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Evaluation of the Antiandrogenic Effects of Flutamide, DDE, and Linuron in the Weanling Rat Assay Using Organ Weight, Histopathological, and Proteomic Approaches

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The Organization for Economic Cooperation and Development (OECD) is currently funding the validation of the Hershberger assay as a rapid *in vivo* means of identifying (anti-) androgens. However, as the assay measures weight changes in the androgen-sensitive tissues of castrated rats, the evaluation of the androgen-stimulated intact weanling as a more ethical model to use in the assay has been requested. As part of the OECD validation exercise two weak antiandrogens, 1,1-dichloro-2,2-bis(4 chlorophenyl)-ethane (DDE) and linuron (LIN), were investigated in our laboratory at several dose levels in the testosterone propionate (TP)–stimulated weanling using flutamide (FM) as a positive control. In addition to weight measurements (sex accessory tissues [SATs], epididymides, and testes), histopathological assessment of the seminal vesicles, prostate, and testes was conducted for vehicle control, TP-stimulated, and TP-stimulated animals treated with FM or the top dose level of DDE or LIN. The modulation of a novel prostate protein associated with apoptosis, L-amino acid oxidase (LAO), was evaluated in these same treatment groups. Our gravimetric data (supported by the histopathology data) indicated that the weanling assay can detect SAT and epididymal weight changes induced by the antiandrogens evaluated. Inconsistent and variable data were recorded for the testicular weight and histopathological effects, suggesting that the testis is of little value in the identification of antiandrogens using this model. Three isoforms of LAO were identified, and all were regulated by TP. Modulation of LAO by the antiandrogens indicated that this protein could be a biomarker for endocrine disruption in male rodents.

Key Words: **antiandrogens; stimulated weanling; Hershberger; L-amino acid oxidase.**

The recognition that certain environmental chemicals can modulate the endocrine system leading to adverse effects on the normal sexual development and reproductive functioning in animals has resulted in several initiatives designed to improve our ability to detect such endocrine disrupting chemicals (EDCs). A key requirement for the inclusion of an assay into any testing strategy is that the candidate assay be extensively validated to ensure its reproducibility, reliability, and relevance. To this end, the Organization for Economic Cooperation and Development (OECD) Endocrine Disruptors Testing and Assessment (EDTA) Validation Management Group has been responsible for managing the validation programs of such *in vivo* rodent assays as the enhancement of the test guideline 407 (OECD, 2000), the uterotrophic assay (Kanno *et al*., 2001, 2003) and, more recently, the Hershberger assay (Owens *et al*., 2006, 2007). Although the validation of TG407 and the uterotrophic assay are nearing completion, additional studies, such as those described herein have been requested to complete the process for the Hershberger assay.

The Hershberger assay is a relatively rapid *in vivo* means of assessing the (anti-) androgenic potential of a chemical by measuring changes in the weights of the androgen-responsive sex accessory tissues (SATs, i.e., Cowper's glands, levator ani/bulbocavernosus muscle [LA/BC], seminal vesicles [SVs; with coagulating gland and dorsolateral prostate {DPs}], and ventral prostate {VP}). Unfortunately, as surgically castrated adult rats are required for the assay, the ethics of such a model have been questioned. Consequently, and, in line with the uterotrophic assay evaluations, in which both intact immature and ovariectomized mature female rats were investigated, the OECD EDTA Validation Management Group has requested the evaluation of a stimulated intact weanling male rat as an alternative to the castrated model in the Hershberger assay. While the use of an intact immature male assay to identify androgens is not novel (Greene and Burrill, 1940, 1941), the use of such a model to detect antiandrogens was only recently explored (Ashby and Lefevre, 1997, 2000, Ashby *et al*., 2002, 2004; Kelce *et al*., 1995, Monosson *et al*., 1999; Stoker *et al*., 2000; Marty *et al*., 2001).

To evaluate this model using noncastrated immature animals, several laboratories have investigated coded compounds, which were either negative controls or antiandrogens, using the testosterone propionate (TP)–stimulated weanling rat. However, before undertaking such a study, we elected to validate the weanling model in our laboratory by assessing

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the activity of the weak antiandrogens 1,1-dichloro-2,2-bis(4 chlorophenyl)ethane (DDE) and linuron (LIN). An independent dose-response study was conducted for each compound, and flutamide (FM) was included as a positive control in each study. The study design and dose levels used were chosen to allow us to compare our data with published data for both the classic Hershberger assay (Owens *et al*., 2006, 2007; Kang *et al*., 2004) and the TP-stimulated weanling rat (Ashby *et al*., 2004).

We dosed TP-stimulated weanlings with the appropriate antiandrogen for 10 days and evaluated the weight changes induced in the SATs, epididymides, and testes 24 h after the last dose. In addition to the standard gravimetric measurements, additional evaluations were conducted for certain groups of animals (controls, TP-stimulated animals, and TP-stimulated animals exposed to the top dose of DDE and LIN as well as the TP-stimulated FM-treated animals). Specifically, histopathological assessment of the testes, SVs, and DP and proteomic profiling of the VP were performed. For the purpose of this paper, our proteomic evaluations focused exclusively on one particular protein, L-amino acid oxidase (LAO). This protein was originally identified in snake venom (Du and Clemetson, 2002) and was recently observed in the adult rat prostate (Cayatte *et al*., 2006) following treatment with the antiandrogens FM and finasteride but was absent in control prostates. Thus, as this novel rat protein appears to be sensitive to antiandrogens, it may prove useful as a biomarker for endocrine disruption and so was considered an ideal candidate to investigate in our studies with the weanling rat.

MATERIALS AND METHODS

Chemicals

TP ($\geq$ 98% purity), LIN (99% purity), FM (> 99% purity), and DDE (99% purity) were purchased from the Sigma-Aldrich (Saint Quentin Fallavier, France). All four compounds were suspended in tocopherol-stripped corn oil (TSCO), which was purchased from MP Biomedicals (Strasbourg, France). The suppliers of all additional compounds are mentioned in the text.

Animals and Housing

All investigations were conducted in accordance with French laws for animal experimentation. Immature male Sprague-Dawley Crl:CD rats were purchased from Charles River (St Germain-sur-l'Arbresle, France). They were 20 days old on arrival and were allowed three days to acclimatize. All animals were group housed (six per cage) in polycarbonate cages with wood chips and were allowed A04C-10 pelleted diet (Scientific Animal Food and Engineering, Augy, France) and water *ad libitum* for the duration of the experiment.

Dose Level Selection

The dose level of TP (1 mg/kg/day by sc injection) was as recommended by the OECD EDTA Validation Management Group (Owens, personal communication). The dose level of FM (3 mg/kg/day; oral) and the doses of DDE (5, 16, 50, and 160 mg/kg/day; oral) and of LIN (3, 10, 30, 100 mg/kg/day; oral) were originally recommended by the OECD for validation of the classic Hershberger assay and were also the same dose levels as those used by Ashby *et al*. (2004) in the initial investigations of the TP-stimulated weanling assay. A dose volume of 2 ml/kg body weight was used for all sc injections and a volume of 5 ml/kg body weight was used for all oral doses.

Study Design

Two independent dose-response studies were conducted, one of which investigated the activity of DDE (experiment 1) in the stimulated weanling rat and the other investigated the activity of LIN (experiment 2). Both assays were conducted as previously described (Ashby *et al*., 2004). Briefly, 23-day-old rats were exposed to a single sc injection of either vehicle (TSCO) or TP as well as a single oral gavage of TSCO, DDE, or LIN at the appropriate dose level or FM on each of 10 consecutive days. The animals were killed 24 h after the last dose by an overdose of isoflurane (T.E.M., Lormont, France), followed by exsanguination. The liver, kidneys, adrenals, testes, epididymides, and SATs were removed, trimmed free of fat and connective tissue, and weighed.

Histopathological Analyses

Histopathological assessment of the testes, SVs, and DP of vehicle control animals, TP-stimulated animals, and TP-stimulated animals coexposed to 160 mg/kg/day DDE, 100 mg/kg/day LIN, or 3 mg/kg/day FM was conducted. The SVs and DP were fixed in 10% neutral buffered formalin. In experiment 1, the testes were fixed in modified Davidson's buffer; however, to improve the fixation, the testes in the second study were fixed in Davidson's buffer. The organs were embedded in paraffin wax, and 4-mm histological sections were prepared and stained with hematoxylin (Sigma-Aldrich) and eosin (Merck, Fontenay-sous-Bois, France).

