1 ml buffer/g tissue. Homogenates were centrifuged at 4°C for 30,000 × g for 30 min. Supernatant (containing the low-salt cytosolic androgen receptor) was collected, pooled, aliquoted, and stored at −80°C until needed (not longer than 6 months). Protein concentration in the cytosol was measured using commercially available reagents (Bio-Rad) according to the method of Bradford (1976).

Binding assay procedures were modified from previously described protocols (Lambright et al., 2000). Briefly, ventral prostate cytosol was diluted with ice-cold low-salt TEDG buffer to a protein concentration of 1.2 mg per 300 µl. Increasing concentrations of TB (0.1, 0.316, 1.0, 3.16, 10, 31.6, and 100 nM) were incubated on a rotary mixer at 4°C overnight (20 h) in the presence of 1 nM [3H]R1881 and 10 µM triamcinolone acetonide (binds and blocks progesterone and glucocorticoid receptors). Inert R1881 (100 X) was added to assess nonspecific binding. After incubation, radioligand bound and free receptors were separated using 500 µl of 60% hydroxyapatite (HAP) slurry in 50 mM Tris buffer. Samples were washed 3 times with 50 mM Tris (centrifuged at 1000 × g) to assure complete removal of unbound ligand. Receptor-bound ligand was recovered using 2 ml ethanol. Counts were determined using liquid scintillation counting.
reporter gene construct. Cells were maintained in L-15 media (Gibco BRL) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37°C without CO2. AR-dependent transcriptional activation assays were conducted as previously described (Wilson et al., 2002). Briefly, cells were plated at 1 × 10^5 cells per well in 100 µl media in 96-well luminometer plates (Costar) and allowed to attach. Cells were then dosed with fresh media containing increasing concentration of TB, TB plus 1 µM hydroxysteroid (OHF), or DHT. Each plate also contained vehicle (ethanol) control wells and 1.0 nM DHT and DHT/plus 1 µM OHF as agonist and antagonist controls, respectively. Cells were incubated in a humidified atmosphere overnight at 37°C without CO2. After incubation, cells were washed once with phosphate buffered saline at room temperature and then lysed by the addition of 25 µl of lysis buffer (Ligand Pharmaceuticals). Luciferase activity was determined using an MLX microtiter plate luminometer (Dynex, Chantilly, VA) and quantified as relative light units (RLU). Data are the mean of three replicate assays (four wells per treatment per assay).

**GR dependent transcriptional activation assay in CV-1 cells.** The ability of TB to either activate GR-mediated transcription or to inhibit dexamethasone -induced activity, was examined as previously described using CV-1 cells cotransfected with 1 µg of pCMVhGR and 5 µg MMTV-lucifere reporter (Parks et al., 2001). Briefly, cells were plated at 200,000 cells/60 mm dish and transfected using 5 µl FuGene reagent (Boehringer Mannheim) plus 95 µl serum-free medium per dish using the manufacturer’s protocol. Twenty-four h after transfection, duplicate wells were treated with either vehicle only, 1 nM Dex only, or increasing concentrations of TB both with and without Dex treatment in Dulbecco’s Modified Eagle Medium (DMEM) with 5% Dextran coated charcoal stripped fetal bovine serum (DCC FBS). After 24 h of exposure to test chemicals, cells were washed and harvested with 0.5 ml cell lysis buffer. Luciferase activity was detected using 0.05 ml of cell lysate and relative light units were determined using a Monolight 2010 luminometer (Analytical Laboratories).

**Immunocytochemistry in COS cells.** The following experiments were conducted to visualize, by immunofluorescence, TB-induced nuclear translocation of hAR in COS cells. Two-chambered slides (Nunc) were seeded with 100,000 cells/chamber in 2 ml DMEM (Gibco) supplemented with 10% FBS (HyClone) after which cells were transfected with 0.5 µg pCMVhAR. Following transfection, medium was replaced with 2 ml DMEM containing 5% dextran-coated charcoal stripped FBS (HyClone) plus 1 µM, 1 nM, or 1 µM TB, 100 nM DHT (positive control) or vehicle only (negative control), and incubated for 24 h at 37°C with 5% CO2. Cells were then washed once with Dulbecco’s phosphate buffered saline (DPBS), allowed to dry for 45 min at room temperature, fixed for 10 min with 95% ethanol (~20°C), and then blocked with 5% BSA (Sigma, in DPBS). Cells were incubated overnight with primary AR antibody (Affinity Bioreagents, 1:1000) at 4°C. The following day, cells were washed once with DPBS and then incubated with fluorescently labeled secondary antibody (Molecular Probes) for 30 min at room temperature. To visualize the nuclei, cells were counterstained with DAPI (Sigma), a DNA stain, mounted with fluoromount (Electron Microscopy Sciences) and examined using a Nikon Eclipse E800 microscope at magnification ×20. The location of the AR was visually classified in a blinded manner as either nuclear, perinuclear, or both from either or nine randomly selected fields from each slide by each of two independent observers. The percent nuclear staining was quantified for each field and the resultant data used for statistical analysis.

