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REGULATORY TOXICOLOGY

P. Andrews · A. Freyberger · E. Hartmann · R. Eiben
I. Loof · U. Schmidt · M. Temerowski · M. Becka

# Feasibility and potential gains of enhancing the subacute rat study protocol (OECD test guideline no. 407) by additional parameters selected to determine endocrine modulation. A pre-validation study to determine endocrine-mediated effects of the antiandrogenic drug flutamide

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**Abstract** Groups of five male and five female Wistar rats were treated by gavage with 0, 1, 10, and 100 mg flutamide/kg body weight for at least 28 days to investigate whether proposed enhancements to the current subacute rodent OECD test guideline no. 407 could be included into the testing routine, which of the current and/or additional parameters would detect endocrine-mediated effects of flutamide reliably and sensitively, and to provide information on intra-laboratory variability. Two identical studies were performed concurrently. The enhanced protocol requests the additional determination of the specific hormones triiodothyronine, thyroxine, thyroid stimulating hormone, follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, prolactin, testosterone, corticosterone; of oestrus cyclicity and necropsy of all females in the dioestrus stage; of the number of homogenization-resistant testicular spermatids and the number, motility, viability, and morphology of cauda epididymal spermatozoa; of additional organ weights (pituitary, ovaries, uterus, thyroid, male accessory reproductive organs); and of the histopathology of additional organs (pituitary, epididymides, coagulation glands, pancreas, vagina). From a technical standpoint, it was possible to conduct a study according to the enhanced protocol, however, with substantial additional effort, an increase in costs by some 67%, and logistic problems. In line with the specific pharmacological effect of flutamide, treatment-related changes were mainly found in male rats, while females were hardly affected by 100 mg/kg. In male rats, 100 mg/kg was the maximal tolerated dose resulting in reduced body weight gain, but no or little other effects on clinical, haematological, clinico-chemical, or behavioral parameters, and 1 mg/kg was the no-observed-adverse-effect level. Antagonism of peripheral androgen receptors by flutamide resulted in decreased relative organ weights of male accessory reproductive organs, changes that were reliably detected in both studies at 100 mg/kg, but only in one of both studies at 10 mg/kg. Corresponding histopathological changes were also detected reliably at 100 mg/kg. Antagonism of central androgen receptors by flutamide increased LH and FSH levels. LH stimulation of testicular Leydig cells in turn increased testosterone and estradiol levels. Again, all these changes were detected reliably at 100 mg/kg, but only in one of both studies at 10 mg/kg. Corresponding histopathological alterations (increase of LH- and FSH-secreting cells, Leydig cell hypertrophy) were detected reliably and sensitively at 10 mg/kg. Studies on liver enzymes performed outside the scope of the enhanced protocol showed that flutamide at 100 mg/kg generally induced hepatic enzyme activcities, but decreased the activity of the sex-specific testosterone-dependent liver enzyme CYP2C11 in male rats. The laboratory methods employed yielded reliable results, i.e., 93.6% of the quantitative measurements obtained in both studies were in agreement. Doubling the animal number from five to ten per sex and dose does not increase the sensitivity of detection of endocrine-mediated effects above the level already provided by histopathological examination of groups of five animals. Some of the proposed enhancements evaluated (additional organgravimetry and histopathology) were helpful in detecting the endocrine-mediated effects of flutamide reliably, while others did not contribute towards this aim (spermatology resulted in doubtful effects, female cyclicity was not affected, hormone determinations provided mechanistic information). Ongoing testing according to the revised version of the enhanced OECD test guideline no. 407 protocol and using ten compounds interfering with the endocrine system by different mechanisms will result in the identification of the most appropriate enhancements.

P. Andrews (✉) · A. Freyberger · E. Hartmann · R. Eiben
I. Loof · U. Schmidt · M. Temerowski
BAYER AG, PH PD Toxicology, D-42096 Wuppertal, Germany
E-mail: peter.andrews.pa@bayer-ag.de
Fax: +49-202-368407

M. Becka
BAYER AG, PH PD Biometry and Pharmacometry, D-42096 Wuppertal, Germany

**Key words** Enhanced OECD test guideline no. 407 · Feasibility · Detection of endocrine effects · Flutamide · Antiandrogenicity

## Introduction

The detection of interactions of chemicals with the endocrine system has become a topic of major priority in recent years. In response to concerns that current toxicological test guidelines may not address this issue sufficiently, EMSG (the endocrine modulator study group) of CEFIC (council of the European chemical industry) has proposed enhancements to the current subacute rodent OECD test guideline no. 407 (OECD 1995) by parameters thought to be effective in detecting endocrine modulation. The enhancements have been discussed at the international level under the umbrella of OECD, and the resulting final version of the enhanced protocol (OECD 1999) was the basis for this pre-validation study. The enhanced protocol requests the additional determination of specific hormones: triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, prolactin, testosterone, corticosterone; oestrus cyclicity from vaginal smears starting in exposure-week 3 and necropsy of all females in the dioestrus stage of the oestrus cycle; the number of homogenization-resistant testicular spermatids and the number, motility, viability, and morphology of cauda epididymal spermatozoa; organ weights of ovaries, uterus, thyroid, male accessory reproductive organs and pituitary; and histopathological investigation of pituitary, epididymides, coagulation glands, pancreas, and vagina.