The SVs and the DP were evaluated for glandular growth (glandular size, presence and size of a lumen, presence of intraluminal secretions, and epithelial maturation) with the maturation process being graded from 1 (minimal amount of glandular parenchyma, with no or minimal lumen and intraluminal secretions) to 4 (nearly mature parenchyma, with well-developed glands filled with secretions) for each animal.

The testes were evaluated for impaired tubular growth and apoptosis. Impaired tubular growth was defined as a reduced tubular diameter (based on the number of tubules observed in the diameter of a field of vision at $\times$20 magnification) and cellular depletion of the germ cells within the tubules. A grading from 0 (considered to be normal morphology with four to five tubules per diameter and the presence of normal tubular germ cells) to 4 (up to 15 tubules per diameter coupled with a high degree of germ cell depletion including the presence of some tubules containing only Sertoli cells) was used to evaluate the degree of tubular impairment. In addition, a semi-quantitative evaluation of the number of apoptotic cells, as defined as those germ cells with shrunken eosinophilic cytoplasm and pyknotic or absent nuclei, was performed. A grading from 0 (considered to be normal with scattered apoptotic cells in few tubules) to 2 (up to 20 apoptotic cells per tubule) was used.

For each tissue/histopathological finding evaluated, a mean severity grade was determined for each treatment group based on the individual animal gradings.

Proteomic Analyses

Prostate tissue protein extraction. The isolation of VP proteins, the conduct of two-dimensional electrophoresis (2-DE), and the subsequent analysis of the protein profiles were conducted based on the procedure described by cayatte *et al*. (2006). Briefly, the VP from each animal from the same groups as the groups used for histological analyses was homogenized in lysis buffer (7M urea, 2M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [wt/vol], 0.24% Triton X-100 [vol/vol]) supplemented by 20mM spermine (Sigma-Aldrich) using a Dounce homogenizer followed by a Tenbroeck homogenizer. After centrifugation (18,000 $\times$ g for 45 min at 20°C), the protein concentration of each resulting supernatant was determined using the modified Bradford protein assay (Ramagli and Rodriguez, 1985) using bovine serum albumin in lysis buffer to generate the standard curve.

As there were no differences in terms of quantity and quality (sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE] profile) between the isolates within a group, the individual prostate protein samples within a group were pooled. All protein samples were kept frozen at $-$ 80°C until used.

Two-dimensional gel electrophoresis. As the 2-DE analysis was performed for both studies at the same time, one gel per study was prepared for

the vehicle control, TP only, and TP/FM groups. Two gels per treatment group were prepared for the TP/DDE and TP/LIN top dose groups. Thus, a total of 10 gels, two per treatment group were prepared and analyzed.

Precast immobilized pH gradient (IPG) strips (pH range 3–10; linear gradient, 17 cm, Bio-Rad, Marnes-la-Coquette, France) were rehydrated with 330-µl lysis buffer supplemented with 100mM 2-hydroxyethyl disulfide (2-HED, Acros Organics, Geel, Belgium) (Olsson *et al*, 2002) and 0.001% bromophenol blue as a tracking dye and incubated for 9 h at 20°C (passive rehydration). Using a cup-loading system (Bio-Rad), 600 µg of each pooled protein extract was supplemented with 100mM 2-HED and loaded onto IPG strips. The isoelectric focusing (IEF) step was carried out using a Protean IEF Cell (Bio-Rad) at 20°C under low viscosity oil, and the proteins were focused successively for 15 min at 250 V, followed by a slow (15 h) voltage ramping to 10,000 V, and focusing was continued at 10,000 V up to 300,000 Vh. Prior to SDS–PAGE, the IPG strips were incubated at room temperature for 30 min in an equilibration buffer (0.375M Tris–HCl [pH 8.8], 6M urea, 20% glycerol, 4% SDS; Sigma-Aldrich) containing 65mM dithiothreitol (DTT) (Sigma-Aldrich), to reduce the proteins. A second equilibration step was carried out for 40 min under the same conditions, except that DTT was replaced by 135mM iodoacetamide (Sigma-Aldrich), and 0.001% bromophenol blue was added as a tracking dye. The equilibrated strips were then loaded onto 10% SDS–PAGE, with 4% SDS–PAGE on top as a stacking gel. IPG strips were sealed with 1% low melting point agarose to ensure good contact between the IPG strips and the gel. The second dimension of 2-DE was carried out using the Ettan Daltsix Electrophoresis Unit (Amersham, Orsay, France) connected to a PowerPac 1000 Power Supply (Bio-Rad). The electrophoresis was performed at 10°C with Tris–glycine buffer (25mM trizma base, 192mM glycine, 0.1% SDS) at a constant power of 5 watt per gel for 30 min followed by 12.5 watt per gel until the tracking dye reached the bottom of the gel. After separation, proteins were visualized by staining the gels with colloidal Coomassie blue stain as recommended by the manufacturer (SafeStain; Invitrogen, Cergy Pontoise, France), and gels were digitalized using the GS-800 Calibrated Densitometer (Bio-Rad), high-resolution scanner.

The protein patterns of each gel were compared using a PD-Quest imaging system (version 7.1, Bio-Rad), and expression levels of the proteins were quantified by analyzing the intensity of each spot. For the purpose of this study, spots considered likely to be LAO, based on their position on the gels as well as their shape, were selected for identification by mass spectrometry.

Protein identification by mass spectrometry. The protein spots of interest were manually excised from the 2-DE gels, processed by enzymatic digestion with trypsin to generate peptide fragments, and analyzed by nano electrospray lonization-tandem mass spectrometry using LCQ DECA XO-PRO mass spectrometers (ThermoQuest, San Jose, CA) (Friry-Santini *et al*., 2007).

Western blot analysis of LAO. Rat VP proteins, separated by 2-DE, were transferred in Tris–ethanol buffer (25mM trizma base, 192mM glycine, 0.1% SDS, 20% ethanol) at 30 V during 15 h at 4°C onto hydrophobic polyvinylidene fluoride membrane Hybond-P (Amersham). After blotting, the membranes were incubated for 7 h at room temperature with blocking buffer Tris-buffered saline (TBS; 2mM Tris–HCl, 50mM NaCl, pH 7.6) containing 5% (wt/vol) nonfat dry milk. They were then incubated overnight at 4°C under slight agitation with anti-LAO antibodies (2 µg/ml; Nordic Immunological Laboratories, Tilburg, The Netherlands) in TBS containing 0.5% (wt/vol) nonfat dry milk. After three washes for 5 min with TBS/0.05% Tween (Sigma-Aldrich), the membranes were incubated for 1 h at room temperature with TBS/0.5% (wt/vol) nonfat dry milk buffer containing a 1:10,000 dilution of horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin secondary polyclonal antibody (Jackson ImmunoResearch Laboratories, Newmarket, Suffolk, UK). They were then washed three times for 5 min in TBS/0.05% Tween and once for 5 min in TBS. Finally, they were incubated for 5 min with ECL plus (Amersham). Detection was performed with autoradiography film (Hyperfilm ECL; Amersham).

Statistical Analyses

The mean and standard deviation for body and tissue weights were calculated for each group. Comparisons between the vehicle control data and the TP-only exposed data were first conducted, according to the following procedures, to confirm the androgenic activity of TP. Comparisons of variances between the vehicle control group and the TP group were performed using the F-test of homogeneity of variances. When the F-test was not significant, means were compared using the *t*-test (two sided). When the F-test was significant, data were transformed using a log transformation, and the *t*-test was applied on transformed data. If the F-test remained significant even after transformation, means were compared using the Mann-Whitney test (two sided). Comparisons between the TP-only data and the data generated due to antiandrogenic/TP treatment were then conducted for each treatment group, according to the following procedures, to determine the antiandrogenic activity of FM and LIN or DDE at the appropriate dose levels. Group variances were intercompared by the use of the Bartlett test for homogeneity of variances. If the Bartlett test indicated homogeneous variances, the antiandrogen/TP group means were compared to the TP mean using a combination of the ANOVA and Dunnett test (two sided). If the Bartlett test indicated heterogeneous variances, the data were transformed using a log transformation to stabilize the variances. If the Bartlett test on transformed data was not significant, the ANOVA and Dunnett test were performed on transformed data. If the Bartlett test indicated heterogeneous variances (even after data transformation), nonparametric approaches were performed.