**In Vivo**

**Hershberger assay.** Castrated-immature Sprague-Dawley (SD) rats were shipped from Charles River Breeding Laboratory (Raleigh, NC) the day after surgery and housed in groups of two or three per cage in clear plastic cages (20 × 25 × 47 cm) with heat-treated (to eliminate resins that induce liver enzymes) laboratory-grade pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Animals were maintained on Purina Rat Chow (5001) and filtered tap water ad libitum. They were kept in a room with a 14:10 h photo-period (light/dark [L/D]; lights off at 1100 h EST), a temperature of 20–22°C, and a relative humidity of 40–50%.

The purpose of the first study was to determine if TB displayed androgenic effects on all androgen (testosterone and DHT)-dependent tissues to the same degree as TP when administered by sc injection in the castrate-immature male rat. A second TB study was conducted in which the animals were dosed with TB by gavage in order to compare the potency of TB administered by po dosing to the activity seen after sc administration. The Hershberger assay used herein was adapted from Hershberger et al. (1953), and was similar to that recommended by the Organization for Economic Co-operation and Development (OECD) for their interlaboratory prevalidation studies of this assay.

Immature SD male rats were castrated at 41–42 days of age, received at 42–43 days of age, and allowed to acclimate for 8–13 days (8 days in the TP study, 13 days in the two TB studies), at which time rats were randomly assigned to treatment groups in a manner that provided each group with similar relative initial body weights. In the TP study, castrate-immature male rats (n = 4 per group) were injected daily with TP (Sigma, Cat. # F1 175, CAS 85-85-2, Lot #98H0566) at 0, 12.5, 25, 50, or 100 µg per rat per day for 10 days and then necropsied. Doses were administered sc on a mg/rat rather than a mg/kg body weight basis so we could directly compare the potency of TP in the Hershberger assay to the developmental effects of TP administered to the dam, since in the past most such studies have dosed rats in this manner (Green et al., 1939). In addition, as shown in the results herein and from other studies, sample sizes of 3–4 are adequate to detect the anabolic effects of potent androgenic substances in this assay. In the sc TB study, rats were injected sc with the vehicle only (0.1 ml corn oil; n = 6), 50 µg TP/0.1 ml corn oil (n = 6) or 50, 100, or 200 µg TB/0.1 ml corn oil (n = 3/group) for 10 consecutive days from 56–65 days of age (Vehicle = corn oil, Sigma, Cat. # 8267, CAS #8001-30-7; LP, Sigma, Cat. # F3925, CAS #57-85-2, Lot #89H0149; TP, Sigma, Cat. # F1 175, CAS #57-85-2, Lot #98H0566; TB, Sigma, Cat. # 3925, CAS #10165-33-8, Lot #40K0596, purity 98%). In the oral TB study, rats were dosed by gavage with either the vehicle only (2.5 ml corn oil/kg), 0.1, 0.5, 1, or 50 mg TB/kg/2.5 ml corn oil (n = 3/group) for 10 consecutive days from 56–65 days of age.

On the day after the last treatment, males were anesthetized using carbon dioxide, euthanized by exsanguination, necropsied, and tissues weighed. The androgen-dependent tissues evaluated included the seminal vesicle plus coagulating gland (including fluid; SVCG), ventral prostate (VP), paired Cowper’s glands, levator ani plus bulbocavernous muscles (LABC), and the glans penis. In addition, body, liver, kidney, and adrenal glands were also weighed. The latter tissues also contain AR and are affected by androgens to some degree, albeit less than the sex accessory tissues. In the TP study, males were anesthetized with Halothane at 57–58 days of age and serum collected for measurement of testosterone via RIA. In the two TB studies, males were necropsied at 66 days of age.

