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 IC_{50} 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 [^{3}H]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 antiglucocorticoid 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 in tissues that lack 5α-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 antiglucocorticoid activity in vivo (Danhaive and Rousseau, 1988), which may explain its effects on adrenal gland morphology and function (Sillence 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 vivo 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 an MMTV-luciferase reporter (Wilson et al., 2002). Via immunofluorescence, we examined the abililty of TB to induce ligand-dependent translocation of the AR into the nucleus of COS cells. Using a transient transfection system, we also examined the ability of TB to either activate or reduce dexamethasone-induced transcriptional activation through the glucocorticoid receptor (GR). The dose dependent effects of TB and testosterone propionate (TP) on androgen-dependent tissues were examined in vivo in castrated-immature male rats using the Hershberger assay. In contrast to testosterone, TB is a C-19 norandrogen and likely has different endocrine activities. Unlike testosterone, C-19 norandrogens cannot be aromatized, some cannot undergo 5-α reduction, and others are inactivated by 5-α reduction. Conversely, testosterone can both be aromatized and is activated to the more potent androgen, DHT, by 5-α reductase. In vivo, the goal was first to determine if TB displayed androgenic effects on all androgen (testosterone and DHT)-dependent tissues to the same degree as testosterone propionate when administered by sc injection. In addition, a second trenbolone study was conducted in which the animals were dosed with TB by gavage in order to compare the potency of trenbolone administered by oral dosing to the activity seen after sc administration. Finally, we wanted to examine the effects of TB in a short-term in utero 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 utero in a similar study in our laboratory (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 rat ventral prostate tissue. Ventral prostate tissue was obtained from 90-day-old Sprague-Dawley rats 24 h after castration. Tissue was homogenized in 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 dinitrotheitol) at 10 ml buffer/g tissue. Homogenates were centrifuged at 4°C at 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 600 × g) to assure 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 human 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 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 CO_2. AR-dependent transcriptional activation assays were conducted as previously described (Wilson *et al.,* 2002). Briefly, cells were plated at 1×10^4 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 hydroxyflutamide (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 CO_2. 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 GR activity, was examined as previously described using CV-1 cells cotransfected with 1 μg of pCMVhGR and 5 μg MMTV-luciferase 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 cotreatment 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 pM, 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% CO_2. 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 eight 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 mean initial body weights. In the TP study, castrate-immature male rats ($n = 4$ per group) were injected daily with TP (Sigma, Cat. #T 1875, CAS #57-85-2, Lot #98H0566) at 0, 12.5, 25, 50, 100, or 200 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. #C 8267, CAS #8001-30-7, Lot #89H0149; TP, Sigma, Cat #T 1875, CAS #57-85-2, Lot #98H0566; TB, Sigma, Cat #T 3925, CAS #10161-33-8, 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, 1, 10, 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 bulbocavernosus 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 anaesthetized 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 utero *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 humidly 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.
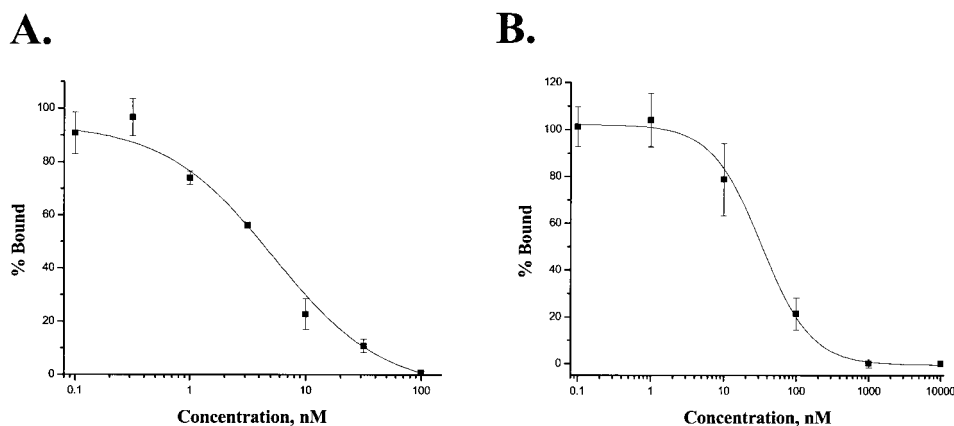
A.

