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EVALUATION OF THE RODENT HERSHBERGER BIOASSAY USING THREE REFERENCE (ANTI)ANDROGENS*

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Under the umbrella of the Organization for Economic Cooperation and Development (OECD) the rodent Hershberger bioassay is being validated as an *in vivo* screen for the detection of (anti)androgens. As part of the validation work we studied trenbolone (TREN), 1,1-bis-(4-chlorophenyl)-2,2-dichloroethylene (*p,p*'-DDE) and vinclozolin (VIN). Oral intubation of castrated rats with TREN [0.3-1.5-8-40 mg kg^{-1} body weight (b.w.)] for ten days increased androgen-sensitive tissue weights (ASTW) at the high dose. *p,p*'-DDE (5-16-50-160 mg kg^{-1} b.w.) and VIN (0-3-10-30-100 mg kg^{-1} b.w.) orally administered for ten days produced a dose-dependent decrease in ASTW in castrated rats subcutaneously supplemented with testosterone propionate (0.4 mg kg^{-1} b.w.). *p,p*'-DDE also strongly increased liver weights and induced hepatocellular hypertrophy and thyroid follicular cell hypertrophy that was most likely mediated by liver enzyme induction. Our data strongly suggest that the OECD protocol of the rodent Hershberger bioassay describes a sensitive *in vivo* screen, capable of detecting weakly active (anti)androgens. Furthermore, our data may also indicate that thyroid effects could be assessed, if the protocol is amended accordingly.

KEY WORDS: in vivo *screen, OECD*, p,p'-*DDE, thyroid, trenbolone, vinclozolin*

The rodent Hershberger bioassay was first described in 1953 by Hershberger and colleagues (1) as a screening assay for androgenic and anabolic agents. *Hershberger et al*. used the increase in the weight of prostate/seminal vesicles and levator ani muscle in castrated rats as indicators of androgenicity and anabolic activity, respectively. Since that time, the Hershberger bioassay has been used primarily by the pharmaceutical industry to screen for strong androgens/anabolic agents and - by using a modified version employing testosterone-supplemented castrated rats - to screen for effective antiandrogens. The characterization of various environmental chemicals as compounds with androgen- or antiandrogen-like activity (2-6) has prompted a need for a reliable *in vivo* screen capable of detecting such compounds. The Hershberger bioassay in rats was considered as the first choice in this respect; however, an internationally accepted assay protocol for toxicological purposes was not available. Thus, OECD took over the lead to develop and validate a robust protocol that can be considered as the basis for an OECD Test Guideline.

The current protocol for this study (phase 2) is based on the standardisation and optimisation work performed under OECD auspices in phase 1 of the Hershberger bioassay validation. The assay was robust and reproducible across laboratories in the presence of several variations, e.g., strain of rat, diet, and modest variations in the age of castration. All laboratories and all protocols were successful in detecting increases in the weights of the accessory sex organs and tissues in response to testosterone propionate, and in detecting the anti-androgenic effects of flutamide, and there was

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a good agreement among laboratories with regard to the dose responses obtained for testosterone propionate and flutamide (7).

The objective of the phase 2 of the validation was to further assess the reliability and relevance of the assay. Specific aims were to (a) demonstrate the reliability of the Hershberger bioassay to respond to and to identify additional androgen receptor agonists, namely 17α-methyltestosterone and trenbolone (TREN) and weak antagonists, namely vinclozolin (VIN), procymidone, linuron, and *p,p'*-DDE using two different dose levels of TP to restore androgen sensitive tissue weights, (b) to investigate the reproducibility of the assay with these additional agonists and antagonists, (c) to study the relative effectiveness of different sex accessory tissues and glands in the assay, (d) to evaluate the capability of the assay to detect the 5α-reductase inhibitor finasteride through differential response of the sex accessory tissues and glands, and (e) to investigate whether optional organ weights of liver, kidneys and adrenals could contribute to the detection of weakly active compounds. Our part in this international effort was to conduct studies on TREN, *p,p'*-DDE, and VIN according to the OECD protocol. In addition to and outside the scope of the OECD protocol, we performed histological examination of the liver and thyroid of *p,p'*-DDE-treated rats. The results of these studies are described below.