**Short-term in vitro androgen-screening assay.** Timed-pregnant SD rats were shipped to our laboratory on GD 2–3 and then housed individually in clear polycarbonate cages. Animals were provided Purina Rat Chow (5008) and filtered (5 microns) water ad libitum in a room with a 14:10 h (L/D) photo-period (lights off at 1100 h EST) and temperature of 20–22°C with relative humidity 40–50%. Thirty pregnant rats (six rats/dose group) were dosed sc with either vehicle alone at 0.1 ml/rat/day or 0.1, 0.5, 1, or 2 mg TB/rat/0.1 ml corn oil from GD 14–19. Dams were assigned to treatments in a manner that provided similar means and variances in body weight before dosing was initiated. Maternal weight during treatment was monitored throughout the dosing period. The number of pups was determined at birth and at 2 days of age (the day after birth = day 1) when individual pup body weights and AGD were measured. AGD was measured in a blind manner using a dissecting microscope with an ocular reticle calibrated with a 1 mm stage (0.01 mm divisions) micrometer at 1.5×. At 13 days of age all pups were weighed and the ventral surface examined (blind as to treatment) and the number of nipples counted. For female pups, which normally have 12 nipples, the nipples were counted after being scored as normal, faint, or absent. As obvious effects on AGD and areola numbers were noted in this study, all F1 offspring were retained for long-term evaluation based upon these triggers. These studies will be ongoing for several months as males are being retained until puberty and females until after puberty into adulthood and mated.
Statistical analysis. In these studies, in vitro data was analyzed using PROC GLM, SAS version 6.08, on the U.S. EPA IBM mainframe computer. Data collected from in vitro binding and transcriptional activation assays were from at least three independent experiments with two or more replicates per experiment. In transcriptional activation assays, relative light units were converted to fold induction over media controls for each replicate for statistical analysis. Fold data was analyzed in a GLM model that included the concentration and replicates. For the immunocytochemistry, data were collected from two independent experiments (each in duplicate) where eight to nine fields from each slide were evaluated by two separate individuals blinded as to treatment. Stained cells in each field were examined and the location of the AR classified as either nuclear, perinuclear, or both. The percent of cells with only nuclear staining in each field was calculated and treatment effects compared by ANOVA. In the Hershberger assay, body and organ weight and serum hormone data were also analyzed using PROC GLM the SAS version 6.08 on the U.S. EPA IBM mainframe. Sex accessory tissue and serum testosterone data were log10 transformed prior to analysis to correct for heterogeneity of variance, the SD being proportional to the means. The regression models for organ weights included body weight at necropsy as a covariant. Statistically significant effects (p ≤ 0.05, F-statistic) were examined using the LSMEANS procedure on SAS (two-tailed t-test) to compare the controls (castrate group without TP) to the TP- and TB-treated groups. In the in utero screening study, maternal weight change and litter sizes were analyzed as above, while AGD length, pup weight, and the numbers of normal nipples were analyzed using litter means for each sex.

RESULTS

In Vitro Assay

The ability of TB to bind to either the rat AR (rAR) or the hAR was assessed (Fig. 1). Increasing concentrations of TB were competed against 1 nM [3H]R1881 using cytosol preparations from rat ventral prostate tissue. In this assay, TB competed with 1 nM [3H]R1881 for rAR binding with an IC50 of about 4 nM. In COS cells transiently transfected with the hAR, TB inhibited 1 nM [3H]R1881 binding with an IC50 of about 33 nM. Differences in IC50 values obtained from the two assays may be due to differing levels of receptor and the fact that the rat ventral prostate cytosol assay is incubated at 4°C, while the COS whole cell binding assay is conducted at 37°C and may metabolize TB.

The potency of TB to activate AR-mediated gene transcription was investigated in the androgen responsive MDA-kb2 cell line that contains endogenous hAR and is stably transfected with an androgen responsive luciferase reporter gene construct. Figure 2 compares dose response curves of the agonist activity of TB to that of the potent androgen, DHT. Similar curves were obtained for both compounds, and at low concentrations (10 pM and 100 pM) responses of TB were significantly greater (p < 0.001) than those obtained with DHT at the same concentrations. Maximum induction for both compounds, however, was attained at 1 nM. In addition, when cells were cotreated with 1 nM of each compound along with 1 μM OHF, a specific androgen receptor antagonist, luciferase activity was significantly decreased by 50–60% compared to activity ob-
To visualize whether TB induced nuclear translocation of hAR, COS cells were transiently transfected with hAR and treated with either 0 (vehicle only, negative control), 100 nM DHT as a positive control, 1 pM, 1 nM, or 1 μM of TB. TB induced nuclear translocation of hAR in a dose dependent manner. Representative photographs depicting fluorescent labeling of AR in the cells are presented in Figure 3. The location of the AR was classified as either nuclear, perinuclear, or both. In untreated cells, 100% of the staining remained perinuclear (Fig. 3A). In cells treated with 100 nM DHT, approximately 90% of AR staining was nuclear (Fig. 3E). By comparison, 1 pM, 1 nM, or 1 μM TB produced statistically significant increases in nuclear AR staining of 22, 56, or 84%, respectively, compared to 0% in the vehicle treated controls, based on the mean of evaluations made by two independent observers (Figs. 3B–3D). The level of nuclear staining of the highest TB treatment of 1 μM, compared to the 100 nM DHT positive control, were not significantly different.