Our laboratory was entrusted with performing a prevalidation study with the antiandrogenic drug flutamide. The doses were selected as stipulated by the test guideline, i.e., the high dose was chosen to be in the range of an MTD (maximum tolerated dose) while the low dose was chosen to result in a NOAEL (no-observed-adverse-effect level).

Main aspects of the study were: (1) to investigate whether it would be feasible to include all enhancements into the testing routine, (2) to determine which of the current and/or additional parameters will detect the endocrine effects of flutamide reliably and sensitively, and (3) to assess intra-laboratory variability by performing two identical studies in parallel using groups of five animals each per dose and sex. In addition and outside the scope of the enhanced protocol, effects of flutamide on liver enzymes were investigated.

## Materials and methods

### Test chemical

Flutamide [4'-nitro-3'-(trifluoromethyl)-isobutyranilide, CAS no. 13311-84-7, purity 99.9%] was obtained from Salutas Pharma, Barleben, Germany. It was formulated in a 2% aqueous solution of Cremophor EL, and the formulations for administration were chemically stable for 4 days.

### Animals

Healthy male and female SPF-bred Wistar rats (Hsd Cpb:WU) were kept individually in polycarbonate cages and fed a commercial pelletized rodent feed (Altromin 1324). They were 7 weeks old at initiation of treatment. The animal room had a 12-h light/dark regime, a temperature of $22 \pm 2°C$, a relative humidity of approximately 55%, and a rate of air exchange of 15 to 20 changes per hour.

### Experimental design

The study design followed the enhanced protocol (OECD 1999). Groups of five male and five female rats were orally gavaged with 0, 1, 10, or 100 mg/kg (mg flutamide/kg body weight per day) using a dose volume of 10 ml/kg for at least 28 days. Two identical studies (designated A and B) were run concurrently in order to verify effects occurring in both studies as being reliable, while effects only occurring in one of both studies were regarded as incipient at best if identical changes were seen at a higher dose level or as doubtful if not corroborated at a higher dose level.

### Laboratory methods

Blood was obtained under $O_2/CO_2$ anesthesia (20%/80%). Blood for hormone determinations and hematology of male rats was taken from the orbital vein plexus in the morning of the day of necropsy (7:40–7:56 a.m.), while blood for clinical chemistry was obtained by cardiac puncture at necropsy. Blood of female rats for all determinations was obtained by cardiac puncture at the time of necropsy (10:06–11:45 a.m.). Blood for glucose determinations was taken from the tail vein of fed, but non-anesthetized animals in the morning of study day 25. Standard hematological and clinical chemical parameters, subject to continuous quality control, were used to determine the hematological parameters, enzymes, electrolytes, substrates, and products of metabolism.

7-ethoxycoumarin (ECOD) and 7-ethoxyresorufin (EROD) dealkylase, aldrinepoxidase (ALD), epoxide hydrolase (EH), glutathione-S-transferase (GS-T), and UDP-glucuronyltransferase (GLU-T) and in vitro testosterone hydroxylation were determined on liver tissue samples of study A using published methods (Ullrich and Weber 1972; Bock 1974; Habig et al. 1974; Burke et al. 1977; Wolff et al. 1979; Guiliano et al. 1980; Oesch et al. 1992).

### Behavioral tests

Fore- and hindlimb grip-strength determinations (Meyer et al. 1979), manipulative tests (approach response, touch response, auditory response, tail pinch response, righting reflex, pupillary reflex; Moser 1989), and motor-activity assessments in a figure-8 maze (Moser 1989) were performed in study week 3.

### Hormone determinations

All hormone determinations were conducted in duplicate from serum samples taken within a narrow time span in order to minimize any circadian effects on the results. Total T3 and T4 and TSH were determined using enzyme luminescence immunometric tests together with the other clinical chemical parameters. Corticosterone, estradiol, FSH, LH, prolactin, and testosterone were determined using radioimmunoassays by Phoenix International McKnight, Hamburg, Germany.

### Oestrus-cycle staging

Vaginal smears were taken daily from day 18 through to day 32, stained with May-Gruenwald stain and classified microscopically as dioestrus, prooestrus, oestrus, or metoestrus.