RESULTS

Body and Tissue Weights

The gravimetric effects of TP treatment on the weanling rats as well as those of DDE, LIN, and FM on the TP-stimulated weanling rat are given in Table 1 (experiments 1 and 2). The effects of TP treatment and TP plus DDE, LIN, or FM on the weights of the prostate, SV, and epididymides are also given in Figure 1.

Body weights. With the exception of 100 mg/kg/day LIN-exposed animals, neither TP nor TP in combination with FM, DDE, or LIN had any effect on terminal body weight. Cotreatment with TP and the top dose level of LIN significantly reduced ($p \leq 0.01$) the terminal body weight of the exposed animals.

Liver, kidney, and adrenal weights. When the TP-exposed animals were compared to the vehicle control animals, statistically significant increases of liver and kidney weight were observed, but the results were not consistent between the two studies. Animals coexposed to TP and DDE showed a statistically significant increased liver weight at 50 and 160 mg/kg/day ($p \leq 0.05$ and $p \leq 0.01$, respectively) when compared to the TP-exposed animals. A significant ($p \leq 0.01$) reduction in kidney weight was recorded for animals coexposed to TP and 100 mg/kg/day LIN, but this decrease could be related to the reduction of terminal body weight observed for these animals. There were no effects on adrenal weight in any of the test groups.

SAT weights. Stimulation of the weanling rat by 1 mg/kg/day TP induced a significant ($p \leq 0.01$) increase in the weights of all SATs compared to the vehicle controls in both studies.

The positive control antiandrogen, 3 mg/kg/day FM, successfully inhibited the stimulatory effect of TP in both

TABLE 1
Analysis of the Weight Changes Induced by DDE and LIN in the TP-Stimulated Weanling Rat Using FM as a Positive Control

		Body weight (g) ± SD		Tissue weights ± SD								
Experiment no	Treatment (mg/kg/day)	Initial	Terminal	Liver (g)	Kidneys (mg)	Adrenals (mg)	Testes (mg)	Epididymides (mg)	LA/BC (mg)	Cowper's (mg)	SV (mg)	Prostate (mg)
1	TSCO[a]	52.7 ± 6.6	104.1 ± 14.2	4.6 ± 0.9	1024.1 ± 137.3	24.3 ± 3.8	751.0 ± 143.8	108.2 ± 20.5	72.9 ± 19.4	5.4 ± 3.2	70.6 ± 17.6	50.9 ± 12.6
	TP (1)	54.4 ± 6.4	113.6 ± 11.7	5.2 ± 0.6	1114.1 ± 136.3	24.8 ± 2.6	365.4 ± 61.0††	153.2 ± 15.0††	209.0 ± 38.4 ††	23.2 ± 2.4 ††	291.7 ± 37.6 ††	134.4 ± 16.4††
	FM (3)	50.1 ± 5.4	110.6 ± 15.4	5.1 ± 1.3	1119.4 ± 187.2	22.2 ± 2.2	563.6 ± 177.2	81.2 ± 25.8**	131.6 ± 33.6 **	11.5 ± 3.9**	120.6 ± 26.2 **	63.4 ± 14.3 **
	DDE (5)	49.0 ± 6.6	109.7 ± 12.1	5.3 ± 0.7	1065.2 ± 116.5	22.7 ± 2.6	357.4 ± 130.4	149.0 ± 18.4	195.5 ± 33.7	21.3 ± 5.0	263.5 ± 51.6	111.8 ± 28.1
	DDE (16)	50.5 ± 5.8	109.8 ± 7.5	5.7 ± 0.5	1109.6 ± 71.9	23.3 ± 2.9	330.3 ± 59.7	141.4 ± 8.7	198.0 ± 31.3	21.6 ± 5.4	268.7 ± 58.6	107.0 ± 14.8
	DDE (50)	50.1 ± 6.7	107.7 ± 7.7	6.3 ± 0.6*	1128.3 ± 104	26.3 ± 2.1	280.3 ± 41.5	121.3 ± 8.2**	157.4 ± 22.5 *	17.4 ± 1.0	215.2 ± 26.0 *	97.2 ± 15.2 **
	DDE (160)	49.7 ± 6.6	105.8 ± 10.9	7.9 ± 1.2**	1164.6 ± 93.5	28.6 ± 6.3	407.1 ± 127.0	95.9 ± 18.1**	116.9 ± 16.9 **	10.3 ± 2.3 *	136.1 ± 29.6 **	78.7 ± 19.2 **
2	TSCO[a]	47.5 ± 5.8	96.2 ± 11.5	4.4 ± 0.8	971.7 ± 90.2	21.5 ± 3.7	665.2 ± 111.7	95.4 ± 15.2	51.5 ± 13.3	4.1 ± 0.9	61.6 ± 7.9	42.0 ± 11.9
	TP (1)[b]	47.8 ± 6.4	110.6 ± 10.6	5.3 ± 0.4 †	1133.0 ± 92.4*	22.3 ± 1.4	320.3 ± 57.6††	130.6 ± 12.0††	156.9 ± 27.7††	20.0 ± 1.1††	261.5 ± 19.9††	102.6 ± 18.1††
	FM (3)	46.8 ± 4.6	104.1 ± 17.2	4.7 ± 0.9	1018.6 ± 128.4	22.3 ± 4.4	406.4 ± 233.1	78.3 ± 15.1**	107.4 ± 14.9 **	7.7 ± 0.6**	87.2 ± 12.1**	60.1 ± 14.7**
	LIN (3)	46.0 ± 5.5	105.4 ± 9.8	4.8 ± 0.6	1042.0 ± 88.5	25.2 ± 4.6	359.3 ± 73.9	128.2 ± 13.5	177.4 ± 29.0	21.4 ± 5.2	285.3 ± 56.6	111.2 ± 19.3
	LIN (10)[b]	48.2 ± 5.7	108.9 ± 10.5	5.0 ± 0.8	1067.5 ± 47.5	23.2 ± 4.5	382.5 ± 89.3	133.9 ± 8.7	176.4 ± 41.8	21.8 ± 3.5	240.4 ± 42.7	102.6 ± 21.3
	LIN (30)	47.2 ± 5.3	101.8 ± 12.4	4.9 ±0.8	1055.3 ± 86.3	21.7 ± 3.4	311.9 ± 121.6	114.5 ± 19.0	151.5 ± 40.7	17.9 ± 1.8	239.8 ± 57.3	91.3 ± 16.4
	LIN (100)	47.3 ± 5.4	88.0 ± 10.5**	4.3 ± 0.7	883.9 ± 109.1**	21.4 ± 4.6	225.1 ± 64.4	88.1 ± 16.3**	125.4 ± 28.8	12.2 ± 1.3*	155.2 ± 14.3**	69.3 ± 7.5*

Note. Animals were 23 days old at the start of treatment. They were dosed on 10 consecutive days with an oral dose of the appropriate test compound at the appropriate dose level and were stimulated by an sc injection of 1 mg/kg/day TP. Data are presented as mean absolute tissue weights ± SD based on six animals per group. Comparisons were performed first between the vehicle control and TP-only groups for statistical significance (†$p \leq 0.05$; ††$p \leq 0.01$) to ensure that TP induced the appropriate androgenic effects. A second comparison was then performed between TP and TP plus DDE, LIN, or FM at the various doses (*$p \leq 0.05$; **$p \leq 0.01$) to evaluate the antiandrogenic efficacy of the three coadministered compounds.

[a]Animals in these groups received TSCO by both sc injection and oral gavage and acted as vehicle controls.

[b]There were five animals per group in these two groups.