TB has been reported to act not only as an AR agonist, but also as a GR antagonist. At concentrations up to 10 μM (the highest concentration tested), TB alone failed to activate GR mediated gene transcription. When TB was coadministered along with 1 nM dexamethasone (DEX), however, TB reduced DEX-induced luciferase induction in a dose dependent manner with statistically significant reductions at 100 nM, 1 μM, and 10 μM of 62.6, 70.2, and 88.4%, respectively.

In Vivo, Hershberger Assay

In the sc TP study, androgen-dependent tissue weights (LABC, VP, SVCG, and glans penis) were significantly increased at all dosage levels, including the lowest dose of 12.5 μg TP/rat/day (Table 1). TP treatment in this study produced serum T levels and seminal vesicle and ventral prostate weights that ranged from subphysiological in the lower dosage groups to values in the 200 μg/rat/day normal for uncastrated control animals of this age (Monosson et al., 1999). Adrenal weights were reduced (linear regression analysis) by TP treatment in this study, while body weight gain was significantly enhanced by TP administration.

The effects of sc TB and sc TP on glans penis, LABC, VP, and SVCG weights in the castrate-immature male rat are shown in Tables 1 and 2. Trenbolone treatment produced a significant increase in VP, SVCG, Cowper’s gland, and glans penis weight only at the highest dose of 200 μg TB/day. LABC weights were significantly increased at all doses of TB while conversely, adrenal weights were decreased by trenbolone treatment. DHT-forming tissues, with 5α-reductase, such as seminal vesicle and VP were much less affected compared to the effects of a similar dose of TP (only showing statistically significant increases at 200 μg TB/day) while the levator ani, which exhibits a testosterone (T)-dependent response lacking this enzyme, is significantly increased in size at 50 μg TB/day with a dose-response curve nearly identical to sc TP (Fig. 4). Hence TB, as compared to TP, differentially induced “anabolic” effects on androgen-dependent muscles, with lower “androgenic” potency on the sex accessory glands. This tissue selective response is likely based upon the ability of 5α-reductase to inactivate TB, while in contrast, testosterone is converted by the same enzyme to DHT, a more potent androgen. In the LABC, sc TB was equipotent with TP, while in the VP, SV, and GP, even 200 μg/day fails to stimulate tissue growth to the same degree as 25 μg TP/day.
The effects of po TB on glans penis, LABC, VP, and SVCG weights in the castrated-immature male rat are shown in Table 3. As with sc dosing of TB, androgen-sensitive tissues containing 5α-reductase were less affected relative to TP than tissues lacking this enzyme. Glans penis, Cowper’s glands, and VP weights were significantly increased at 50 mg/kg/day (using body weight at necropsy as a covariant), while seminal vesicle and LABC weights were significantly increased at 10 and 50 mg/kg/day. Liver and adrenal weights were not significantly affected while body weight gain was significantly reduced in the high dose group. Overall, less response was produced with po dosing than sc dosing. When compared on a mg/kg/day basis, po TB was about 100-fold less effective in increasing LABC (Fig. 5) and glans penis weights and approximately 80-fold less effective inducer of VP and SVCG weights. Both sc TB and sc TP significantly reduced adrenal weights (TB data analysis included necropsy body weight as a covariant). Oral dosing of TB also reduced adrenal weights; however, the reduction was not statistically significant. Body weight gain was enhanced by sc TB treatment, similar to the

TABLE 1
Comparison of Effects of Subcutaneous Dosing with the Anabolic Steroid Testosterone Propionate (TP) on Organ Weights in Castrate-Immature Male Rat