B.

FIG. 1. TB competitively inhibits [³H]R1881 binding to the rat (A) and human (B) androgen receptor. Assays with the rAR used cytosolic preparations prepared from ventral prostate tissue. Binding assays with hAR were conducted using hAR expressed in COS cells. Data are presented as mean ± SE for 3 replicate assays for rAR and 4 replicates assays for hAR.

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 IC$_{50}$ of about 4 nM. In COS cells transiently transfected with the hAR, TB inhibited 1 nM [³H]R1881 binding with an IC$_{50}$ of about 33 nM. Differences in IC$_{50}$ 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 transcrip-

tion was investigated in the androgen responsive MDA-kb2 cell line that contains endogenous hAR and is stably tranfected 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-
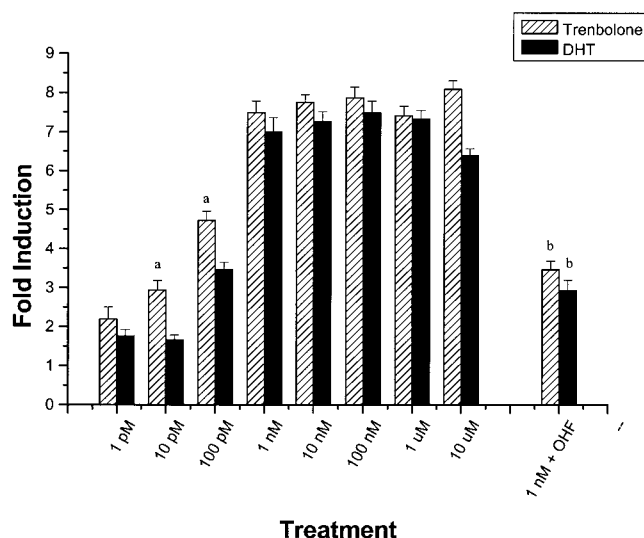

FIG. 2. TB induced AR-dependent gene transcription in the MDA-kb2 stable cell line at a level that was at least as effective as DHT. Data are presented as the mean ± SE of three replicate assays. ᵃIndicates a value significantly different from the same concentration of DHT. ᵇIndicates a statistically significant value compared to the same concentration of test chemical without OHF, an androgen specific competitor.
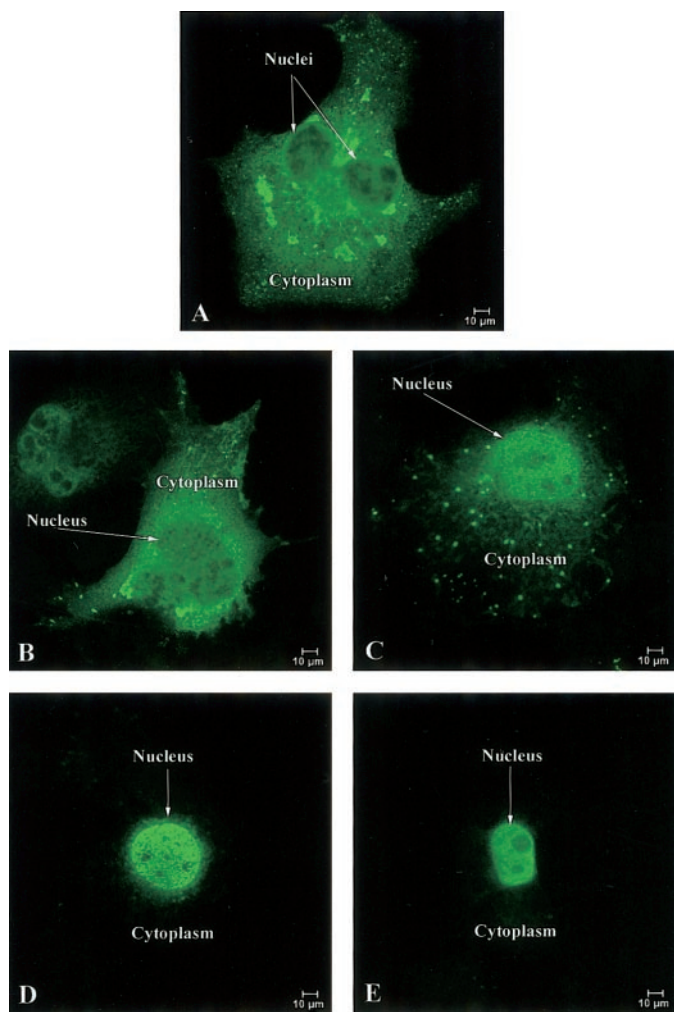
FIG. 3. Representative photos of immunocytochemical localization of fluorescently tagged AR using vehicle (A), 1 pM TB (B), 1 nM TB (C), 1 μM TB (D), or 100 nM DHT (E) in COS cell culture meduim. Translocation of AR from perinuclear to the nuclear region takes place in COS cells after androgenic ligand binding. All staining (100%) remained perinuclear in vehicle controls as AR ligands are absent from these samples. TB induced translocation of the AR into the nucleas in a dose dependent manner and a statistically significant increase in nuclear staining compared to vehicle controls was obtained at 1 pM TB (the lowest concentration tested). Approximately 90% of the cells receiving 1 μM TB treatment showed only nuclear staining that was comparable to the level seen with 100 nM DHT. Scale bars represent 10 μm.

