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# Evaluation of the rodent Hershberger bioassay: Testing of coded chemicals and supplementary molecular-biological and biochemical investigations☆

A. Freyberger*, H. Ellinger-Ziegelbauer, F. Krötlinger

*Bayer HealthCare AG, Pharmaceuticals, GDD Toxicology, D-42096 Wuppertal, Germany*

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**Abstract**

Under the auspices of the Organization for Economic Cooperation and Development (OECD) the Hershberger assay is being validated as an in vivo screen for compounds with (anti)androgenic potential. We participated in the final activity, the testing of coded chemicals. Test compounds included trenbolone (TREN; 1.5, 40 mg/kg), testosterone propionate (TP; 0.4 mg/kg), flutamide (FLUT; 3 mg/kg), linuron (LIN; 10, 100 mg/kg), 1,1-bis-(4-chlorophenyl)-2,2-dichloroethylene (*p*,*p*′-DDE; 16, 160 mg/kg), and two negative reference substances, i.e., compounds not considered to affect androgen-sensitive tissue weights (ASTWs) in the Hershberger assay, namely 4-nonylphenol (NP; 160 mg/kg) and 2,4-dinitrophenol (DNP; 10 mg/kg); TREN, LIN, *p*,*p*′-DDE, NP, and DNP being used under code. Compounds were administered for 10 days by oral intubation or subcutaneous injection (TP). Additional investigations not mandatorily requested by OECD included organ gravimetry of the liver, gene expression analysis in prostate using quantitative RT PCR for prostate specific binding protein polypeptide C3 (PBPC3) and ornithine decarboxylase 1 (ODC1) and determination of testosterone metabolizing and phase II conjugating enzymes in the liver. After submission of all study reports to OECD by participants uncoding revealed the following results: (A) When assessing androgenic potential in castrated rats, administration of TREN increased the weights of ventral prostate (VP), seminal vesicles (SV), glans penis, levator ani and bulbocavernosus muscles, and Cowper's glands at the high dose. A similar or stronger (VP, SV) increase of ASTWs was observed for TP; NP and DNP were ineffective. TREN dose-dependently increased gene expression of ODC1 and PBPC3, TP induced expression of these genes even more strongly (almost) to the level of untreated intact animals, whereas NP and DNP were inactive. Liver enzyme activities depending on physiological androgen levels were lower in castrated than in intact rats and could not be restored by androgen treatment. (B) When assessing antiandrogenic potential in TP-supplemented castrated rats, administration of LIN and *p*,*p*′-DDE decreased ASTWs only at the high dose. FLUT even more effectively decreased ASTWs, NP and DNP were again without effect. Decreases in androgen-responsive gene expression in the prostate corresponding to the organ weight changes were only observed for *p*,*p*′-DDE (high dose) and flutamide (PBPC3 only). *p*,*p*′-DDE dose-dependently induced liver weights and most liver enzyme activities including androgen-dependent ones. Our study accurately reproduced ASTW changes obtained in previous studies also under code suggesting that the Hershberger assay is a robust tool to screen for an (anti)androgenic potential. Assessment of ODC1 and PBPC3 gene expression in prostate, however, may only represent a sensitive tool for the detection of an androgenic potential. Finally, *p*,*p*′-DDE may affect ASTWs by several mechanisms including enhanced testosterone metabolism.


*Keywords:* Hershberger assay; Testing of coded chemicals; Trenbolone; *p*,*p*′-DDE; Linuron; Flutamide; Nonylphenol; 2,4-Dinitrophenol; Androgen-responsive gene expression; Liver enzyme induction

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* Corresponding author. Fax: +49 202 36 4137.
*E-mail address:* alexius.freyberger@bayerhealthcare.com (A. Freyberger).