<table>
<thead>
<tr>
<th>Weights</th>
<th>Oil</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>LABC (mg)</td>
<td>162.9 ± 9.1</td>
<td>226.4 ± 11.7*</td>
<td>280.9 ± 23.0*</td>
<td>376.7 ± 14.9*</td>
<td>483.9 ± 13.1*</td>
<td>512.8 ± 67.2*</td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>16.6 ± 3.0</td>
<td>37.3 ± 2.1*</td>
<td>61.8 ± 6.1*</td>
<td>96.0 ± 11.0*</td>
<td>149.0 ± 10.4*</td>
<td>206.7 ± 21.3*</td>
</tr>
<tr>
<td>SVCG (mg)</td>
<td>46.5 ± 5.6</td>
<td>94.0 ± 12.1*</td>
<td>172.5 ± 8.1*</td>
<td>370.5 ± 17.7*</td>
<td>631.5 ± 43.5*</td>
<td>776.5 ± 116.2*</td>
</tr>
<tr>
<td>Glans penis (mg)</td>
<td>35.8 ± 3.5</td>
<td>50.9 ± 4.6*</td>
<td>65.3 ± 2.0*</td>
<td>74.1 ± 2.4*</td>
<td>76.7 ± 5.2*</td>
<td>89.9 ± 9.3*</td>
</tr>
<tr>
<td>Pituitary gland (mg)</td>
<td>10.4 ± 1.0</td>
<td>11.0 ± 0.8</td>
<td>11.9 ± 0.7</td>
<td>11.1 ± 0.3</td>
<td>11.4 ± 0.6</td>
<td>11.6 ± 0.2</td>
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<tr>
<td>Adrenals (mg)</td>
<td>54.4 ± 2.5</td>
<td>50.1 ± 4.0</td>
<td>50.1 ± 1.8</td>
<td>47.9 ± 4.4</td>
<td>48.0 ± 1.1</td>
<td>41.1 ± 7.8*</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>1.79 ± 0.15</td>
<td>1.75 ± 0.06</td>
<td>1.95 ± 0.03</td>
<td>2.02 ± 0.05</td>
<td>2.00 ± 0.06</td>
<td>1.98 ± 0.17</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>9.97 ± 0.98</td>
<td>10.4 ± 0.61</td>
<td>11.0 ± 0.51</td>
<td>11.1 ± 0.03</td>
<td>11.0 ± 0.04</td>
<td>10.6 ± 0.13</td>
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<tr>
<td>Body (g)</td>
<td>239 ± 16</td>
<td>247 ± 9.8</td>
<td>251 ± 7.2</td>
<td>256 ± 10.2</td>
<td>263 ± 6.5</td>
<td>255 ± 8.5</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>55.3 ± 6.4</td>
<td>61.8 ± 4.1</td>
<td>65.1 ± 1.5</td>
<td>67.2 ± 5.3</td>
<td>73.9 ± 2.6*</td>
<td>66.6 ± 5.7</td>
</tr>
<tr>
<td>Serum testosterone (ng/ml)</td>
<td>Nondetectable</td>
<td>0.27 ± 0.05</td>
<td>0.48 ± 0.07</td>
<td>1.01 ± 0.27</td>
<td>1.28 ± 0.16</td>
<td>2.44 ± 0.18</td>
</tr>
</tbody>
</table>

Note. Testosterone propionate (TP) was administered to castrate-immature SD (CD) male rats at 12.5, 25, 50, 100, and 200 µg/day/rat for 10 days; effects on androgen-dependent and other organ weights were compared at necropsy at 57–58 days of age. Values are means ± SE.

*Differs significantly from oil control values.

TABLE 2
Comparison of Effects of Subcutaneous Dosing of the Anabolic Steroid Trenbolone (TB) to Those of Testosterone Propionate (TP)

<table>
<thead>
<tr>
<th>Weights</th>
<th>Oil</th>
<th>TB (50 µg/day)</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>LABC (mg)</td>
<td>173.7 ± 9.3</td>
<td>453.7 ± 16.1*</td>
<td>380.3 ± 22.9*</td>
<td>397.4 ± 24.2*</td>
<td>458.7 ± 13.9*</td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>19.3 ± 0.6</td>
<td>100.4 ± 6.5*</td>
<td>26.1 ± 4.4</td>
<td>26.6 ± 0.6</td>
<td>38.7 ± 4.2*</td>
</tr>
<tr>
<td>SVCG (mg)</td>
<td>39.3 ± 4.9</td>
<td>302.5 ± 26.2*</td>
<td>59.8 ± 5.0</td>
<td>67.9 ± 2.8</td>
<td>90.9 ± 16.4*</td>
</tr>
<tr>
<td>Cowpers (mg)</td>
<td>6.0 ± 0.6</td>
<td>27.7 ± 2.5*</td>
<td>6.0 ± 1.3</td>
<td>11.9 ± 2.7</td>
<td>16.4 ± 2.0*</td>
</tr>
<tr>
<td>Glans penis (mg)</td>
<td>42.9 ± 2.2</td>
<td>73.8 ± 3.0*</td>
<td>56.2 ± 5.2</td>
<td>55.2 ± 4.1</td>
<td>62.8 ± 1.7*</td>
</tr>
<tr>
<td>Adrenals (mg)</td>
<td>66.3 ± 10.5</td>
<td>46.9 ± 2.7*</td>
<td>51.2 ± 5.4*</td>
<td>54.1 ± 3.2*</td>
<td>42.7 ± 3.2*</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>1.94 ± 0.08</td>
<td>2.24 ± 0.06</td>
<td>2.08 ± 0.05</td>
<td>2.18 ± 0.12</td>
<td>2.19 ± 0.04</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>10.6 ± 0.25</td>
<td>12.6 ± 0.3</td>
<td>12.2 ± 0.5</td>
<td>11.4 ± 0.5</td>
<td>11.4 ± 0.4</td>
</tr>
<tr>
<td>Body (g)</td>
<td>288.3 ± 16.0</td>
<td>304.5 ± 8.5</td>
<td>309.0 ± 14.0</td>
<td>303.8 ± 5.2</td>
<td>305.0 ± 6.8</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>49.5 ± 3.4</td>
<td>72.7 ± 5.3*</td>
<td>70.3 ± 1.9*</td>
<td>61.7 ± 1.9*</td>
<td>61.6 ± 2.4*</td>
</tr>
</tbody>
</table>