tained with the individual compounds. Overall, TB was as least as potent as an AR agonist as DHT.

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 dependant 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 dexamathasone (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/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.

TABLE 1

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

Weights	Oil	TP (μg/day)				
		12.5	25	50	100	200
LABC (mg)	162.9 ± 9.1	226.4 ± 11.7*	280.9 ± 23.0*	376.7 ± 14.9*	483.9 ± 13.1*	512.8 ± 67.2*
Ventral prostate (mg)	16.6 ± 3.0	37.3 ± 2.1*	61.8 ± 6.1*	96.0 ± 11.0*	149.0 ± 10.4*	206.7 ± 21.3*
SVCG (mg)	46.5 ± 5.6	94.0 ± 12.1*	172.5 ± 8.1*	370.5 ± 17.7*	631.5 ± 43.5*	776.5 ± 116.2*
Glans penis (mg)	35.8 ± 3.5	50.9 ± 4.6*	65.3 ± 2.0*	74.1 ± 2.4*	76.7 ± 5.2*	89.9 ± 9.3*
Pituitary gland (mg)	10.4 ± 1.0	11.0 ± 0.8	11.9 ± 0.7	11.1 ± 0.3	11.4 ± 0.6	11.6 ± 0.2
Adrenals (mg)	54.4 ± 2.5	50.1 ± 4.0	50.1 ± 1.8	47.9 ± 4.4	48.0 ± 1.1	41.1 ± 7.8*
Kidneys (g)	1.79 ± 0.15	1.75 ± 0.06	1.95 ± 0.03	2.02 ± 0.05.	2.00 ± 0.06	1.98 ± 0.17
Liver (g)	9.97 ± 0.98	10.4 ± 0.61	11.0 ± 0.51	11.1 ± 0.03	11.0 ± 0.04	10.6 ± 0.13
Body (g)	239 ± 16	247 ± 9.8	251 ± 7.2	256 ± 10.2	263 ± 6.5	255 ± 8.5
Body weight gain (g)	55.3 ± 6.4	61.8 ± 4.1	65.1 ± 1.5	67.2 ± 5.3	73.9 ± 2.6*	66.6 ± 5.7
Serum testosterone (ng/ml)	Nondetectable	0.27 ± 0.05	0.48 ± 0.07	1.01 ± 0.27	1.28 ± 0.16	2.44 ± 0.18
n	4	4	4	4	4	2

Note. Testosterone priopionate (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.