MATERIAL AND METHODS

General

Investigations were performed in compliance with good laboratory practice and in agreement to the German law of animal experimentation. All parts of the study, i.e., study protocol, in-life phase, raw data and the study report were subjected to quality assurance inspections.

Chemicals

Trenbolone (≥95 %) and corn oil were purchased from Sigma (Taufkirchen, Germany), Vinclozolin (99.2 %), *p,p'*-DDE (99.5 %), flutamide (99.9 %) and testosterone propionate (97 %) were obtained through the OECD Chemical Repository at TNO (Nederlandse organisatie voor toegepast-natuurwetenschappelijk oenderzoek, Delft, The Netherlands)

Animal treatment

Investigations were performed according to the OECD phase 2 protocol (8). Forty day old (breeder´s information) SPF-bred male Wistar rats (strain Hsd/Cpb:WU) were obtained from Harlan-Winkelmann (Borchen, Germany) and were maintained under controlled conditions. The animal room had a 12 h light/dark regime, a temperature of 22±2 °C, relative humidity of approximately 55 % and a rate of air exchange of ≥10-fold per hour. Animals had free access to NAFAG No. 9439 mouse and rat pellet diet (supplied by Eberle Nafag AG, Gossau, Switzerland) and to drinking water. Five days after their arrival, the animals were castrated. Thereafter they were acclimatised to laboratory conditions for one week until treatment began. Their state of health was also monitored during this period. Only healthy animals showing no clinical signs were used for the study. One day before the treatment began, castrated rats were randomly assigned to vehicle and treatment groups (n=6 each) in the way that mean body weights were similar in all groups. Test compounds were formulated in corn oil at the beginning of the study and were stored in a refrigerator throughout the study. Except for higher concentrations of TREN, clear solutions were obtained. Suspensions of TREN were thoroughly stirred before administration. Test compounds were administered for 10 days using dosing volumes of 5 mL kg^{-1} b.w. for oral and 0.5 mL kg^{-1} b.w. for subcutaneous (s.c.) application. TREN, *p,p'*-DDE and VIN were studied in separate experiments. TREN was administered by oral intubation at dose levels of 0.3, 1.5, 8, and 40 mg kg^{-1} b.w., whereas control animals received only the vehicle. TP (0.4 mg kg^{-1} b.w., s.c.) served as positive control. *p,p'*-DDE and VIN were administered orally at the doses of 5, 16, 50, and 160 mg kg^{-1} (*p,p'*-DDE) and 3, 10, 30, and 100 mg kg^{-1} b.w. (VIN) to castrated rats supplemented with TP (0.4 mg kg^{-1} b.w., s.c.). Orally applied flutamide (3 mg kg^{-1}) was used as a reference antiandrogen. In addition, a vehicle control group served as a measure for maximal weight reduction of androgen-sensitive tissues. Animals were inspected for clinical signs at least once a day, body weights were recorded daily.

Necropsy

One day after the last treatment, animals were killed in deep anaesthesia by exsanguination. Liver, kidneys, adrenals, ventral prostate (VP), seminal vesicles (SV), glans penis (GP), levator ani and bulbocavernosus muscles (LABC), and Cowper's glands (CG) were rapidly removed, trimmed free of fat and adjacent tissue and weighed.

Histology

Livers and thyroids from all animals of the study with *p,p'*-DDE (with the exception of the flutamide and vehicle control groups) were fixed in a 10 % neutral buffered formalin. Paraffin sections were prepared by routine techniques, stained with haematoxylin and eosin, and examined microscopically.

Statistics

For statistical evaluation, Dunnett's test was used. The criterion for significance was set at $p \leq 0.05$.

RESULTS

TRENBOLONE STUDY

Appearance, behaviour, and mortality

No clinical signs were observed and no animal died throughout the entire treatment period.

Body weight development

No significant effects on food intake were observed and final bodyweights of the treated groups were not significantly different from those of the castrated control group (Table 1). However, body weight gain decreased in the group receiving 40 mg kg^{-1} b.w. of TREN and increased in the TP group.