Note. The anabolic steroid trenbolone (TB) was administered to castrate-immature SD (CD) male rats at 50, 100, and 200 µg/day/rat for 10 days; the effects on androgen-dependent and other organ weights were compared to the effects of testosterone propionate (TP) at 50 µg/day/rat for 10 days. The effects were compared at necropsy at 66 days of age. Values are means ± SE.

*Differs significantly from oil control values.
The biological basis for this is unclear, as there is no evidence of overt systemic toxicity of TB administered by the po route based upon any cage-side observations or organ weights.

**Short-Term in Utero Screening Study**

In the in utero screening study, maternal body weight gain was reduced by TB treatment in a dose-related fashion; however, weight gain was only significantly decreased in the two highest treatment groups. Body weight gain by GD 19 was 59.8 ± 3.4 g in control dams but only 40.8 ± 6.7 and 35.7 ± 4.6 in the 1 mg/rat/day and 2 mg/rat/day dose groups, respectively. However, dam body weight at GD 19 was not significantly lowered in any dose group when compared to controls by ANOVA. Maternal body weight at birth was reduced in the 2.0 mg TB dose group, but this effect was not statistically significant (303 ± 7.0 in controls and 291 ± 11 in 2.0 mg/rat/day group). The number of live pups at birth was not reduced by TB treatment. AGD in the female pups was significantly increased by TB treatment at doses of 0.5 mg/rat/day and above in a dose-related manner (Fig. 6). Male AGD was

![FIG. 4.](image)

Comparison of the mean effect of subcutaneous dosing of TB or TP on weights of LABC and VP in the Hershberger assay. In LABC, which exhibits a testosterone-dependent androgenic response (does not contain 5α-reductase), the effects of sc TB treatment was nearly identical to that of sc TP. However, in tissues containing 5α-reductase such as the VP, the effect of TB was much less than that seen with TP. Effects of sc TB on increased VP weights were statistically significant only at the highest dose tested of 200 μg/day. Thus, sc TB differentially induced anabolic effects on androgen-dependent muscles with lower androgenic potency on the sex accessory glands.

![FIG. 5.](image)

Comparison of the relative potency of po vs. sc dosing of TB on LABC weights in the Hershberger assay. SC dosing with TB was approximately 100-fold more effective in increasing LABC weight than po TB. Relative potency estimate was based on the magnitude of the differences between the dose response curves where each route of exposure produced approximately equivalent effects.

### TABLE 3

<table>
<thead>
<tr>
<th>Weights</th>
<th>Oil</th>
<th>0.1</th>
<th>1.0</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>LABC (mg)</td>
<td>177.3 ± 15.4</td>
<td>185.3 ± 10.3</td>
<td>197.7 ± 15.7</td>
<td>318.8 ± 29.1*</td>
<td>478.8 ± 53.7*</td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>17.5 ± 1.53</td>
<td>9.5 ± 3.7</td>
<td>12.9 ± 3.2</td>
<td>17.2 ± 1.0</td>
<td>57.5 ± 6.9*</td>
</tr>
<tr>
<td>SVCC (mg)</td>
<td>49.5 ± 5.5</td>
<td>46.7 ± 3.3</td>
<td>46.7 ± 2.8</td>
<td>74.1 ± 3.9*</td>
<td>133.5 ± 17.3*</td>
</tr>
<tr>
<td>Cowpers (mg)</td>
<td>9.0 ± 2.3</td>
<td>8.3 ± 2.4</td>
<td>6.9 ± 0.7</td>
<td>11.7 ± 1.1</td>
<td>17.7 ± 1.2*</td>
</tr>
<tr>
<td>Glans penis (mg)</td>
<td>46.13 ± 4.0</td>
<td>41.1 ± 3.1</td>
<td>43.4 ± 1.9</td>
<td>53.1 ± 4.5</td>
<td>62.7 ± 1.0*</td>
</tr>
<tr>
<td>Adrenals (mg)</td>
<td>54.3 ± 4.2</td>
<td>52.0 ± 6.1</td>
<td>43.7 ± 6.1</td>
<td>42.7 ± 3.4</td>
<td>44.0 ± 2.6</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>2.0 ± 0.03</td>
<td>2.0 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.04</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>10.5 ± 0.16</td>
<td>11.1 ± 0.4</td>
<td>11.1 ± 0.3</td>
<td>11.0 ± 0.8</td>
<td>10.8 ± 0.7</td>
</tr>
<tr>
<td>Body (g)</td>
<td>302.7 ± 7.0</td>
<td>307.7 ± 4.4</td>
<td>295.5 ± 6.2</td>
<td>296.6 ± 7.2</td>
<td>277.7 ± 14.6</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>45.6 ± 2.6</td>
<td>49.5 ± 2.1</td>
<td>40.9 ± 0.9</td>
<td>33.3 ± 3.9</td>
<td>18.7 ± 9.2*</td>
</tr>
</tbody>
</table>