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## 1. Introduction

Concerns have been raised that man-made and natural compounds may interfere with the endocrine system and thus may affect wildlife and humans and/or its progeny. The characterization of various environmental chemicals as compounds with affinity for the androgen receptor (AR) showing androgen- or antiandrogen-like activity (Kelce et al., 1994, 1995; Lambright et al., 2000; Wilson et al., 2002; Jenkins et al., 2003; Soto et al., 2004) had prompted a need for a reliable in vivo screen being capable of detecting compounds with affinity for the androgen receptor (AR). The rodent Hershberger bioassay using weight changes of androgen-sensitive tissues in castrated rats as an indicator for an (anti)androgenic potential has been proposed for this purpose both by the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) and by the Task Force on Endocrine Disrupters Testing and Assessment (EDTA) of the Organization for Economic Cooperation and Development (OECD), and this assay is currently being validated in an international effort under the auspices of OECD. Following the demonstration of assay protocol transferability in phase 1 of the validation work using the reference AR agonist testosterone propionate and the reference AR antagonist flutamide (OECD, 2002; Owens et al., 2006), dose response studies on a variety of compounds with weak (anti)androgenic potential were performed in phase 2. The outcome of these studies clearly indicated that the Hershberger assay was capable of reliably detecting weakly active substances (OECD, 2003a; Yamasaki et al., 2003a; Kennel et al., 2004; Freyberger et al., 2005; Owens et al., 2007; Shin et al., 2007). Accordingly, OECD recently initiated "blind testing" as the phase 3 final experimental activity of the validation process in order to further demonstrate the reliability of the Hershberger bioassay, including its sensitivity and specificity with coded samples.

Our laboratory actively engaged in phase 3 of the validation. In addition to the mandatory parameters of the Hershberger assay, i.e., body weight development, clinical signs, and final body and androgen-sensitive tissue weights, we performed several investigations not requested by OECD. Gene expression analysis of androgen-responsive ornithine decarboxylase 1 (ODC1) and prostate specific binding protein polypeptide C3 (PBPC3) in prostate as potential markers of (anti)androgenicity was performed. Recent investigations had indicated that changes in the expression of these genes sensitively reflected interactions with androgenic signalling (Vinggaard et al., 2002). Furthermore, we studied the effects of castration and *p*,*p*′-DDE treatment on liver metabolising enzymes. The results of these studies – as evident after decoding of dose groups by OECD – are described herein.

## 2. Material and methods

### 2.1. Chemicals

Testosterone propionate (purity ≥ 97%), flutamide (≥99%) and coded test chemicals linuron (99%), *p*,*p*′-DDE (≥98.5%), 2,4-dinitrophenol (97%) and 4-nonylphenol (95.6%) were observed through the OECD Chemical Repository at TNO (Nederlandse organisatie voor toegepast-natuurwetenschappelijk oenderzoek, Delft, The Netherlands). For legal reasons, a specific lot (123K1402, 96.6%) of trenbolone had to be obtained from Sigma (Taufkirchen, Germany) and was coded internally.

### 2.2. Animal treatment

Investigations were performed according to the OECD phase 3 protocol (OECD, 2003b). 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, a relative humidity of approximately 55% and a rate of air exchange of ≥10 $h^{-1}$. 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 the laboratory conditions for 1 week until start of treatment. 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 onset of treatment, castrated rats were assigned to vehicle and treatment groups ($n = 6$ each) by a randomized procedure in a way that mean body weights were similar in all groups. Test compounds included trenbolone (TREN; 1.5, 40 mg/kg), testosterone propionate (TP; 0.4 mg/kg), flutamide (FLUT; 3 mg/kg), linuron (LIN; 10, 100 mg/kg), 1,1-bis-(4-chlorophenyl)-2,2-dichloroethylene (*p*,*p*′-DDE; 16, 160 mg/kg), 4-nonylphenol (NP; 160 mg/kg), and 2,4-dinitrophenol (DNP; 10 mg/kg). TP and FLUT, as in previous phases of the validation, served as positive control substances, TP was also used to provide the necessary androgenic stimulus when testing for antiandrogenic potential. TREN, LIN, and *p*,*p*′-DDE had been chosen as coded test compounds, as they had turned out in the phase 2 study to be the least active ones. The two selected doses represented a no/low effect level and a clear effect level, respectively. NP and DNP, considered to be challenging negative reference compounds (for further details see discussion), were tested in a single dose. Test compounds were formulated in corn oil at the beginning of the study according to the instructions provided by TNO and were stored in a refrigerator throughout the study. Formulations which were suspensions were thoroughly