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 2

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

Weights	Oil	TP (50 μg/day)	TB (μg/day)		
			50	100	200
LABC (mg)	173.7 ± 9.3	453.7 ± 16.1*	380.3 ± 22.9*	397.4 ± 24.2*	458.7 ± 13.9*
Ventral prostate (mg)	19.3 ± 0.6	100.4 ± 6.5*	26.1 ± 4.4	26.6 ± 0.6	38.7 ± 4.2*
SVCG (mg)	39.3 ± 4.9	302.5 ± 26.2*	59.8 ± 5.0	67.9 ± 2.8	90.9 ± 16.4*
Cowpers (mg)	6.0 ± 0.6	27.7 ± 2.5*	6.0 ± 1.3	11.9 ± 2.7	16.4 ± 2.0*
Glans penis (mg)	42.9 ± 2.2	73.8 ± 3.0*	56.2 ± 5.2	55.2 ± 4.1	62.8 ± 1.7*
Adrenals (mg)	66.3 ± 10.5	46.9 ± 2.7*	51.2 ± 5.4*	54.1 ± 3.2*	42.7 ± 3.2*
Kidneys (g)	1.94 ± 0.08	2.24 ± 0.06	2.08 ± 0.05	2.18 ± 0.12	2.19 ± 0.04
Liver (g)	10.6 ± 0.25	12.6 ± 0.3	12.2 ± 0.5	11.4 ± 0.5	11.4 ± 0.4
Body (g)	288.3 ± 16.0	304.5 ± 8.5	309.0 ± 14.0	303.8 ± 5.2	305.0 ± 6.8
Body weight gain (g)	49.5 ± 3.4	72.7 ± 5.3*	70.3 ± 1.9*	61.7 ± 1.9*	61.6 ± 2.4*
n	3	6	3	3	3

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 necropy at 66 days of age. Values are means ± SE.
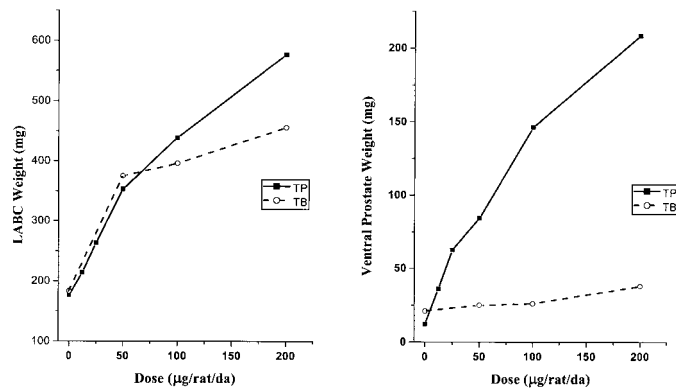
*Differs significantly from oil control values.

FIG. 4. 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.

effect of sc TP, while in contrast, po TB treatment retarded growth rather than stimulating it. 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
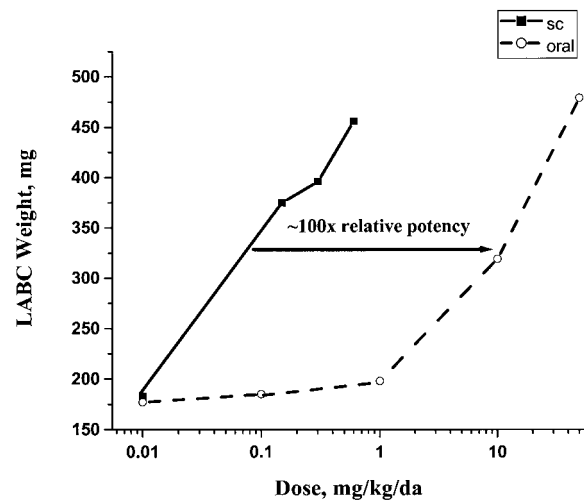

FIG. 5. Comparison of the relative potency of po vs. sc dosing of TB on LABC weights in the Hershberger assay. SC dosing with TB was approximately 100-fold more effective in increasing LABC weight than po TB. Relative potency estimate was based on the magnitude of the differences between the dose response curves where each route of exposure produced approximately equivalent effects.