**Note.** Castrate-immature SD (CD) male rats were dosed orally with the anabolic steroid trenbolone (TB) at 0, 0.1, 1, 10, and 50 mg/kg/day/rat for 10 days; effects on androgen-dependent and other organ weights were compared at necropy at 66 days of age. Values are means ± SE.

*Differ significantly from oil control values (p < 0.05).
unaffected by TB administration. In the analysis of the AGD data, pup weight was not used as a covariant in the analysis because it was not a significant factor in the ANOVA model. Pup viability to 13 days of age was unaffected by TB administration, while pup weight was reduced at day 2 in the high dose group, but not at 13 days of age. The numbers of normal and total (normal + faint) nipples was reduced in TB-treated female offspring (Fig. 6). Maternal administration of sc TB on GD 14–19 at 0.5 mg/rat/day and greater with no normal areolas seen in the two higher dose groups. *p < 0.05; **p < 0.001.

FIG. 6. Effects on female offspring after sc maternal administration of TB on GD 14–19 at 0.1, 0.5, 1.0, or 2.0 mg/rat/day. TB at doses of 0.5 mg/rat/day and greater produced dose-dependent increases in AGD in female pups when measured on PND 2. The total number of areolas per female pup was significantly reduced in the 2 mg/rat/day dose group at PND 13. However, the number of normal areolas present on female pups was significantly reduced at 0.5 mg/rat/day and greater with no normal areolas seen in the two higher dose groups.

DISCUSSION

In the current study, we demonstrated in vitro that TB is a high affinity AR ligand that induces AR-dependent gene transcription with potency equal to or greater than DHT. We also found that this steroid is active in vivo both in the Hershberger assay and during sexual differentiation. In the castrated-immature male rat, TB displays tissue-selective androgenic activity, exhibiting potency similar to TP in stimulating growth of the LABC muscles, while in contrast, tissues high in 5α-reductase activity such as the VP and seminal vesicles were stimulated only slightly as compared to TP. Such tissue-specific androgenicity is not unusual; for example, this has been described for 7α-methyl-19-nortestosterone, which cannot be metabolized by 5α-reductase, and 19-nortestosterone, which is inactivated by 5α-reductase (Sundaram et al., 1995; Toth and Zakar, 1986). In contrast to the ventral prostate and seminal vesicles, the effect of androgens in the LABC muscles is normally mediated via testosterone rather than conversion of testosterone to DHT. It is interesting to note, however, that maternal administration of TB during the critical period of sexual differentiation had marked effects on both AGD length and nipple regression, which are DHT-dependent processes. In the present study, TB administration during this period increased AGD and caused complete atrophy of nipple anlagen in female offspring. In comparison to the effects of TP administered at identical dosages levels during the same period of gestation, TB was about half as potent as TP in inducing malformations in these androgen-dependent tissues. Studies in our laboratory (Gray et al., 1999; Hotchkiss et al., submitted manuscript; Wolf et al., 2002) and in other laboratories (McIntyre et al., 2001, 2002) have documented that AGD and nipple formation in newborn and infant male and female rats are not only permanent effects but also are highly correlated with other serious reproductive malformations. With this in mind, we have developed a short-term in utero protocol to screen for androgens and antiandrogens using AGD and infant nipple formation as triggers. Reduced AGD at birth and increased infant nipple formation in male rats at birth is indicative of antiandrogenic activity and triggers an extensive evaluation of all F1 male offspring after puberty. On the other hand, as seen here, increased AGD in newborn female rats and reduced nipple formation triggers an extensive evaluation of all F1 female offspring after puberty. Evaluation of F1 offspring from this study is still ongoing and will determine what other adverse effects are associated with administration of TB during sexual differentiation. Based upon previous studies with TP,
We anticipate retained male sex accessory tissues, vaginal agenesis, and hydrometrocolpos in addition to other effects (Wolf et al., 2002).