### Spermatology

In week 5, motility (100 spermatozoa in HAM's F10 tissue culture medium at 37°C), viability (decrease in motility during 5 min), morphology (200 formalin-fixed, Eosin-G-stained spermatozoa), and number (heated to 70°C) of spermatozoa were determined from the right epididymis. The number of homogenization-resistant (0.9% NaCl solution plus Triton-X100) spermatid heads was determined from the right testis.

### Necropsy

Animals were necropsied after exsanguination under deep anesthesia induced by inhalation of $O_2/CO_2$ from day 28–32. Females were killed beginning at day 28, provided they were in the dioestrus stage of the oestrus cycle. One male rat per group and study was necropsied per day from day 28 through 32.

### Organgravimetry

Organ weights were taken of fresh organs at necropsy [brain, heart, lungs, kidneys, liver, spleen, thymus, thyroid (left, the right lobe was left untouched for histopathology), adrenals, testis (left, right testis weight taken during spermatology), epididymis (left), male accessory sex organs (seminal vesicles plus coagulation glands plus ventral and dorso-lateral prostatic lobes), prostate (ventral lobe), ovaries and uterus], or after organ fixation [pituitary gland, seminal vesicles plus coagulation glands, and ventral prostatic lobe].

### Histopathology

Eyes, kidneys, and right testes were fixed in Davidson's solution. Other organs were fixed in 10% neutral buffered formalin (lungs after instillation). Routine techniques were employed to prepare paraffin sections that were stained with hematoxylin and eosin. Sections of the pituitary gland and the liver were additionally stained with periodic acid Schiff reaction or oil red O, respectively. The following organs were examined: brain, liver, pancreas, kidneys, left testis, left epididymis, dorso-lateral and ventral prostate, coagulation glands, seminal vesicles, ovaries, uterus, vagina, pituitary gland, thyroid gland, parathyroid glands, adrenal glands, thymus, mammary gland, and skin.

### Statistics

Dunnett's test was used for body weight, feed intake, water consumption, organ weight, and liver tissue data. The Kruskal-Wallis test followed by the adjusted U test, analysis of variance (ANOVA) followed by Dunnett's test or the adjusted Welsh test were used for hematological and clinical chemical data. Grip strength data and locomotor activity measurements were analyzed by ANOVA. A repeated-measurements analysis approach was used for the individually determined locomotor-activity habituation curves. After transformation, the corresponding profile for each animal was characterized by a straight line, its slope and intercept. Slopes and intercepts were investigated for treatment differences, individually and together, using a multivariate ANOVA. Likewise, hormone data were analyzed by ANOVA. In order to compare oestrus-cycle patterns, the individual time axes were shifted providing each animal with a dioestrus starting stage at a virtual day 0, followed by different stages on subsequent relative virtual days. Spermatological data (spermatozoa per mg epididymis, spermatid heads per mg testis, spermatozoa viability) were analyzed by ANOVA. Viability was analyzed in terms of percentage change in motility from minute 1 to minute 5.

The results of the manipulative behavioral tests and the incidences of abnormal spermatozoa and of histopathological findings were not subjected to statistical analysis.

## Results

All animals survived to necropsy. Comparable clinical signs that were attributable to the treatment (piloerection, reduced motility, increased and bright colored feces) were only observed at 100 mg/kg in studies A and B. Feed consumption (g/day or g/kg body weight) was reduced in all high-dose groups during week 1. In addition, feed consumption (g/day) was also reduced in high-dose males of study A in week 4. Mean water consumption (g/kg body weight) was increased in high-dose

**Table 1** Effect of flutamide treatment on body weights and relative organ weights in male rats. *ASO* Male accessory sex organs (prostate, seminal vesicles, coagulating glands), *remainder of ASO* ASO without ventral prostate. Mean values and standard deviations are given. Tissues were weighed fresh (pituitary: after fixation)