Organ and tissue weights

Liver and kidney weights were unaffected by treatment with TREN or TP. In comparison to the control group, the absolute and relative adrenal weights decreased at doses 0.3 mg kg^{-1} b.w. of TREN and above; the differences were partly statistically significant, but not dose-related. Adrenal weights also increased in TP-treated animals (Tables 1 and 2).

The high dose of TREN induced an increase in absolute and relative weights of prostate, seminal vesicles together with coagulating gland, Cowper's glands, levator ani and bulbocavernosus muscles,

Table 1 *Effects of orally administered trenbolone (TREN) and subcutaneously administered testosterone propionate (TP) on body, absolute organ and tissue weights of castrated rats. Castrated rats were treated either with TREN or TP for ten days. One day after the last administration, terminal body and organ and tissue weights were recorded.*

Treatment	N	Dose	Body weight/g		Optional organ weights			Androgen-sensitive tissue weights/mg				
		mg kg^{-1}	Initial	Terminal	Liver g	Kidneys g	Adrenals mg	Ventral prostate	Seminal vesicles	Glans penis	LABC	Cowper's gland
Vehicle	6		201±15	239±18	10.38±0.95	1.60±0.19	56±8	26±13	63±19	48±4	196±40	5.7±1.9
TREN	6	0.3	203±11	243±11	10.33±0.76	1.52±0.11	46±7	30±16	58±9	48±4	186±37	5.2±1.3
TREN	6	1.5	206±12	248±19	10.67±1.34	1.49±0.12	48±8	32±15	77±16	52±6	219±31	7.3±1.2
TREN	6	8.0	210±9	237±12	10.56±0.87	1.58±0.06	43±7**	29±16	68±31	51±7	224±37	5.5±0.7
TREN	6	40.0	207±16	224±24	10.36±2.26	1.501±0.24	45±6*	48±23	155±72	70±11**	395±51**	11.2±2.4**
TP, s.c.	6	0.4	205±10	259±17	11.01±1.12	1.57±0.12	45±5*	157±31**	744±164**	82±10**	578±38**	30.3±4.5**

N - number of animals; LABC - levator ani and bulbocavernosus muscles.
*Statistical significance: * - p<0.05; ** - p<0.01 (compared to vehicle control).*

Table 2 *Effects of orally administered trenbolone (TREN) and subcutaneously administered testosterone propionate (TP) on relative organ and tissue weights of castrated rats. For experimental details see Table 1. To obtain relative organ and tissue weights, the recorded absolute weights were divided by the corresponding body weights (b.w.).*

Treatment	N	Dose	Optional organ weights / mg per 100 g b.w.			Androgen-sensitive tissue weights / mg per 100 g b.w.				
		mg kg^{-1}	Liver	Kidneys	Adrenals	Ventral prostate	Seminal vesicles	Glans penis	LABC	Cowper's gland
Vehicle	6		4347±193	669±49	23±3	11±6	26±8	20±2	82±15	2.4±0.8
TREN	6	0.3	4240±179	626±30	19±3	12±6	24±4	20±2	76±17	2.2±0.5
TREN	6	1.5	4299±307	601±486	19±3	13±7	31±7	21±2	89±15	3.0±0.6
TREN	6	8	4457±298	670±415	18±4*	12±7	29±13	22±3	94±13	2.3±0.3
TREN	6	40	4582±527	669±67	20±3	21±9*	69±30**	31±4**	177±26**	5.1±1.2**
TP, s.c.	6	0.4	4238±217	606±36	17±1**	61±12**	288±65**	32±5**	223±15**	11.7±2.0**

N - number of animals; LABC - levator ani and bulbocavernosus muscles.
*Statistical significance: * - p<0.05; ** - p<0.01 (compared to vehicle control).*

and glans penis. These differences from controls were predominantly statistically significant. Similarly, TP significantly increased the weights of all these androgen sensitive tissues. Whereas 40 mg kg^{-1} b. w. TREN and TP were similarly effective in increasing the (relative) weights of LABC and glans penis, TP was more effective than TREN in Cowper's glands and even more effective in the ventral prostate and in the seminal vesicles plus coagulating glands (Tables 1 and 2).