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

TABLE 3
Comparison of Effects of Oral Dosing of the Anabolic Steroid Trenbolone (TB)

Weights	Oil	TB (mg/kg/day)			
		0.1	1.0	10	50
LABC (mg)	177.3 ± 15.4	185.3 ± 10.3	197.7 ± 15.7	318.8 ± 29.1*	478.8 ± 53.7*
Ventral prostate (mg)	17.5 ± 1.53	9.5 ± 3.7	12.9 ± 3.2	17.2 ± 1.0	57.5 ± 6.9*
SVCG (mg)	49.5 ± 5.5	46.7 ± 3.3	46.7 ± 2.8	74.1 ± 3.9*	133.5 ± 17.3*
Cowpers (mg)	9.01 ± 2.3	8.3 ± 2.4	6.9 ± 0.7	11.7 ± 1.1	17.7 ± 1.2*
Glans penis (mg)	46.13 ± 4.0	41.1 ± 3.1	43.4 ± 1.9	53.1 ± 4.5	62.7 ± 1.0*
Adrenals (mg)	54.3 ± 4.2	52.0 ± 6.1	43.7 ± 6.1	42.7 ± 3.4	44.0 ± 2.6
Kidneys (g)	2.0 ± 0.03	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.04	2.0 ± 0.2
Liver (g)	10.5 ± 0.16	11.1 ± 0.4	11.1 ± 0.3	11.0 ± 0.8	10.8 ± 0.7
Body (g)	302.7 ± 7.0	307.7 ± 4.4	299.5 ± 6.2	296.6 ± 7.2	277.7 ± 14.6
Body weight gain (g)	45.6 ± 2.6	49.5 ± 2.1	40.9 ± 0.9	33.3 ± 3.9	18.7 ± 9.2*
n	3	3	3	3	3

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.

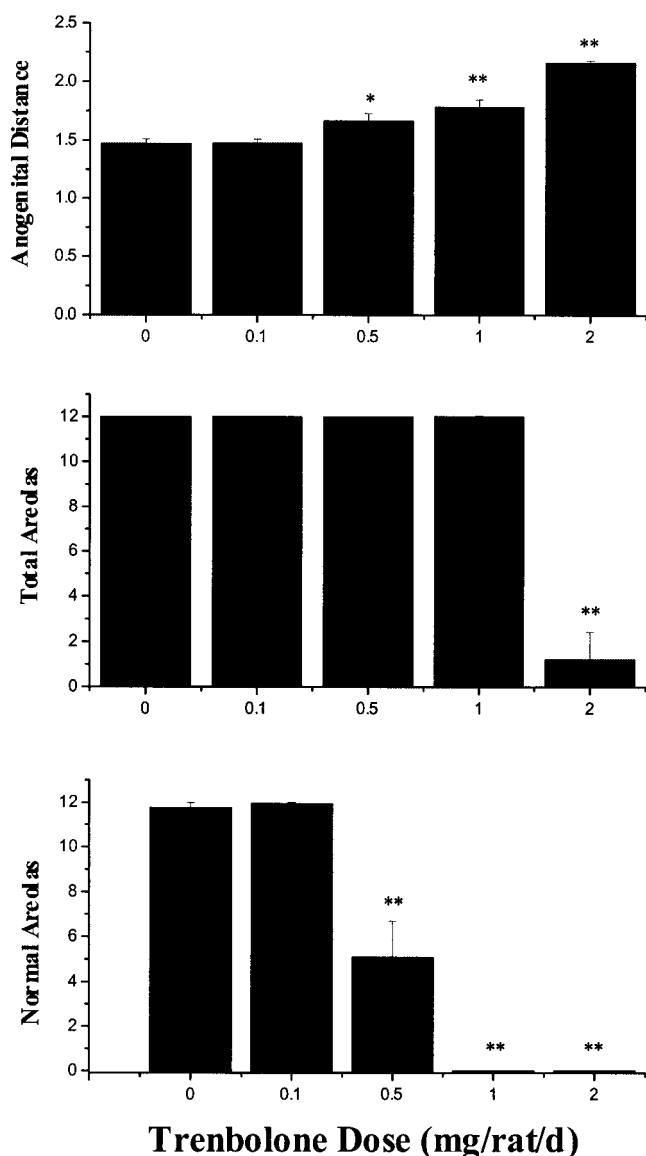*Differs significantly from oil control values ($p < 0.05$).

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. $*p < 0.05$; $**p < 0.001$.