| | Control | | 1 mg/kg | | 10 mg/kg | | 100 mg/kg | |
|---|---|---|---|---|---|---|---|---|
| | Study A | Study B | Study A | Study B | Study A | Study B | Study A | Study B |
| Body weights (g) | | | | | | | | |
| Initial | 215 ± 8 | 212 ± 5 | 211 ± 7 | 207 ± 8 | 212 ± 10 | 207 ± 10 | 213 ± 7 | 214 ± 9 |
| Terminal | 352 ± 15 | 327 ± 19 | 318 ± 32 | 311 ± 36 | 338 ± 38 | 313 ± 32 | 308 ± 17 | 288 ± 20 |
| Organ weights (mg/100 g) | | | | | | | | |
| Liver | 4348 ± 296 | 4124 ± 330 | 4110 ± 142 | 4267 ± 262 | 4332 ± 204 | 4284 ± 339 | 5359 ± 593** | 5383 ± 182** |
| Pituitary | 3 ± 0.2 | 3 ± 0.4 | 3 ± 0.1 | 3 ± 0.5 | 3 ± 0.3* | 3 ± 0.4 | 4 ± 0.3** | 3 ± 0.8 |
| Adrenals | 20 ± 3 | 19 ± 2 | 22 ± 1 | 21 ± 3 | 21 ± 2 | 24 ± 5 | 24 ± 3 | 24 ± 3* |
| Testis, left | 469 ± 12 | 485 ± 24 | 494 ± 41 | 421 ± 96 | 462 ± 39 | 499 ± 42 | 490 ± 5 | 496 ± 39 |
| Epidydimis, left | 185 ± 20 | 198 ± 12 | 186 ± 18 | 179 ± 29 | 152 ± 20 | 166 ± 19 | 133 ± 35** | 160 ± 18** |
| ASO | 597 ± 10 | 590 ± 32 | 526 ± 97 | 553 ± 110 | 426 ± 47* | 485 ± 106 | 137 ± 26** | 132 ± 36** |
| Ventral prostate | 124 ± 16 | 121 ± 26 | 92 ± 14 | 90 ± 11 | 92 ± 21 | 93 ± 10 | 32 ± 14** | 30 ± 21** |
| Remainder of ASO | 473 ± 104 | 467 ± 37 | 433 ± 86 | 462 ± 102 | 333 ± 38* | 391 ± 108 | 103 ± 14** | 101 ± 21** |

*$P < 0.05$, **$P < 0.01$

**Table 2** Plasma hormone levels in male animals treated with flutamide. Least square means and confidence intervals are given. *LH* Luteinizing hormone, *FSH* follicle stimulating hormone, *TSH* thyroid stimulating hormone, *T3* triiodothyronine, *T4* thyroxine

| | Control | | 1 mg/kg | | 10 mg/kg | | 100 mg/kg | |
|---|---|---|---|---|---|---|---|---|
| | Study A | Study B | Study A | Study B | Study A | Study B | Study A | Study B |
| LH (ng/ml) | 0.37 | 0.60 | 0.32 | 0.73 | 1.06 | 1.40* | 2.52* | 2.71** |
| | (0.20–0.69) | (0.44–0.81) | (0.17–0.60) | (0.54–0.99) | (0.57–2.00) | (1.03–1.90) | (1.34–4.73) | (2.00–3.68) |
| FSH (ng/ml) | 1.03 | 1.54 | 1.59 | 2.26 | 4.80* | 5.13 | 8.14** | 9.19* |
| | (0.58–1.83) | (0.83–2.87) | (0.90–2.81) | (1.21–4.22) | (2.71–8.49) | (2.75–9.56) | (4.60 –14.40) | (4.93–17.13) |
| Prolactin (ng/ml) | 21.0 | 17.0 | 22.4 | 19.7 | 25.7 | 29.1 | 25.3 | 21.2 |
| | (10.1–43.4) | (9.4–30.7) | (10.8–46.4) | (10.9–35.7) | (12.4–53.1) | (16.1–52.6) | (12.2–52.4) | (11.7–38.4) |
| TSH (µg/l) | 1.46 | 3.01 | 2.32 | 2.40 | 3.25 | 2.25 | 3.98 | 1.93 |
| | (0.85–2.52) | (1.25–7.24) | (1.34–4.01) | (1.00–5.78) | (1.88–5.62) | (0.94–5.43) | (2.31–6.88) | (0.80–4.65) |
| Testosterone (ng/ml) | 0.58 | 1.27 | 1.82 | 2.44 | 6.23** | 4.95 | 9.62** | 11.53* |
| | (0.30–1.12) | (0.62–2.58) | (0.94–3.54) | (1.20–4.98) | (3.20–12.10) | (2.43–10.10) | (4.95–18.69) | (5.65–23.53) |
| Estradiol (pg/ml) | 93.1 | 99.4 | 95.5 | 104.1 | 96.2 | 100.8 | 111.2** | 115.2* |
| | (88.7–97.6) | (95.2–103.9) | (91.1–100.2) | (99.7–108.7) | (91.7–100.9) | (96.5–105.3) | (106.0–116.6) | (110.34–120.4) |
| T3 (nmol/l) | 1.89 | 1.85 | 1.86 | 1.84 | 1.87 | 1.89 | 1.66 | 1.66 |
| | (1.75–2.04) | (1.74–1.97) | (1.72–2.01) | (1.73–1.96) | (1.73–2.02) | (1.77–2.01) | (1.54–1.79) | (1.56–1.77) |
| T4 (nmol/l) | 52.1 | 46.2 | 51.3 | 55.6 | 56.7 | 50.7 | 38.4* | 38.2 |
| | (46.7–58.0) | (41.3–51.6) | (46.0–57.1) | (49.7–62.2) | (50.9–63.2) | (45.3–56.7) | (34.4–42.8) | (34.10–42.68) |
| Corticosterone (ng/ml) | 159.6 | 183.9 | 165.2 | 116.3 | 66.6 | 268.9 | 162.0 | 136.6 |
| | (77.2–329.9) | (93.3–362.5) | (79.9–341.4) | (59.0–229.4) | (32.2–137.8) | (136.4–530.2) | (78.4–334.7) | (69.3–269.4) |