STUDY WITH p,p'*-DDE*

Appearance, behavior, and mortality

Clinical signs were only observed in TP-supplemented rats treated with 160 mg kg^{-1} b.w. *p,p*'-DDE, whose water intake and urine excretion increased. No animal died throughout the treatment period.

Body weight development

No significant effects on food intake were observed. In TP-treated animals, treatment with *p,p*'-DDE or flutamide did not result in a statistically significant change in the final body weights. However, at the dose of 160 mg kg^{-1} b.w. of *p,p*'-DDE, body weight gain considerably decreased. As in the study with trenbolone, body weight gain in TP-supplemented rats was higher than in the castrated control group (Table 3).

Organ and tissue weights

Compared to TP-treated animals, enlarged livers were observed in DDE-treated animals, whose incidence (2-4-6-6 animals per treatment group) increased in a dose-dependent manner. Concomitantly, an increasing liver weight was observed in *p,p*'-DDE-treated animals. This increase was marked, resulting in a 50 % liver weight increase at the high dose of *p,p*'-DDE. Liver weights of flutamide-treated and castrated control groups were similar to those of the TP group. In general, weights of kidneys and adrenals were similar for all treatment groups. Only at the high dose of *p,p*'-DDE, relative kidney and adrenal weights increased, presumably due to reduced body weight (Tables 3 and 4).

Compared to the TP-treated group, *p,p*'-DDE produced a dose-dependent decrease in the weights of all investigated androgen-sensitive tissues. Marked and predominantly statistically significant weight reductions were observed at 50 mg kg^{-1} b.w. *p,p*'-DDE and above. At the high dose, *p,p*'-DDE was roughly as effective in reducing androgen-sensitive tissue weight as the applied dose of flutamide. Neither the high dose of *p,p*'-DDE nor 3 mg kg^{-1} b.w. flutamide were able to decrease androgen-sensitive tissue weight to the extent observed in the castrated control group (Tables 3 and 4).

Histology

These investigations were performed outside the scope of the OECD protocol. No findings were observed in the livers of TP-treated animals. Histological correlates of the increased liver weights in *p,p*'-DDE-treated animals were a strong hepatocellular hypertrophy and cytoplasmic change, the incidence and severity of which increased with the dose. Furthermore, in *p,p*'-DDE-treated animals, Kupffer cell foci decreased in a dose-dependent manner.

Table 3 *Effects of orally administered* p,p'*-DDE and flutamide (FLUT) on body, absolute organ and tissue weights of testosterone propionate (TP, 0.4 mg* kg^{-1} *subcutaneously) supplemented castrated rats. Castrated rats were concomitantly treated with TP and* p,p'*-DDE or flutamide for ten days. A vehicle group was tested for comparison. One day after the last administration, terminal body and organ and tissue weights were recorded.*

Treatment	N	Dose	Body weight/g		Optional organ weights			Androgen-sensitive tissue weights/mg				
		mg kg^{-1}	Initial	Terminal	Liver g	Kidneys g	Adrenals mg	Ventral prostate	Seminal vesicles	Glans penis	LABC	Cowper's gland
TP	6		203±13	259±21	12.21±0.99	1.74±0.11	45±7	153 ±16	539±78	80 ±10	529±84	29.3±5.1
+ DDE	6	5	205±19	261±34	12.93±2.31	1.76±0.26	43±8	156±32	601±134	81±10	498±76	27.4±1.7
+ DDE	6	16	211±17	274±20	15.52±1.516*	1.78±0.20	44±4	147±20	524± 86	76±6	451±28	23.5±4.3*
+ DDE	6	50	206±14	257±22	16.73±2.26**	1.78±0.16	46±4	94±22**	402± 98*	73±8	370±41**	17.3±2.5**
+ DDE	6	160	209±13	229±32	17.97±2.89**	1.68±0.24	51±8	40±23**	128± 54**	54±8**	194±36**	10.7±2.9**
+ FLUT	6	3	209±13	262±18	11.91±1.21	1.77±0.14	50±7	35±13**	146± 46**	68±9	253±34.**	10.7±2.1**
Vehicle only	6		209±17	254±26	11.00±1.64	1.63±0.11	51±12	21±17**	65± 17**	44±11**	175±16**	6.2±1.6**