The toxicity of TB in the environment recently became an issue when it was recognized that this chemical and one of its metabolites, 17α-trenbolone, which is a weaker androgen in vitro, are excreted into feedlots at concentrations that might be expected to be physiologically active (Schiffer et al., 2001). In fact, Ankley et al. (in preparation) recently reported that environmentally relevant concentrations of TB in the low ng/l (ppt) range masculinized female fathead minnows in the laboratory and reduced fecundity in the fish. Here, we show that low ppt (nM and below) concentrations of TB also stimulate gene expression in vitro via the human AR. To date, regulatory agencies have not conducted an environmental risk assessment of this compound; in spite of the fact that, as shown here and by Ankley et al. (in preparation), ppt concentrations are active in vitro and in vivo in two vertebrate classes. Schiffer et al. (2001) found concentrations of TB ranging from 5 to 75 ng/g and from 22 to 49 times higher levels of 17α-trenbolone in the manure canal. In addition, it has been shown that feedlot effluent from a concentrated animal feedlot operation (CAFO) displays a high level of androgenic activity (Gray et al., 2001; Jegou et al., 2001). Furthermore, altered endocrine physiology was detected in fathead minnows collected from streams near this CAFO (Jegou et al., 2001). While there are no data on the concentrations of TB in biota from different trophic levels around these CAFO sites, one would speculate that fish would be at greater risk, absorbing the chemical from the water across the gills, than would mammals that might ingest contaminated fish or water. The potential risk to lower vertebrates like amphibians, reptiles, and birds at the CAFO site, is too speculative to comment on at this time due to lack of data. Our hypothesis that mammals would be at less risk after oral ingestion of TB is based upon our results from the two Hershberger assays which demonstrate that TB was about 80–100 fold less effective via the oral route than via injection in the Hershberger assay. Similar results were cited by the WHO from unpublished Hershberger assay studies (Escuret and Bas, 1978; Schroder, 1971a,b). Such speculation, however, should be confirmed by data on this point because long-term dietary TBA treatment has adverse effects on reproduction at μg/kg/day dosage levels (Hunter et al., 1976, 1981, 1982). Although the oral route was less effective than was sc injection, trenbolone acetate (TBA) and TB have been shown to disrupt the reproductive system of humans, pigs, mice, rats, and other mammalian species at relatively low dosage levels when administered orally (Hess, 1983, 1984; Hunter et al., 1981, 1982; Kruskemper et al., 1967, Lopez-Bote et al., 1994).

The developmental and reproductive effects of TBA were extensively studied in the 1970s and 1980s (Trenbolone acetate: WHO Food Additives Series 25). However, none of these studies are published and only brief summaries of the results are available from WHO documents. In those studies, reproductive effects were seen in multigenerational studies using rats at doses ranging from 0.5–18 ppm in the diet. However, those studies did not report any effects that were clearly related to alterations of sexual differentiation. It is possible that some developmental effects were missed because they involve non-standard endpoints (e.g., looking for the ventral prostate in females or reduced nipple numbers) or confused with direct effects on the adult because treatments were continued throughout life. Furthermore, several teratology studies cited in this review failed to observe any malformations in TBA or TB-treated fetuses and measurement of AGD in males also revealed no effect. In our study, it is clear that sc TB administration does induce malformations and it is likely that oral TB also will be “teratogenic,” albeit at higher dosage levels.

In summary, this study confirms that in vitro TB is a high affinity ligand for both the rat and human AR and also induces AR-dependent gene expression with a potency equal to or greater than DHT. Along with its AR agonist activity, TB also acted as a GR antagonist in vitro. In the castrated immature rat, TB displays selective androgenic activity as compared to testosterone, affecting tissues that lack 5α-reductase more than those with this enzyme. Conversely, administration of TB during the critical period of sexual differentiation increased AGD and attenuated nipple formation in female offspring, both of which are DHT-dependent tissues. These types of malformations have been shown to be indicators of more serious reproductive malformations later in life after exposure during sexual differentiation. Given the extensive use of TB in certain types of livestock feeding operations, its persistence in the environment, and the fact that it does induce reproductive malformations, further studies would be warranted. Similar to the problems with estrogenic effects seen with steroidal estrogens found in sewage effluents, one would predict that fish residing downstream of feedlot operations where TB is used would be masculinized. In addition, as relatively little is known about the fate and transport of TB in such systems, it seems reasonable that an ecological risk assessment should be conducted.

ACKNOWLEDGMENTS

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REFERENCES