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 mg/rat/day reduced the display of areolas and

nipples at PND 13 in female rat offspring (similar to the response demonstrated by Wolf *et al.,* 2002 for TP). Mean number of total nipples was reduced in the highest dose group only. The numbers of normal areolas/nipples, however, was significantly reduced at doses of 0.5 mg/rat/day and above based on litter mean values.

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 7 α-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 hydrometrocolpus 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, repro-

ductive 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 nonstandard 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 is 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 tranport of TB in such systems, it seems reasonable that an ecological risk assessment should be conducted.

REFERENCES

Bauer, E. R., Daxenberger, A., Petri, T., Sauerwein, H., and Meyer, H. H. (2000). Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor. *APMIS* **108,** 838–846.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Danhaive, P. A., and Rousseau, G. G. (1988). Evidence for sex-dependent anabolic response to androgenic steroids mediated by muscle glucocorticoid receptors in the rat. *J. Steroid Biochem.* **29,** 575–581.

Escuret, P., and Bas, M. N. (1978). DL_50 chez le rat et chez le souris. Unpublished report: Recherches Veterinaires R.U._essai 5.77.71 R. Submitted to WHO by Roussel Uclaf, Paris, France.

Gray, L. E., Jr., Lambright, C., Wilson, V., Ostby, J., Guillette, L., and Wilson, E. (2001). *In vivo* and *in vitro* androgenic effects of β trenbolone, a potential feedlot effluent contaminant. SETAC 22nd Annual Meeting, Baltimore, MD. Abstract, p. 334.

Gray, L. E., Jr., Wolf, C. J., Lambright, C., Mann, P., Cooper, R. L., and Ostby, J. (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p′-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169 and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health* **15,** 94–118.

Green, R. R., Burill, M. W., and Ivy, A. C. (1939). Experimental intersexuality. The effect of antenatal androgens on sexual development of female rats. *Am. J. Anat.* **65,** 415–469.

Hershberger, L., Shipley, E., and Meyer, R. (1953). Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. *Proc. Soc. Exp. Biol. Med.* **83,** 175–180.

Hess, D. L. (1983). Determination of a hormonal no-effect dose level for 17-β-trenbolone, Rhesus Macaque. Unpublished report from Oregon Regional Primate Research Center, Beaverton, OR. Submitted to WHO by Roussel Uclaf, Paris, France.

Hess, D. L. (1984). Determination of the hormonal no-effect dose level for trenbolone acetate in the female Rhesus Macaque. Unpublished report from Oregon Regional Primate Research Center, Beaverton, OR. Submitted to WHO by Roussel Uclaf, Paris, France.

Hunter, B., Batham, P., Heywood, R., Street, A. E., Gibson, W. A., Prentice, D., Gregson, R., and Offer, J. (1982). Trenbolone acetate potential tumorigenic and toxic effects in prolonged administration to rats following "*in utero*" exposure (final report). Unpublished report volumes I, II, III and IV, No. RSL 295_G/80974 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by Roussel Uclaf, Paris, France.

Hunter, B., Bathman, P., Heywood, R., Street, A. E., Prentice, D. E., and Majeed, S. K. (1976). Trenbolone acetate oral toxicity to rats by dietary administration for 13 weeks. Unpublished report No. RSL 237/76426 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by Roussel Uclaf, Paris, France.

Hunter, B., Graham, C., Gibson, W. A., Read, R., Gregson, R., Heywood, R., Offer, J., Prentice, D., and Woodhouse, R. (1981). Long-term feeding of trenbolone acetate to mice (final report). Unpublished report volumes I, II and III, No. RSL/267/80122 from Huntingdon Research Centre, Huntingdon, England. Submitted to WHO by Roussel Uclaf, Paris, France.

Jegou, B., Sots, A., Sundlof, S., Stephany, R., Meyer, H., and Leffers, H. (2001). Existing guidelines for the use of meat hormones and other food additives in Europe and USA. *APMIS* **103**(Suppl.), S551–S556.

Kruskemper, H. L., Morgner, K. D., and Noell, G. (1967). Klinische pharmakologie von Trienolonen, eine Gruppe neuartiger anabol wirksamer Oestran-Derivate. [Clinical pharmacology of trienolones, a group of new anabolically actine aestrane-derivatives (4,9,11-oestratrien-17beta-ol-3-on)]. *Arzneimittelforschung* **17,** 449–454.