N - number of animals; LABC - levator ani and bulbocavernosus muscles.
*Statistical significance: * - p<0.05; ** - p<0.01 (compared to TP-treated castrated rats).*

Table 4 *Effects of orally administered* p,p'*-DDE and flutamide on relative organ and tissue weights of testosterone propionate-supplemented (TP, 0.4 mg kg^{-1} subcutaneously) castrated rats. For experimental details see Table 3. To obtain relative organ and tissue weights, the recorded absolute weights were divided by the corresponding body weights (b.w.).*

Treatment	N	Dose mg kg^{-1}	Optional organ weights / mg per 100 g b.w.			Androgen-sensitive tissue weights / mg per 100 g b.w.				
			Liver	Kidneys	Adrenals	Ventral prostate	Seminal vesicles	Glans penis	LABC	Cowper's gland
TP	6		4707±91	671±17	17±2	59±6	210±40	31±5	203±19	11.2±1.2
+ DDE	6	5	4930±341	672±44	16±1	61±14	231±46	31±2	194±43	10.6±1.2
+ DDE	6	16	5672±343**	650±33	16±1	54±9	192±35	28±3	166±12	8.7±2.0*
+ DDE	6	50	6493±502**	693±29	18±2	37±9	157±38	28±3	144±17**	6.7±0.9**
+ DDE	6	160	7866±573**	735±26*	23±4*	17±9**	55±20**	24±4*	86±16**	4.7±0.9**
+ FLUT	6	3	4537±264	674±40	19±3	13±5**	55±16**	26±3	96±10**	4.1±0.9**
Vehicle only	6		4316±217	642±31	20±4	8±7**	25±8**	17±5**	69±8**	2.4±0.6**

N - number of animals; LABC - levator ani and bulbocavernosus muscles
*Statistical significance: * - p<0.05; ** - p<0.01 (compared to TP-treated castrated rats).*

Compared to the TP-treated group, animals treated with 50 mg kg^{-1} *p,p'*-DDE, had a higher incidence of mild hypertrophy of the thyroid follicular epithelium (Table 5).

Table 5 *Histological findings in the liver and thyroid of testosterone propionate-supplemented,* p,p'*-DDE-treated castrated rats. For experimental details see Table 3.*

Histological findings	Number of animals with findings/ Number of animals investigated				
	p,p'-DDE / mg kg^{-1}				
	0	5	16	50	160
Liver					
Hepatocellular hypertropy	0/6	0/6	4/6	6/6	6/6
Grade 1	0/6	0/6	1/6	0/6	0/6
Grade 2	0/6	0/6	3/6	2/6	0/6
Grade 3	0/6	0/6	0/6	4/6	3/6
Grade 4	0/6	0/6	0/6	0/6	3/6
Cytoplasmatic change	0/6	4/6	6/6	6/6	5/6
Grade 1	0/6	4/6	3/6	0/6	0/6
Grade 2	0/6	0/6	3/6	4/6	0/6
Grade 3	0/6	0/6	0/6	2/6	3/6
Grade 4	0/6	0/6	0/6	0/6	2/6
Granuloma	0/6	0/6	0/6	1/6	0/6
Grade 2	0/6	0/6	0/6	1/6	0/6
Kupffer cell foci	4/6	3/6	2/6	1/6	1/6
Grade 1	4/6	3/6	2/6	1/6	1/6
Thyroid					
Hypertrophy of follicular epithelium	1/6	1/6	1/6	4/6	1/6
Grade 1	1/6	1/6	1/6	4/6	1/6

STUDY WITH VINCLOZOLIN

Appearance, behavior, and mortality

No clinical signs were observed and no animal died throughout the treatment period.

Body weight development

No significant effects on food intake were observed. In TP-treated animals, treatment with *p,p'*-DDE or flutamide did not result in any statistically significant change in the final body weight. However, mild reductions in body weight gain were observed in all VIN groups and, more pronouncedly, in the flutamide group. As in the other studies, body weight gain in TP-supplemented rats was higher than in the castrated control group (Table 6).