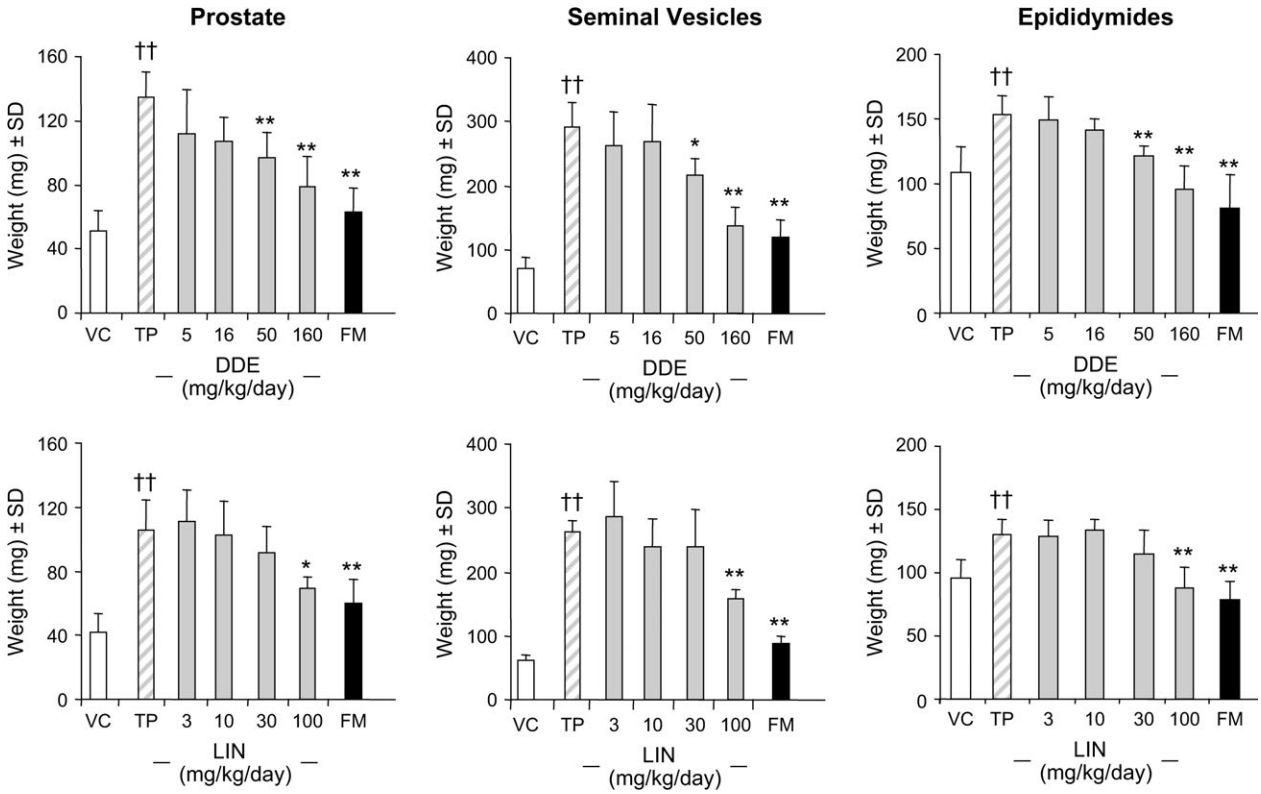

FIG. 1. Effects of TP (1 mg/kg/day) and TP plus DDE, LIN, or FM (3 mg/kg/day) on the weights of the prostate, SVs, and epididymides of the weanling rat. VC, vehicle (TSCO) control; ††$p \leq 0.01$ due to TP-only treatment; *$p \leq 0.05$; **$p \leq 0.01$ due to treatment with TP plus antiandrogen.

studies, leading to a significant reduction in the weights of all SATs compared to the corresponding tissues from TP-only–treated animals.

DDE induced a dose-related decrease in the weights of all SATs in the TP-stimulated weanling. All SAT weight reductions were statistically significant following cotreatment with TP and the top dose of 160 mg/kg/day DDE. The SAT weight reductions recorded at this dose of DDE were similar in magnitude to those recorded for FM. Statistically significant weight reductions were also observed at the 50 mg/kg/day DDE dose level for the LA/BC, SV, and prostate tissues.

The antiandrogenic effects of LIN were not as marked as those induced by either DDE or FM. Compared to the weights of TP-stimulated tissues, LIN induced a statistically significant decrease in the weights of the prostate, SV, and Cowper's glands at the top dose level (100 mg/kg/day) only. A marginal, though nonsignificant, reduction in the weights of these tissues was also apparent at 30 mg/kg/day and, in the case of the SVs, at 10mg/kg/day too. Although a reduction in the LA/BC weight was recorded at 100mg/kg/day, it was not statistically significant.

Epididymides and testicular weights. Effects on epididymal weights were similar to those observed for the SATs. Thus, TP induced a significant increase in epididymal weight, whereas FM, DDE, and LIN reduced the weight of this tissue in the stimulated weanling. This tissue appeared to be highly sensitive to the effects of the antiandrogens as treatment with FM and either DDE or LIN at the top dose level led to epididymal weights, which were lower than that recorded for the vehicle control animals.

The effects on testicular weight were inconsistent, and a high degree of interanimal variability was recorded. The mean testicular weight was significantly reduced ($p \leq 0.01$) due to TP treatment; however, the corresponding increase in weight following treatment of the stimulated weanlings with FM was not statistically significant. Similarly, a nonsignificant increase in testicular weight was induced by 160 mg/kg/day DDE. In contrast to the FM and DDE data, LIN at 30 mg/kg/day and 100 mg/kg/day induced a reduction (nonsignificant) rather than an increase in testicular weight compared to the TP-stimulated tissues.

Histopathology

The data generated from the histopathological assessment of the SVs, DP, and testes of vehicle control animals, TP-stimulated animals, and TP-stimulated animals coexposed to 160 mg/kg/day DDE, 100mg/kg/day LIN, or 3mg/kg/day FM are presented in Table 2. Representative photomicrographs of the SVs, DP, and testes for each treatment group are given in Figures 2A–C. For convenience, the data generated from each

TABLE 2

Analysis of the histopathological changes induced in the SVs, prostate, and testes of vehicle control animals, TP-stimulated animals, and TP-stimulated animals treated with 3 mg/kg/day FM, 160 mg/kg/day DDE, or 100 mg/kg/day LIN

			1 mg/kg/day TP +		
	Vehicle control	1 mg/kg/day TP	3 mg/kg/day FM	160 mg/kg/day DDE	100 mg/kg/day LIN
SVs	Induced glandular growth				
Mean severity grade	1.1	3.1	1.25	1.5	2.5
No. animals affected/no. evaluated	12/12	11/11	12/12	6/6	6/6
Prostate	Induced glandular growth				
Mean severity grade	1.2	2.4	1.3	1.2	1.7
No. animals affected/no. evaluated	12/12	11/11	12/12	6/6	6/6
Testes	Impaired tubular growth				
Mean severity grade	0.08	1.4	1.2	2.0	2.5
No. animals affected/no. evaluated	1/12	10/11	8/12	5/6	6/6
	Increased apoptotic germ cells				
Mean severity grade	0.25	0.7	0.75	0.5	1.3
No. animals affected/no. evaluated	3/12	7/11	6/12	2/6	6/6

Note. The data for the vehicle control, TP-stimulated, and TP + FM animals have been pooled from both experiments. The mean severity grade was calculated for each finding based on the gradings for individual animals within a group. Specifically, glandular growth (i.e., glandular size, presence and size of a lumen, presence of intraluminal secretions, and epithelial maturation) was graded from 1 (minimal amount of glandular parenchyma, with no or minimal lumen and intraluminal secretions) to 4 (nearly mature parenchyma, with well-developed glands filled with secretions). Impaired tubular growth (i.e., reduced tubular diameter, based on the number of tubules observed in the diameter of a field of vision at ×20 magnification, and cellular depletion of the germ cells within the tubules) was graded from 0 (considered to be normal morphology with four to five tubules per diameter and the presence of normal tubular germ cells) to 4 (up to 15 tubules per diameter coupled with a high degree of germ cell depletion including the presence of some tubules containing only Sertoli cells). A semi-quantitative evaluation of the number of apoptotic cells (i.e., germ cells with shrunken eosinophilic cytoplasm and pyknotic or absent nuclei) used a grading from 0 (normal with scattered apoptotic cells in few tubules) to 2 (up to 20 apoptotic cells per tubule).

individual experiment were combined for the vehicle controls, TP-stimulated, and TP/FM groups.