*$P < 0.05$, **$P < 0.01$

animals (by 14% and 27% in males and females of study A, and by 25 % and 34% in males and females of study B). In study A, body weights were reduced throughout the study in high-dose males and in week 1 in high-dose females. In study B, body weights were only transiently reduced (weeks 1–3) in high-dose males. Body-weight gains were biologically significantly and consistently reduced in high-dose males (–44% in study A, –38% in study B). In females, a comparable effect (–18%) was only seen in study A. Initial and terminal body weight group means of male rats are shown in Table 1.

## Behavioral testing

Reflex testing, grip-strength measurements, and locomotor-activity profiles did not provide any indication of treatment-related effects in studies A or B up to 100 mg/kg. At this dose, there were minor effects on grip strength (reductions in studies A and B) and locomotor activity (more rapid habituation in study A) of males that had experienced a decrement in body weight gain. Thus, these minor changes were regarded as sequelae of general toxic effects (data not shown).

## Hematological and clinical chemical findings

The determination of hematological and clinical-chemical parameters required by the enhanced protocol resulted only in a few changes. Changes in hematological parameters were marginal in extent and restricted to the red blood at 100 mg/kg. They were statistically significant only in either male or female rats and only in either study A or study B. Changes in clinical chemical parameters that were regarded as treatment-related occurred in studies A and B: increase in total bilirubin (not in males of study A) and albumin levels in both sexes at 100 mg/kg and reduction in inorganic phosphorus in males at ≥10 mg/kg (data not shown).

## Changes in hormone levels

Analysis of variance of hormone data did not reveal treatment-related effects in female rats. In male rats, levels of LH, FSH, and testosterone were elevated at ≥10 mg/kg or at 100 mg/kg in either study A or study B. Estradiol levels were elevated at 100 mg/kg in both studies, and T4 levels were reduced at 100 mg/kg in study A. Tables 2 and 3 show the least square mean values calculated during analysis of variance.

## Effects on oestrus cyclicity

The general aim of oestrus-cycle staging performed from study day 18 until necropsy was to assess possible effects of treatment on the female cycle. Analysis of the oestrus-

**Table 3** Plasma hormone levels in female animals treated with flutamide. Least square means and confidence intervals are given. *Dashes* indicate that values were below the limit of quantification. *LH* Luteinizing hormone, *FSH* follicle stimulating hormone, *TSH* thyroid stimulating hormone, *T3* triiodothyronine, *T4* thyroxine

| | Control | | 1 mg/kg | | 10 mg/kg | | 100 mg/kg | |
|---|---|---|---|---|---|---|---|---|
| | Study A | Study B | Study A | Study B | Study A | Study B | Study A | Study B |
| LH (ng/ml) | 1.77 | 1.88 | 1.92 | 1.87 | 1.57 | 1.68 | 2.33 | 1.72 |
| | (1.13–2.76) | (1.49–2.38) | (1.23–2.99) | (1.48–2.36) | (1.01–2.45) | (1.33–2.12) | (1.50–3.64) | (1.36–2.17) |
| FSH (ng/ml) | 1.28 | 2.14 | 1.09 | 2.38 | 0.63 | 1.78 | 1.52 | 1.71 |
| | (0.79–2.09) | (1.59–2.86) | (0.67–1.78) | (1.78–3.19) | (0.38–1.02) | (1.33–2.38) | (0.93–2.48) | (1.23–2.37) |
| Prolactin (ng/ml) | 111.0 | 122.8 | 128.0 | 85.8 | 103.8 | 71.2 | 89.1 | 71.2 |
| | (72.1–170.7) | (80.8–186.6) | (83.2–196.9) | (56.5–130.4) | (67.5–159.7) | (46.8–108.2) | (57.9–137.0) | (46.9–108.2) |
| TSH (µg/l) | 1.46 | 1.85 | 1.60 | 2.61 | 1.23 | 1.88 | 2.32 | 1.92 |
| | (0.99–2.15) | (1.24–2.76) | (1.09–2.37) | (1.75–3.90) | (0.83–1.82) | (1.26–2.80) | (1.57–3.43) | (1.29–2.86) |
| Testosterone (ng/ml) | – | – | – | – | – | – | – | – |
| Estradiol (pg/ml) | 120.2 | 118.8 | 123.0 | 128.7 | 115.5 | 127.9 | 114.9 | 133.2 |
| | (112.5–128.4) | (109.3–128.9) | (115.1–131.4) | (118.6–139.8) | (108.1–123.4) | (117.8–138.9) | (107.6–122.8) | (122.7–144.6) |
| T3 (nmol/l) | 1.86 | 2.04 | 1.84 | 2.02 | 1.89 | 2.04 | 1.86 | 1.96 |
| | (1.73–1.99) | (1.84–2.26) | (1.72–1.98) | (1.82–2.24) | (1.76–2.03) | (1.84–2.26) | (1.73–1.99) | (1.77–2.18) |
| T4 (nmol/l) | 37.2 | 34.7 | 28.8 | 40.7 | 32.5 | 36.4 | 29.3 | 34.9 |
| | (30.8–44.9) | (29.7–40.5) | (23.8–34.7) | (34.9–47.5) | (26.9–39.2) | (31.2–42.5) | (24.3–35.4) | (29.9–40.7) |
| Corticosterone (ng/ml) | 94.2 | 66.9 | 83.2 | 91.9 | 66.2 | 68.6 | 91.6 | 83.6 |
| | (42.5–209.2) | (38.4–116.5) | (37.5–184.7) | (52.7–160.1) | (29.8–147.0) | (39.4– 119.6) | (41.3–203.2) | (48.0–145.7) |