Organ and tissue weights

Compared to TP-treated animals, increases in relative liver weight and relative adrenal weight at doses ≥30 mg kg^{-1} b.w. of VIN were observed, whereas kidney weights remained unchanged in all dose groups (Tables 6 and 7).

Compared to the TP-treated group, VIN decreased the weights of all investigated androgen-sensitive tissues in a dose-dependent manner. Marked and predominantly statistically significant weight reductions were observed at 30 mg kg^{-1} b.w. VIN for ventral prostate, seminal vesicles and LABC. At the high dose of VIN, the weight of all androgen-sensitive tissues significantly dropped, but the effect was less pronounced than the one induced by flutamide. As in the study on *p,p'*-DDE, the reference antiandrogen flutamide at 3 mg kg^{-1} b.w. was unable to decrease

Table 6 *Effects of orally administered vinclozolin (VIN) and flutamide (FLUT) on body, absolute organ and tissue weights of testosterone propionate-supplemented (TP, 0.4 mg kg^{-1} subcutaneously) castrated rats. Castrated rats were concomitantly treated with TP and VIN or flutamide for ten days. A vehicle group was tested for comparison. One day after the last administration, terminal body and organ and tissue weights were recorded.*

Treatment	N	Dose	Body weight/g		Optional organ weights			Androgen-sensitive tissue weights / mg				
		mg kg^{-1}	Initial	Terminal	Liver g	Kidneys g	Adrenals mg	Ventral prostate	Seminal vesicles	Glans penis	LABC	Cowper's gland
TP	6		250±15	309±18	13.19±1.71	1.90±0.18	49±6	179±25	814±25	91±8	653±81	31.7±4.4
+ VIN	6	3	246±16	301±25	13.00±1.99	1.90±0.18	54±8	183±56	914±151	90±12	700±81	36.4±7.7
+ VIN	6	10	244±14	300±17	12.98±0.916	1.86±0.21	60 ±.8	172±38	764±98	84±3	634±57	33.4±4.0
+ VIN	6	30	241±17	295±17	13.75±0.90	1.89±0.13	63±8	130±23	533±117**	92±11	483±24**	29.8±2.4
+ VIN	6	100	244±15	298±19	13.99±1.39	1.86±0.20	62±14	83±26**	347±50**	74±7**	369±24**	20.7±2.9**
+ FLUT	6	3	240±14	288±18	11.96±1.28	180±0.21	53±4	56±7**	218±72**	67±6**	312±53**	14.9±2.6**
Vehicle only	6		249±14	289±20	11.75±0.85	1.70±0.16	54±10	36±20**	110±24**	56±7**	270±19**	8.3±2.1**

N - number of animals; LABC - levator ani and bulbocavernosus muscles.
*Statistical significance: * - p<0.05; ** - p<0.01 (compared to TP-treated castrated rats).*

Table 7 *Effects of orally administered vinclozolin (VIN) and flutamide on relative organ weights of testosterone propionate-supplemented (TP, 0.4 mg kg^{-1} subcutaneously) castrated rats. For experimental details see Table 6. To obtain relative organ and tissue weights, the recorded absolute weights were divided by the corresponding body weights (b.w.).*

Treatment	N	Dose	Optional organ weights/mg per 100 g b.w.			Androgen-sensitive tissue weights / mg per 100 g b.w.				
		mg kg^{-1}	Liver	Kidneys	Adrenals	Ventral prostate	Seminal vesicles	Glans penis	LABC	Cowper's gland
TP	6		4256±314	613±30	16±1	58± 10	266±55	29±2	213±38	10.3±1.56
+ VIN	6	3	4309±303	632±37	18±2	61±18	308±65	30±4	235±40	12.2±2.5
+ VIN	6	10	4330±153	618±38	20±2*	57±11	256±35	28±2	212±22	11.2±1.3
+ VIN	6	30	4665±178*	641±31	21±2**	44±7	182±47*	31±4	165±16*	10.1±1.2
+ VIN	6	100	4689±242*	622±38	21±4**	28±10**	118±248**	25±4*	124±11**	7.0±1.1**
+ FLUT	6	3	4150±248	623±58	18±2	19±3**	75±22**	23±2**	108±15**	5.2±0.9**
Vehicle only	6		4070±89	588±43	19±2	12±7**	38± 8**	20±2**	94±12**	2.9±0.7**

N - number of animals; LABC - levator ani and bulbocavernosus muscles.
*Statistical significance: * - p<0.05; ** - p<0.01 (compared to TP-treated castrated rats).*

androgen-sensitive tissue weights to the extent observed in the castrated control group (Tables 6 and 7).