The SVs and DP. The morphologies of the SVs (Fig. 2A) and the DP (Fig. 2B) of the vehicle control animals were considered to be normal for this age (33 days) of animal. Specifically, the mean severity grades for these tissues were 1.1 and 1.2, respectively (Table 2), reflecting the minimal glandular growth recorded in the majority of control SVs and DP. In contrast, the SVs and DP of TP-exposed animals were of a more mature morphology with enhanced glandular development resulting in mean severity grades for these tissues of 3.1 and 2.4, respectively.

As expected, based on the gravimetric findings, the positive control, FM, inhibited the maturation of the TP-stimulated SVs and DP. Thus, both tissues were poorly developed and of similar morphology to the control tissues (Figs. 2A and 2B). Consequently, the mean severity grades for the SVs (1.25) and DP (1.3) were similar to those calculated for the control tissues (Table 2).

Treatment of stimulated weanlings with 160 mg/kg/day DDE resulted in SVs (Fig. 2A) and DPs (Fig. 2B), which were of similar morphology to those observed for the vehicle control and FM-treated groups, i.e., poorly developed with only minimal to slight glandular growth. The mean severity grades calculated for the DDE/TP group (1.5 for SV and 1.2 for DP) were, therefore, similar to those calculated for the control and FM groups (Table 2). The histopathological findings were, therefore, in accordance with the weight changes observed for these two tissues and reinforced the similarity between DDE and FM in their ability to inhibit the stimulatory effects of TP.

In contrast, treatment of the TP-stimulated weanling rat with 100 mg/kg/day LIN had only a slight impact on the morphology of the SVs and DP compared to the morphology of the TP-only–treated tissues (Figs. 2A and 2B). Thus, while the severity grading for the SVs was 3.1 for the TP-stimulated group, it was only marginally reduced to 2.5 for the TP group treated with LIN (Table 2). Similarly, while TP gave rise to a mean severity grading of 2.4 for the prostate, the grading for LIN was 1.7 (Table 2).

Testes. The vehicle control animals exhibited a testicular morphology, which was considered to be normal for immature animals. Specifically, meiotic divisions were observed within the seminiferous tubules, with the most differentiated germ cells present being the round spermatids. Sertoli cells, spermatogonia, and spermatocytes were also observed within the seminiferous tubules, and only a few apoptotic cells were

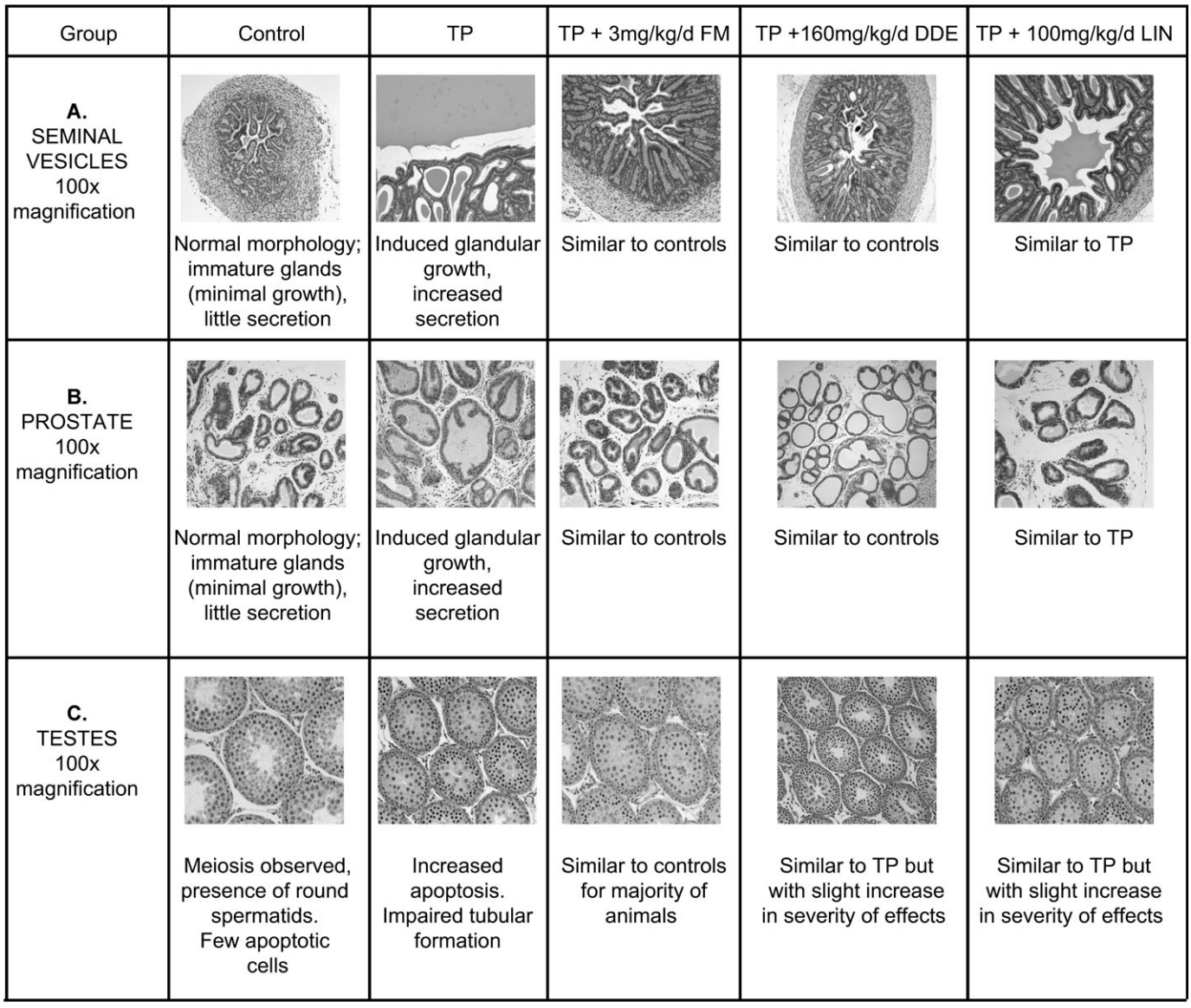

FIG. 2. Representative images of microscopic findings for (A) SVs, (B) DP, and (C) testes for each of the treatment groups evaluated.

recorded (Fig. 2C). This resulted in a mean severity grading of 0.08 for impaired tubular growth and of 0.25 for the presence of apoptotic germ cells (Table 2).

The severity grading increased to 1.4 for impaired tubular growth and to 0.7 for the occurrence of apoptosis following treatment of the weanlings with 1 mg/kg/day TP.

The interanimal variability recorded for the weights of the testes isolated from stimulated weanlings treated with the antiandrogens was also observed during the histopathological assessments. For example, although FM reduced the testicular weight for the majority of treated animals, the testes from two treated animals were approximately 70% lighter than the testes from the rest of the TP/FM group, and they were also approximately 50% lighter than the TP-only testicular weights. The histopathological findings were in accordance with these observations inasmuch as FM inhibited the effects of TP on the testes (i.e., impaired tubular growth and apoptosis) in the majority of the treated weanlings. However, the same two TP/FM–treated animals showing unexpectedly reduced testicular weights exhibited moderate to severe tubular damage coupled with an absence of germ cells in some tubules. In addition, these two animals exhibited an increased number of apoptotic germ cells, with the incidence of apoptosis being comparable in both animals to that of the animals exposed to TP only. Consequently, the mean severity gradings for the testes of TP/FM–treated animals (1.2 for impaired tubular growth and 0.75 for apoptotic germ cells) were similar to those of the TP-stimulated weanling (Table 2). The weight and

histopathological observations recorded for these two animals do not, however, appear to be correlated with an error in their dosing (e.g., nontreatment with FM) as their SAT weights were significantly reduced compared to those of the TP group (Table 1, experiment 2). In addition, there were no differences in the morphology of the SVs and DP of these two animals compared to the rest of the TP/FM group.

Cotreatment with either DDE or LIN increased the mean severity grade for tubular degeneration from 1.4 recorded for the TP-stimulated weanling to 2.0 for the TP/DDE group and to 2.5 for the TP/LIN group (Table 2; Fig. 2C). In the case of LIN cotreatment, an increased occurrence of apoptotic germ cells was also observed in all treated animals resulting in a mean severity grade of 1.3 compared to 0.7 for the TP group.