Lambright, C., Ostby, J., Bobseine, K., Wilson, V., Hotchkiss, A. K., Mann, P. C., and Gray, L. E., Jr. (2000). Cellular and molecular mechanisms of action of linuron: An antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol. Sci.* **65,** 389–399.

Lopez-Bote, C., Sancho, G., Martinez, M., Ventanas, J., Gazquez, A., and Roncero, V. (1994). Trenbolone acetate induced changes in the genital tract of male pigs. *Zentralbl. Veterinarmed. [B]* **41,** 42–48.

McIntyre, B. S., Barlow, N. J., and Foster, P. M. (2001). Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: Permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol. Sci.* **62,** 236–249.

McIntyre, B. S., Barlow, N. J., and Foster, P. M. (2002). Male rats exposed to linuron *in utero* exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicol. Sci.* **65,** 62–70.

Meyer, H. H. D., and Rapp, M. (1985). Reversible binding of the anabolic steroid trenbolone to steroid receptors. *Acta Endocinol.* **267**(Suppl.), 129.

Monosson, E., Kelce, W. R., Lambright, C., Ostby, J., and Gray, L. E., Jr. (1999). Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. *Toxicol. Ind. Health* **15,** 65–79.

Parks, L. G., Lambright, C. S., Orlando, E. F., Guillette, L. J., Jr., Ankley, G. T., and Gray, L. E., Jr. (2001). Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway river water as associated with androgen receptor agonist activity. *Toxicol. Sci.* **62,** 257–267.

Pottier, J., Cousty, C., Heitzman, R. J., and Reynolds, I. P. (1981). Differences in the biotransformation of a 17β-hydroxylated steroid, trenbolone acetate, in rat and cow. *Xenobiotica* **11,** 489–500.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., and Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **8,** 275–285.

Schiffer, B., Daxenberger, A., Meyer, K., and Meyer, H. H. (2001). The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: Environmental studies. *Environ. Health Perspect.* **109,** 1145–1151.

Schroder, R. (1971a). Prüfung der androgenen und muskelbildenden Wirkung bei oraler Verabreichung. R 1967. Unpublished report from Hoechst A.G. Submitted to WHO by Roussel Uclaf, Paris, France.

Schroder, R. (1971b). Untersuchung auf androgene und anabole Wirkung, Untersuchung auf oestrogene Wirkung. R 1697. Unpublished report from Hoechst A.G. Submitted to WHO by Roussel Uclaf, Paris, France.

Sillence, M. N., and Rodway, R. G. (1990). Effects of trenbolone acetate and testosterone on growth and on plasma concentrations of corticosterone and ACTH in rats. *J. Endocrinol.* **126,** 461–466.

Sundaram, K., Kumar, N., Monder, C., and Bardin, C. W. (1995). Different patterns of metabolism determine the relative anabolic activity of 19-norandrogens. *J. Steroid Biochem. Mol. Biol.* **53,** 253–257.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D., and Servos, M. (1999). Behavior and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ.* **225,** 81–90.

Thomas, K. M., and Rodway, R. G. (1983). Effects of trenbolone acetate on adrenal function and hepatic enzyme activities in female rats. *J. Endocrinol.* **98,** 121–127.

Toth, M., and Zakar, T. (1986). Classification of anabolic steroids using the method of competitive metabolism. *Exp. Clin. Endocrinol.* **87,** 125–132.

Wilson, V. S., Bobseine, K., Lambright, C. R., and Gray, L. E., Jr. (2002). A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicol. Sci.* **66,** 69–81.

Wolf, C. J., Hotchkiss, A., Ostby, J. S., LeBlanc, G. A., and Gray, L. E., Jr. (2002). Effects of prenatal testosterone propionate on the sexual development of male and female rats: A dose-response study. *Toxicol. Sci.* **65,** 71–86.

Wong, C., Kelce, W. R., Sar, M., and Wilson, E. M. (1995). Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J. Biol. Chem.* **270,** 19998–20003.