DISCUSION

Following the standardisation and optimisation of the OECD Hershberger bioassay protocol in the validation phase 1, which showed that castrated rats robustly and reproducibly responded to testosterone propionate (TP) and that the anti-androgenic effects of flutamide were sensitively detected in TP-supplemented rats, the testing of weak (anti)androgens in phase 2 was an important part of the validation.

Trenbolone (TREN) acetate has extensively been used to promote cattle growth. However, data on the androgen receptor binding and transactivating properties of TREN have become available only recently (5, 9, 10). TREN is a potent androgen and anabolic agent *in vitro* and *in vivo* after subcutaneous application; but about 100 times less active if administered orally (5). This points towards a strong first-pass metabolism at the17β-hydroxyl group that is not protected from metabolic activity by the presence of a 17α-methyl or 17α-ethinyl group. In line with the expected strong metabolism, weight increase of all measured androgen-dependent tissues was detected only at the high dose of TREN and, in contrast to TP, TREN was unable to increase the body weight gain of castrated rats. Compared to the effects of TP, the high dose of TREN was most active in levator ani and bulbocavernosus muscles (LABC) and the glans penis, while it was the least active in the ventral prostate and seminal vesicles. These findings are in line with previously published data (5) and seem to reflect the strong dependency of ventral prostate and seminal vesicle growth on 5α-reductase-catalysed

formation of dihydrotestosterone from testosterone, a metabolic activation step that is not possible with TREN. Accordingly, LABC weight appears to be the most sensitive parameter, especially for androgens that cannot be activated by 5α-reductase.

The DDT metabolite *p,p'*-DDE was shown to interfere with androgenic signalling at the receptor level (3). In androgen receptor (AR) binding studies, moderate affinity for AR was observed (3, 9, 10). Like *p,p'*-DDE, vinclozolin (VIN) was demonstrated to interfere with androgenic signalling at the receptor level (2). VIN itself has little or no affinity for the androgen receptor and acts through its metabolites (2, 9, 10). Both compounds decreased androgen sensitive tissue weight even at lower doses, *p,p'*-DDE typically at 50 mg kg^{-1} and above, and VIN at 30 mg kg^{-1} and above. Not all tissues responded equally. Based on relative weight changes, most sensitive tissues were again LABC and Cowper's gland for *p,p'*-DDE and LABC and seminal vesicles for VIN. The clear effect of VIN indicates the efficient formation of active metabolites. In two independent experiments, the reference antiandrogen flutamide effectively decreased androgen sensitive tissue weight to the same extent, providing further evidence of the robustness of the protocol.

Another important aspect of the validation phase 2 was to compare the sensitivity of the assay when using a lower (0.2 mg kg^{-1}) dose of TP to restore androgen sensitive tissue weight. In theory, using the lower dose should allow compounds with affinity for the androgen receptor to more readily displace (dihydro)testosterone from its receptor. On the other hand, in the presence of the lower dose of TP, androgen sensitive tissues would be smaller and trimming would be more difficult. A comparison of our data for *p,p'*-DDE and vinclozolin with the published data on validation using 0.2 mg kg^{-1} TP (11) does not suggest differences in sensitivity between the two approaches.

Finally, the contribution of optional organ weight detection and characterisation has to be considered. In line with the findings of the validation phase 1, TP slightly decreased adrenal weights in castrated rats. A comparable change was observed for TREN, but without any dose dependency. The massive increase in the liver weight of *p,p'*-DDE-treated animals reflected the known and strong liver enzyme-inducing properties of this organochlorine compound (12, 13), whereas the increased relative kidney weight in high-dose animals corresponded to the reduced final body weight. The high dose of *p,p'*-DDE, like flutamide, increased adrenal weights in TP-supplemented rats to the level observed in vehicle controls, but the effect was not significant. On the other hand, VIN increased adrenal weights beyond the level of flutamide-treated and vehicle control rats, but only slightly affected liver weights. These findings suggest that potentially specific changes in adrenal weight seem to be small and eventually superimposed by other effects, and that, in general, optional organ weight greatly reflect substance-specific findings and thus cannot contribute to a sensitive and specific detection of compounds with affinity for the androgen receptor.