Identification and Modulation of LAO (proteomic analysis of prostate tissue)

The modulation of LAO was assessed by 2-DE in the VP of vehicle control animals, TP-stimulated animals as well as TP-stimulated weanlings cotreated with 160 mg/kg/day DDE, 100mg/kg/day LIN, or 3mg/kg/day FM. The conduct of Western blot analysis to identify LAO indicated that the antibody cross-reacted with proteins in at least three neighboring but distinct areas of the gel (data not shown). As the antibody used in the Western blot was derived from snake venom, the observed cross-reactivity could have been indicative of either the use of a nonspecific antibody or the presence of several isoforms of LAO on the gel. To further investigate these possibilities, as well as to determine whether or not the LAO, as described by Cayatte *et al.* (2006), was present on our gels, seven spots (Fig. 3) located in the areas corresponding to the three areas of cross-reactivity recorded on the Western blot were identified by nano ESI-MS/MS. Of these seven spots, four were identified as LAO (Fig. 3), one of which was the protein spot previously published as LAO by Cayatte *et al.* (2006).

The spot intensity ratios for TP treatment versus vehicle control and for antiandrogen/TP treatment versus TP treatment are given in Figures 4 and 5 for the protein confirmed as being LAO according to Cayatte *et al.* (2006). The data indicated that LAO was clearly present in the VP of vehicle control weanling rats and that TP treatment led to a downregulation of this protein by ~60%. Treatment of the stimulated weanling with the antiandrogens led to an upregulation of LAO to varying degrees compared to the TP-only–treated animals. Thus, FM induced an approximately 7-fold increase in the spot intensity of LAO compared to that recorded for the stimulated weanling. Similarly, DDE stimulated a 4.4-fold increase in the production of LAO. A marginal increase in LAO spot intensity (1.42-fold) was recorded due to LIN treatment.

A similar modulation (Fig. 5) was recorded for two of the three additional proteins identified as LAO (spots A and B, Fig. 3). Thus, TP downregulated both proteins by 60–70%

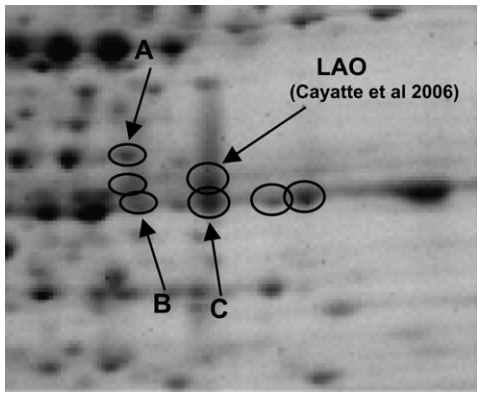

FIG. 3. Based on the Western blot observations, seven spots (ringed) in three neighboring but distinct areas of the 2-DE gels were chosen for identification by nano ESI-MS/MS. One protein was confirmed as being the LAO previously identified by Cayatte *et al.* (2006) and three further proteins (A–C) were also identified as LAO.

compared to the vehicle control, whereas FM led to a two- to threefold increase in spot intensity compared to TP. DDE marginally increased the spot intensity of both proteins. LIN, however, had no effect on the modulation of one of the spots (spot B, Fig. 5) and appeared to downregulate the other (spot A) compared to TP. Insufficient data were available for the third spot (spot C, Fig. 3) as this spot was not present on all gels.

DISCUSSION

The Hershberger assay validation programme has indicated that this model can detect a range of reference EDCs including biochemical modulators, antiandrogens, and androgens (Owens *et al.*, 2007). As part of the validation process, several laboratories are currently assessing the sensitivity of the TP-stimulated, intact weanling model as a more ethical means of identifying EDCs. Before conducting the OECD validation study, we validated the weanling model using compounds and doses levels for which previously published data were available for both the castrated adult and the immature intact animal. At the same time, we assessed the histopathological changes occurring in the SVs, DP, and testes in some of the groups. Furthermore, as we had recently shown that a protein, LAO, which is found in snake venom (Du and Clemetson, 2002) and is associated with apoptosis (Ande *et al.*, 2006), was modulated in the adult rat VP due to treatment with antiandrogenic compounds (Cayatte *et al.*, 2006), we took the opportunity to further investigate this protein in the weanling animal.

Our gravimetric data correlated well with published data generated in both the castrated adult (Owens *et al.*, 2006, 2007; Yamasaki *et al.*, 2006, Takeyoshi *et al.*, 2005) and the TP-stimulated intact weanling (Ashby *et al.*, 2004). In addition, our histopathological observations recorded for the SVs and prostate were in agreement with the weight changes. Thus, TP

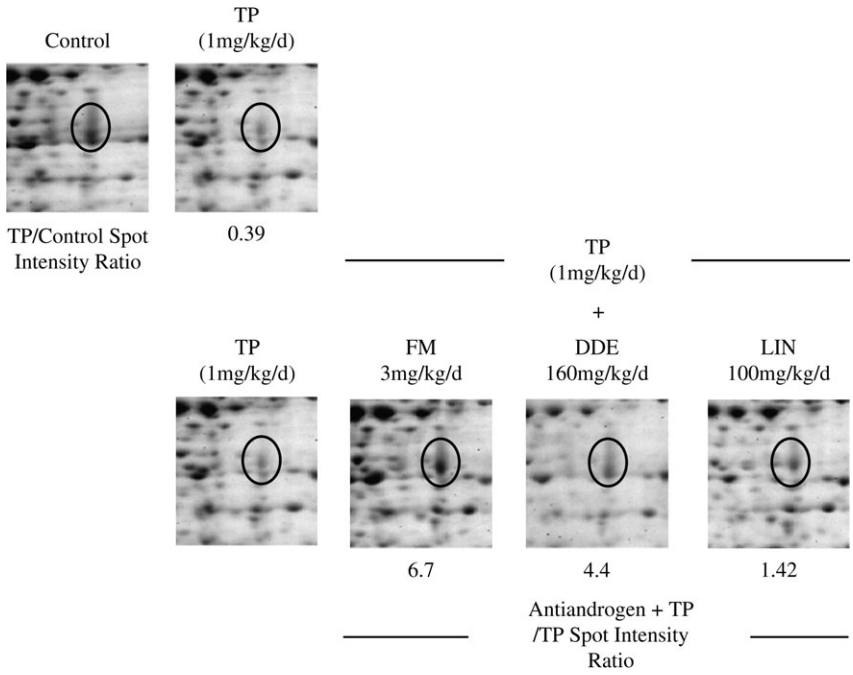

FIG. 4. Modulation of the LAO protein (ringed) was investigated in the VP of vehicle control animals and those animals exposed to either TP only or TP and 3 mg/kg/day FM (positive control), 160 mg/kg/day DDE, or 100 mg/kg/day LIN. The images are representative of the 2-DE analyses performed and illustrate the LAO previously identified by Cayatte *et al.* (2006). The spot intensities were compared first between the vehicle control and TP-only–treated groups. The intensities were then compared between the antiandrogen/TP and the TP-only–treated groups.

induced a characteristic stimulation of the SATs leading to significant weight increases and a precocious maturity of the SVs and prostate. Our positive control compound, FM, clearly inhibited the stimulatory effects of TP as evidenced by the significant SAT weight reductions and the control-like morphologies of the SVs and prostate.

Our DDE dose-response data for both liver and SAT weights were in agreement with earlier published data. Thus, our dose-related increase in liver weight has been observed by several investigators (Freyberger *et al.*, 2005; You *et al.*, 1999) and has previously been shown to be related to increased cytochrome P450 enzyme activity (Hart and Fouts, 1963; Nims and Lubet, 1995) and hepatocellular hypertrophy (You *et al.*, 1999; Freyberger *et al.*, 2005). Similarly, our dose-related reductions in SAT weights have been reported for the stimulated weanling (Ashby *et al.*, 2004) as well the castrated adult rat (Freyberger *et al.*, 2005; Owens *et al.*, 2007). The control-like morphologies of the SVs and prostate of the TP/DDE (160 mg/kg/day) animals also confirmed the antiandrogenic activity of DDE.