cycle staging data did not reveal statistically significant changes between treatment groups in studies A and B. The specific aim of the oestrus-cycle staging was to ensure that all females were in the dioestrus stage when sacrificed and when blood samples were taken for hormone determinations. Achievement of this aim was corroborated by histopathology of the vaginal epithelium for all but two females of study B, which were histologically classified as being in oestrus or in prooestrus (data not shown).

## Spermatological findings

Matching results were obtained in studies A and B. There were no treatment-related changes in spermatological parameters in males up to 10 mg/kg. The only statistically significant change at 100 mg/kg was reduced spermatozoa viability in study B. Qualitatively, there was an increase in the frequency of abnormal spermatozoa at 100 mg/kg, which was more clearly seen in study B than in study A. The abnormalities observed (curved sperms, isolated or amorphous sperm heads, loops or sharps at various positions along the tail, wriggled or snaky tails, and also the lack of a yolk sac at the middle of the tail) all belong to the changes normally found in rats, albeit with a lower frequency (Table 4).

## Observations at necropsy

There was a trend towards size reductions of prostate and seminal vesicles at 1 mg/kg in studies A and B. The livers of 100 mg/kg females of study B were enlarged.

## Effects on organ weights

Only weight-normalized (relative) organ weights were considered. Liver weights were increased in high-dose males and females. All other changes were restricted to male rats.

Comparable statistically significant reductions in prostate weights (by approximately 75%) were obtained in studies A and B only at 100 mg/kg, but a non-dose-dependent trend was already recognizable at ≥1 mg/kg (reduction by approximately 25%). Statistically significant reductions in the weights of the male accessory-reproductive-organ complex were observed in all high-dose males and in study A at 10 mg/kg, which also had a statistically significantly reduced weight of seminal vesicles plus coagulation glands. The apparently statistically significant increase in pituitary-gland weight of male rats of study A at ≥10 mg/kg was considered a spurious result not reliably attributable to the treatment. Changes in male relative organ weights are given in (Table 1).

**Table 4** Effect of flutamide treatment on spermatological parameters. For abnormal spermatozoa and motility in min 1 and 5, mean values are given. All other data represent least square means and confidence intervals

| | Control | | 1 mg/kg | | 10 mg/kg | | 100 mg/kg | |
|---|---|---|---|---|---|---|---|---|
| | Study A | Study B | Study A | Study B | Study A | Study B | Study A | Study B |
| Abnormal spermatozoa (%) | | | | | | | | |
| Percent change | 1.3 | 1.3 | 1.0 | 1.3 | 1.6 | 1.4 | 3.1 | 7.9 |
| Sperm viability | | | | | | | | |
| Motility in min 1 (%) | 80 | 90 | 81 | 87 | 78 | 78 | 47 | 46 |
| Motility in min 5 (%) | 78 | 77 | 78 | 97 | 69 | 71 | 51 | 14 |
| Percent change | +3.0 | –13.4 | –2.1 | –9.0 | –11.0 | –8.8 | +1.4 | –60.9* |
| | (–18.1–24.1) | (–35.1–8.2) | (–23.2–19.0) | (–30.7–12.6) | (–32.1–10.1) | (–30.5–12.8) | (–19.7–22.5) | [–82.5–(–39.2)] |
| Epididymal spermatozoa counts ($10^3$ spermatozoa/mg) | | | | | | | | |
| Right epididymis | 602 | 687 | 705 | 701 | 780 | 901 | 498 | 270 |
| | (398–805) | (460–915) | (502–909) | (473–928) | (577–984) | (673–1128) | (294–701) | (43–498) |
| Testicular spermatid counts ($10^3$ spermatids/mg testis) | | | | | | | | |
| Right testis | 55.5 | 54.2 | 56.9 | 55.5 | 49.8 | 53.5 | 49.9 | 47.7 |
| | (47.3–63.8) | (47.5–60.8) | (8.7–65.2) | (48.9–62.2) | (41.5–58.1) | (46.8–60.1) | (41.6–58.1) | (41.0–54.3) |