Histological examination of the liver and thyroid of *p,p'*-DDE-treated animals was performed outside the scope of the OECD protocol. In line with massive liver weight increase, strong hepatocellular hypertrophy was diagnosed, corroborating thus the known liver enzyme-inducing properties of *p,p'*-DDE. The observed thyroid follicular cell hypertrophy in animals treated with 50 mg kg^{-1} *p,p'*-DDE is thus considered a secondary effect due to increased metabolic disposition of thyroid hormones as the consequence of liver enzyme induction (14). *Yamada et al*. (15) reported similar effects for both liver enzyme inducers phenobarbital and *p,p'*-DDE on rat thyroid under conditions of Hershberger assay. Surprisingly, at the high dose of *p,p'*-DDE, we did not observe thyroid follicular cell hypertrophy. This phenomenon remains to be explained. One may speculate that the initial toxic effect of the highest-dose *p,p'*-DDE may have hampered necessary induction of the relevant liver enzyme glucuronyltransferase.

CONCLUSIONS

Our data suggest that the Hershberger bioassay performed in accordance with the OECD protocol is a suitable *in vivo* screen for compounds with the affinity for the androgen receptor: Even weakly active compounds - such as orally administered TREN, moderate androgen receptor antagonist *p,p'*-DDE and VIN, a compound requiring metabolic activation - were readily detected. LABC weight was always among the most sensitive parameters in these studies. Our data also confirm recent findings demonstrating the Wistar rat to be a sensitive rat strain for this assay [16]. Further testing with 17α-methyltestosterone, linuron, procymidone and finasteride and coded testing of reference compounds on an international scale will

provide comprehensive information on the properties of the rodent Hershberger bioassay. Furthermore, our data may also indicate that thyroid effects could be assessed, if the Hershberger bioassay protocol is amended accordingly.

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Sažetak

EVALUACIJA HERSHBERGOVA BIOTESTA NA GLODAVCIMA S POMOĆU TRI REFERENTNA (ANTI) ANDROGENA

Hershbergerov biotest na glodavcima potvrđen je u sklopu OECD-ove međunarodne studije kao in vivo test probira za otkrivanje tvari s afinitetom za androgene receptore. Našem laboratoriju povjereno je provođenje istraživanja s anaboličkim agensom trenbolonom (TREN) te s 1,1-bis-(4-klorfenil)-2,2-dikloretilenom (p,p'-DDE) i vinklozolinom (VIN). Samo visoka doza TREN primijenjena na kastriranim štakorima oralnom intubacijom [0,3 - 1,5 - 8 - 40 mg kg-1 tjelesne mase (t. m.)] tijekom deset dana, jako povećava masu tkiva osjetljivih na androgen. Oralno primijenjeni p,p'-DDE (5 - 16 - 50 - 160 mg kg-1 t. m.) i VIN (0 - 3 - 10 - 30 - 100 mg kg-1 t. m.) tijekom deset dana u štakora koji su dodatno primali testosteron propionat (0,4 mg kg-1 t. m., sc.), izazvali su ovisno o dozi smanjenje težine svih tkiva osjetljivih na androgen. Uz to je p,p'-DDE, također ovisno o dozi, izazvao izrazito povećanje težine jetre i hipertrofiju stanica jetre i folikularnih stanica štitnjače, što je najvjerojatnije posljedica indukcije enzima jetre. Podaci u radu pokazuju da je opisani protokol osjetljiv za probir in vivo te sposoban za otkrivanje slabih (anti)androgena. Nadalje, podaci također upućuju na to da bi se mogli procjenjivati i učinci na štitnjaču ako se ovaj protokol prikladno unaprijedi.

KLJUČNE RIJEČI: *metoda probira in vivo, OECD,* p,p'*-DDE, štitnjača, trenbolon, vinclozolin*

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