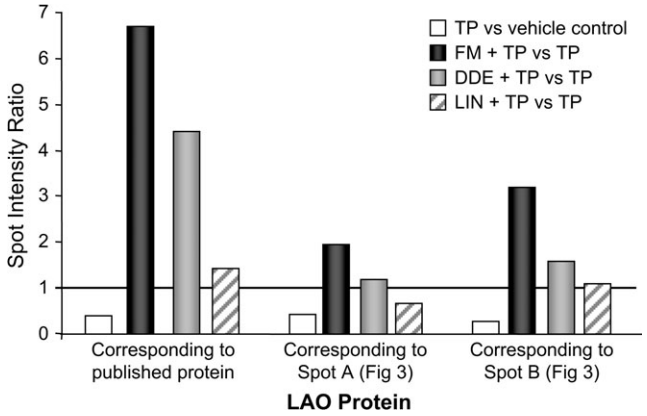

FIG. 5. Spot intensity ratios are given for each protein identified as LAO as shown in Figure 3. A ratio below 1 indicates a downregulation of LAO and a ratio above 1 indicates an upregulation of LAO due to treatment. No data are presented for spot C of Figure 3 as this spot was not present on all gels.

Of the three antiandrogens evaluated, LIN had the greatest impact on body and kidney weight parameters with the significant decreases in both parameters following treatment with 100 mg/kg/day LIN, indicating that this dose was slightly toxic to the stimulated weanling. In addition, this compound was only weakly active as an antiandrogen as evidenced by the marginal weight reductions recorded for the SVs, VP, and Cowper's glands, which were statistically significant at the top dose level only (100mg/kg/day), the inability to reduce the weights of the stimulated LA/BC muscles and the lack of effect on the morphology of the stimulated SVs and prostate. Similar marginal weight effects were recorded by Ashby *et al.* (2004) for the TP-stimulated weanling model and by Owens *et al.* (2007) in the castrated adult rat. The LA/BC observations are of particular interest as there are conflicting reports concerning the effects of LIN on this tissue. For example, our data and those of

Ashby *et al*. (2004) demonstrated that LIN had no effect on the LA/BC. However, Lambright *et al*. (2000) observed a significant reduction in LA/BC weight in both the immature and adult castrated rat following 7 days treatment with 100 mg/kg/day LIN but found no effect if castrated adults were exposed for only 4 days. While these differences may merely be a reflection of the overall weak activity of LIN or a difference in the relative sensitivities of the castrated and intact animal, it could also be indicative of a mechanism of action other than AR antagonism for this compound as previously implied by Wolf *et al*. (1999). For example, the 5α-reductase inhibitor, finasteride, can reduce the weights of the SVs and prostate, which are dependent on 5α-dihydrotestosterone for growth, but it is unable to inhibit LA/BC growth (Blohm *et al*., 1986).

Apart from the ethics, an apparent advantage of using the stimulated weanling rat rather than the castrated rat is that it should allow the evaluation of the testes and epididymides. Our data indicated that the epididymides were as sensitive as the SATs to the agonistic and antagonistic effects of TP and the antiandrogens, respectively. In contrast to the increase in SAT and epididymal weights following TP stimulation, the testes of the TP-treated animals were reduced in size and this atrophy corresponded with mild, though noticeable histological changes. These observations have been reported by several other investigators. For example, Yamasaki *et al*. (2000) reported a dose-related decrease in testicular weight, which was associated with histological changes in the testes in response to treatment of weanling rats with 17-methyl testosterone (17MT). Similarly, Wason *et al*. (2003) noted a reduction in testicular weight and associated histological changes following treatment of adult rats to 17MT. These inhibitory effects of testosterone on testicular growth may readily be explained by the fact that exposure to exogenous testosterone leads to a reduction in circulating LH, which in turn causes a decline in testosterone production by the Leydig cells and, therefore, a reduction in intratesticular testosterone concentration (Steinberger, 1971).

We recorded somewhat conflicting data for the effects of antiandrogens on the testes. Whereas interanimal variability was low for most of the tissues weighed, a high degree of interanimal variability was recorded for the testicular weights of the antiandrogen-treated stimulated weanlings, which meant that any weight increases were not statistically significant. Similar variability in testicular weight has also been noted by other investigators (Ashby *et al*., 2004; Lefevre, personal communication). In addition to the weight variability, the severity of the testicular lesions was also contradictory. For example, although FM increased the testicular weight and reduced the severity of the lesions of most stimulated weanlings, the weights and lesions of two treated weanlings were more affected than the TP-only–exposed animals. Similarly, although DDE marginally increased the testicular weights, the severity of the lesions was marginally greater than those observed in the TP-only–exposed animals. Finally, the weakest antiandrogen, LIN, reduced testicular weight and increased the severity of the testicular lesions compared to the TP-only–exposed tissues. Based on these observations, therefore, it would appear that the testis is not a suitable tissue to use for identifying EDCs in the stimulated weanling assay.

The use of molecular tools such as genomics and proteomics is becoming increasingly more commonplace in EDC investigations. For example, several studies investigating EDCs have included gene profiling of the VP (Rosen *et al*., 2005; Yamada *et al*., 2005) and the testes (Gye and Ohsako, 2003). More recently, we have conducted proteomic assessment of the VP (Cayatte *et al*., 2006) and testes (Friry-Santini *et al*., 2007) of adult rats exposed to antiandrogens. We demonstrated that a protein, LAO, found in snake venom (Du and Clemetson, 2002), was modulated in the adult prostate due to FM or finasteride treatment (Cayatte *et al*., 2006). In these present studies, we extended this database by demonstrating the presence of LAO in the VP of our weanling control animals and its subsequent downregulation following treatment with TP (Figs. 3–5). Taking our control and TP weanling data and the control adult data of Cayatte *et al*. (2006) together, it would appear that LAO is negatively controlled by testosterone. Further evidence for this negative control is derived from the observation that treatment of the stimulated weanling with FM, DDE, or LIN led to an upregulation of LAO (compared to the TP-only observations) in the weanling VP, just as FM and finasteride led to the appearance of this protein in the adult VP (Cayatte *et al*., 2006). As LAO is associated with apoptosis (Ande *et al*., 2006), it seems reasonable to assume that the inhibition of such a protein would be required for the maturation and growth of the prostate during testosterone stimulation. Our weanling data also indicated that at least three additional isoforms of rat prostate LAO exist, which were similarly modulated by TP and the antiandrogens. The existence of isoforms of LAO derived from other species is well documented (Hayes and Wellner, 1969; Sun *et al*., 2002). However, this is the first time that isoenzymes have been reported for the rat prostate protein.

In conclusion, our gravimetric (and histopathological) data indicate that in a study using only six animals per group, the TP-stimulated weanling assay is able to detect SAT and epididymal weight changes induced by both potent and weak antiandrogens. Our data also demonstrate that the sensitivity of the weanling assay is comparable to that of the adult rat Hershberger assay. Specifically, DDE induces significant antiandrogenic effects at the same dose level (50 mg/kg/day) in both the castrated adult (Owens *et al*., 2007) and the intact weanling rat. Similarly, the weak antiandrogenic effects of LIN are only consistently detected in both animal models at 100 mg/kg/day. Our limited data also indicate that the weanling assay is reproducible as the magnitudes of the tissue weight increases induced by TP and the tissue weight decreases due to cotreatment with FM were similar between the two experiments described herein. However, the inconsistent testicular

data suggest that this tissue is of little value in the evaluation of EDCs in the stimulated weanling assay. Our LAO data gave us valuable insight into the modulation of this protein. The observation of at least three isoforms of this protein clearly demonstrates the need for further investigations to fully characterize LAO in the rat prostate. However, the rodent LAO data indicate that this protein has the potential to be a biomarker for endocrine disruption.

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REFERENCES

Ande, S. R., Kommoju, P. R., Draxl, S., Murkovic, M., Macheroux, P., Ghisla, S., and Ferrando-May, E. (2006). Mechanisms of cell death induction by L-amino acid oxidase, a major component of ophidian venom. *Apoptosis* **11,** 1439–1451.

Ashby, J., and Lefevre, P. (1997). The weanling rat as an assay for endocrine disruption: Preliminary findings. *Regul. Toxicol. Pharmacol.* **31,** 92–105.

Ashby, J., and Lefevre, P. (2000). The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators. *J. Appl. Toxicol.* **20,** 35–47.

Ashby, J., Lefevre, P. A., Tinwell, H., Odum, J., and Owens, W. (2004). Testosterone stimulated weanlings as an alternative to castrated male rats in the Hershberger anti-androgen assay. *Regul. Toxicol. Pharmacol.* **39,** 229–238.