*$P < 0.05$

### Histopathological findings

There were no treatment-related effects in organs other than those specifically mentioned below. Results from both studies were almost identical. At 100 mg/kg, centrilobular hepatocellular hypertrophy and cytoplasmic change were found in both sexes of studies A and B. In addition, in study A, a slightly increased mitotic rate was observed in hepatocytes of male rats (data not shown). All other changes referred to endocrine tissues and are summarized in Table 5. The most sensitive alterations observed in male rats at ≥10 mg/kg in both studies were diffuse Leydig cell hypertrophy and increased numbers of PAS-positive cells in the pituitary gland. At 100 mg/kg, spermatic debris were found in the epididymides only in study A, whereas all other changes occurred both in studies A and B: hypertrophy of basophilic pituitary cells (adenohypophysis) and microvesicular cytoplasm of the adrenal zona fasciculata in males; reduction of secretory products and flattened epithelia in the prostate (dorsolateral and ventral), the coagulation glands, and the seminal vesicles; increased interstitial tissue in the epididymes and a minimal increase in interstitial ovarian glands.

### Effects on liver enzymes

Although not required by the enhanced protocol, the effect of flutamide on hepatic metabolizing enzymes was studied in study A. The high dose of flutamide induced cytochrome P-450-dependent monooxygenases, such as CYP 1A1 (EROD) and 2B1 and 2D1 (ECOD), EH, and conjugating enzymes (GS-T, GLU-T) in the liver. The activities of ECOD, EROD, EH, GS-T, and GLU-T were increased in males (to 164, 382, 373, 143, and 193%) and in females (to 376, 247, 126, 555, 222, and 449%), whereas ALD (partly reflecting CYP2C11) was strongly reduced in males (data not shown). The effect of flutamide treatment on liver-mediated in vitro testosterone hydroxylation in males is shown in Fig. 1. Treatment with 100 mg/kg resulted in strongly decreased 2α- and 16α-hydroxylation and less pronounced reduction of 6ß-hydroxylation and androstenedione formation. In females (data not shown), 6α-, 16ß-, 2α-, and 2ß-hydroxylation was typically undetectable at ≤10 mg/kg, but it was clearly demonstrable at 100 mg/kg. Relevant changes of 7α-, 6ß-, and 16α-hydroxylation were only observed at the high dose with enzyme activities being increased to 127, 479, and 260% of corresponding controls (data not shown).

## Discussion

The main objective of this study was to investigate on a quantitative and qualitative basis whether the proposed enhancements to the OECD test guideline no. 407 could,

**Table 5** Sensitivity of selected endocrine-related parameters in studies A and B ($n = 5$ each) and a combined approach ($n = 10$). *Arrows* indicate increase or decrease of a given parameter. *ASG* Accessory sex organs, ⊕ changes detected

| Dose (mg/kg body weight) | | 1 | | | 10 | | | 100 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Study | | A | B | A and B | A | B | A and B | A | B | A and B |
| Changes in relative organ weights | | | | | | | | | | |
| Male pituitary (↑) | | | | | * | | | ** | | |
| Male accessory sex organs (↓) | | | | | * | | ** | ** | ** | ** |
| Ventral prostate (↓) | | | | | | | | ** | ** | ** |
| Remainder of ASG (↓) | | | | | * | | ** | ** | ** | ** |
| Epididymis (↓) | | | | | | | * | * | ** | ** |
| Male adrenal glands (↑) | | | | | | | * | | * | ** |
| Histology | | | | | | | | | | |
| Male pituitary | PAS-positive cells | | | | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| | Intracytoplasmic inclusions and hypertrophic basophilic cells | | | | | | | ⊕ | ⊕ | ⊕ |
| Testis | Leydig cell hypertrophy | | | | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| Prostate (all parts) | Reduction of secretory products, flattened epithelium, and reduced acinar size | | | | | | | ⊕ | ⊕ | ⊕ |
| Coagulating glands | As in prostate | | | | | | | ⊕ | ⊕ | ⊕ |
| Seminal vesicles | Reduction of secretory products, flattened epithelium, and reduced luminal size | | | | | | | ⊕ | ⊕ | ⊕ |
| Epididymis | Decreased tubular size and increased interstitial tissue | | | | | | | ⊕ | ⊕ | ⊕ |
| | Increased spermatic debris | | | | | | | ⊕ | | |
| Male adrenals | Microvesicular cytoplasmic vacuoles in zona fasciculata | | | | | | | ⊕ | ⊕ | ⊕ |
| Ovaries | Minimally increased incidence of increased interstitial glands | | | | | | | ⊕ | ⊕ | ⊕ |
| Hormone determinations in males | | | | | | | | | | |
| Luteinizing hormone (↑) | | | | | | * | * | * | ** | ** |
| Follicle stimulating hormone (↑) | | | | | * | | ** | ** | * | ** |
| Testosterone (↑) | | | | | ** | | ** | ** | * | ** |
| Estradiol (↑) | | | | | | | | ** | * | ** |
| Thyroxine (↓) | | | | | | | | * | | * |