Ashby, J., Owens, W., and Lefevre, P. (2002). Concept evaluation: Androgen-stimulated immature intact male rats as an assay for antiandrogens. *Regul. Toxicol. Pharmacol.* **35,** 280–285.

Blohm, T. R., Laughlin, M. E., Benson, H. D., Johnston, J. O., Wright, C. L., Schatzman, G. L., and Weintraub, P. M. (1986). Pharmacological induction of 5α-reductase deficiency in the rat: Separation of testosterone mediated and 5α-dihydrotestosterone-mediated effects. *Endocrinology* **119,** 959–966.

Cayatte, C., Pons, C., Guigonis, J. M., Pizzol, J., Elies, L., Kennel, P., Rouquie, D., Bars, R., Rossi, B., and Samson, M. (2006). Protein profiling of rat ventral prostate following chronic finasteride administration: Identification and localization of a novel putative androgen-regulated protein. *Mol. Cell Proteomics* **5,** 2031–2043.

Du, X.-Y., and Clemetson, K. J. (2002). Snake venom l-amino acid oxidases. *Toxicon* **40,** 659–665.

Freyberger, A., Hartmann, E., and Krotlinger, F. (2005). Evaluation of the rodent Hershberger bioassay using three reference (anti-)androgens. *Arh. Hig. Rada. Toksikol.* **56,** 131–139.

Friry-Santini, C., Rouquie, D., Kennel, P., Tinwell, H., Benahmed, M., and Bars, R. (2007). Correlation between protein accumulation profiles and conventional toxicological findings using a model antiandrogenic compound, flutamide. *Toxicol. Sci.* **97,** 81–93.

Greene, R. R., and Burrill, M. W. (1940). Twenty-four hour response to androgens in the immature male rat. *Toxicol. Ind. Health* **15,** 94–118.

Greene, R. R., and Burrill, M. W. (1941). Forty-eight hour response of the immature male rat to androgens. *Endocrinology* **29,** 402–408.

Gye, M. C., and Oshako, S. (2003). Effects of flutamide in the rat testis on the expression of occluding, an integral member of the tight junctions. *Toxicol. Lett.* **143,** 217–222.

Hart, L. G., and Fouts, J. R. (1963). Effects of acute and chronic DDT administration on hepatic microsomal drug metabolism in the rat. *Proc. Soc. Exp. Biol. Med.* **114,** 388–392.

Hayes, M. B., and Wellner, D. (1969). Microheterogeneity of L-amino acid oxidase. *J. Biol. Chem.* **244,** 6636–6644.

Kang, I. H., Kim, H. S., Shin, J. H., Kim, T. S., Moon, H. J., Kim, I. Y., Choi, K. S., Kil, K. S., Park, Y. I., Dong, M. S., *et al.* (2004). Comparison of antiandrogenic activity of flutamide, vinclozolin, procymidone, linuron, and p,p′-DDE in rodent 10-day Hershberger assay. *Toxicology* **199,** 145–159.

Kanno, J., Onyon, L., Haseman, J., Fenner-Crisp, P., Ashby, J., and Owens, W. (2001). The OECD program to validate the rat uterotrophic bioassay to screen compounds for *in vivo* estrogenic responses: Phase 1. *Environ. Health Perspect.* **109,** 785–794.

Kanno, J., Onyon, L., Peddada, S., Ashby, J., Jacob, E., and Owens, W. (2003). The OECD program to validate the rat uterotrophic bioassay. Phase 2: Dose-response studies. *Environ. Health Perspect.* **111,** 1530–1549.

Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A., and Wilson, E. M. (1995). Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature* **375,** 581–585.

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.* **56,** 389–399.

Marty, M. S., Crissman, J. W., and Carney, E. N. (2001). Evaluation of the male rat pubertal onset to detect testosterone and steroid inhibitors in CD rats. *Toxicol. Sci.* **60,** 285–295.

Monosson, E., Kelce, W. R., Lambright, C. R., 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.

Nims, R. W., and Lubet, R. A. (1995). Induction of cytochrome P-450 in the Norway rat, *Rattus norvegicus*, following exposure to potential environmental contaminants. *J. Toxicol. Environ. Health* **46,** 271–292.

Organization for Economic Cooperation and Development (OECD). (2000). OECD protocol for investigating the efficacy of the enhanced TG407 test guideline (phase 2); rationale for the investigation and description of the protocol. Organisation for Economic Co-operation and Development, Paris.

Olsson, I., Larsson, K., Palmgren, R., and Bjellqvist, B. (2002). Organic disulfides as a means to generate streak-free two-dimensional maps with narrow range basic immobilized pH gradient strips as first dimension. *Proteomics* **2,** 1630–1632.

Owens, W., Gray, L. E., Jr, Zeiger, E., Walker, M., Yamasaki, K., Ashby, J., and Jacob, E. (2007). The OECD program to validate the rat Hershberger bioassay to screen compounds for *in vivo* androgen and antiandrogen responses. Phase 2: Dose response studies. *Environ. Health Perspect.* **115,** 671–678.

Owens, W., Zeiger, E., Walker, M., Ashby, J., Onyon, L., and Gray, L. E., Jr (2006). The OECD program to validate the rat Hershberger bioassay to screen compounds for *in vivo* androgen and antiandrogen responses. Phase 1: Use of a potent agonist and a potent antagonist to test the standardized protocol. *Environ. Health Perspect.* **114,** 1259–1265.

Ramagli, L. S., and Rodriguez, L. V. (1985). Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* **6,** 559–563.

Rosen, M. B., Wilson, V. S., Schmid, J. E., and Gray, L. E. (2005). Gene expression analysis in the ventral prostate of rats exposed to vinclozolin or procymidone. *Reprod. Toxicol* **19,** 367–379.

Steinberger, E. (1971). Hormonal control of mammalian spermatogenesis. *Physiol. Rev.* **51,** 1–22.

Stoker, T. E., Parker, L. G., Gray, L. E., and Cooper, R. L. (2000). Endocrine disrupting chemicals: Prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on EDSTAC recommendations. *Crit. Rev. Toxicol.* **30,** 197–252.

Sun, Y., Nonobe, E., Kobayashi, Y., Kuraishi, T., Aoki, F., Yamamoto, K., and Sakai, S. (2002). Characterization and expression of L-amino acid oxidase of mouse milk. *J. Biol. Chem.* **277,** 19080–19086.

Takeyoshi, M., Mitoma, H., and Yamasaki, K. (2005). Changes in serum alpha2u-globulin levels in castrated male rats treated with testosterone propionate in a Hershberger assay. *J. Appl. Toxicol.* **25,** 176–178.

Wason, S., Pohlmeyer-Esch, G., Pallen, C., Palazzi, X., Espuna, G., and Bars, R. (2003). 17α-methyltestosterone: 28-day oral toxicity study in the rat based in the "enhanced OECD test guideline 407" to detect endocrine effects. *Toxicology* **192,** 119–137.

Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R. L., Ostby, J., and Gray, L. E., Jr (1999). Administration of potentially antiandrogenic pesticides (procymidone, iprodione, chlozolinate, p,p′-DDE and ketoconazole) and toxic substance (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.

Yamada, T., Sumida, K., Saito, K., Ueda, S., Yabushita, S., Sukata, T., Kawamura, S., Okuno, Y., and Seki, T. (2005). Functional genomics may allow accurate categorization of the benzimidazole fungicide benomyl: Lack of ability to act via steroid receptor mediated mechanisms. *Toxicol. Appl. Pharmacol.* **205,** 11–30.

Yamasaki, K., Ashby, J., Lefevre, P. A., and Sawaki, M. (2000). Comparison of reproductive tissue weights in the enhanced Hershberger assay of 17α-methyltestosterone between prepubertal and castrated rats. *J. Toxicol. Pathol.* **13,** 173–178.

Yamasaki, K., Ohta, R., and Okuda, H. (2006). OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals. *Toxicol. Lett.* **163,** 121–129.

You, L., Chan, S. K., Bruce, J. M., Archibeque-Engle, S., Casanova, M., Corton, J. C., and Heck, H.d'A. (1999). Impaired male sexual development in perinatal Sprague-Dawley and Long Evans hooded rats exposed in utero and lactationally to p,p'-DDE. *Toxicol. Sci.* **45,** 162–173.