*$P < 0.05$; **$P < 0.01$

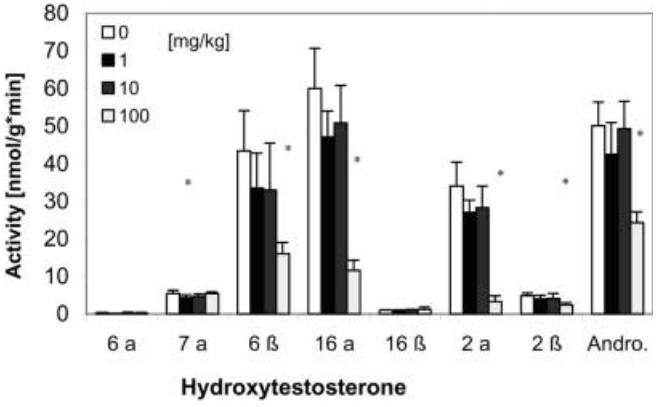


**Fig. 1** Effect of flutamide treatment on in vitro testosterone hydroxylation and androstenedione formation by male rat liver tissue. *Asterisks* indicate a significant changes at the $P < 0.05$ level. *Andro,* Androstenedione

firstly, be included into the testing routine and, secondly, which of the current and/or additional parameters would detect the endocrine-mediated effects of flutamide reliably and sensitively. Thirdly, as the studies were performed in duplicate, this provided information on intra-laboratory variability and enabled us to assess what might be gained by using more than five animals per sex and dose as stipulated by the guideline.

It was possible to conduct a study according to the enhanced protocol, however, with substantial additional effort, costs (an increase by approximately 2/3), and logistic problems. The main logistic draw back was the requirement to necropsy females in the dioestrus stage; thus, necropsy was spread over five days, which about doubled the time required for necropsy, as preparatory activities and subsequent clearing work have to be taken into account.

Stress is a major factor modulating hormone levels. Animals were anesthetised with 20% oxygen in carbon dioxide for blood sampling in order to minimize stress. Evidence that the procedure employed did not stress the animals (as does diethylether anesthesia) can be obtained by comparing the control levels obtained in this investigation (prolactin 20–29 ng/ml, corticosterone 69–269 ng/ml) with published data for levels of the stress-prone hormones of unstressed male rats (prolactin range: 5–30 ng/ml, Conybeare et al. 1988; Hagmüller et al. 1992; corticosterone approximately 130 ng/ml, Gärtner et al. 1980).

Organgravimetry and preservation of delicate structures for histopathological examination were sometimes difficult. Therefore, it is suggested to isolate, trim, and weigh organs after fixation that are delicate and/or difficult to isolate, especially the thyroids (to be fixed attached to the trachea), the pituitary gland, and the complex of prostate, seminal vesicles and coagulation gland, which can easily be separated after fixation.

Flutamide, and more pronouncedly its metabolite hydroxy-flutamide, antagonize the actions of testosterone at the androgen receptor level (Simard et al. 1986). In line with this specific pharmacological effect of flutamide, treatment-related changes were mainly found in male rats. Antagonism of peripheral androgen receptors resulted in decreased relative organ weights of androgen-sensitive tissues, namely epididymis and male accessory sex organs (ventral prostate and seminal vesicles plus coagulation glands). These changes were reliably detected in both studies at 100 mg/kg, while at 10 mg/kg significant changes were only observed in one of both studies (Table 1). Histopathological changes in these tissues were also detected reliably at 100 mg/kg (Table 5). Toyoda et al. (2000) had reported flutamide-induced histological changes in the male mammary gland of Crj:CD(SD)IGS rats as the consequence of androgen-receptor antagonism. However, such changes were not observed in this study, thus suggesting a strain-specific alteration.

In correlation with antagonism of central androgen receptors and with the expected activation of the hypothalamic-hypophysal-gonodal axis (Viguier et al. 1983; Chandolia et al. 1991), flutamide increased LH and concomitantly FSH levels. Subsequent to increased LH stimulation of testicular Leydig cells, increased levels of testosterone were observed. As testosterone is the precursor of estradiol, increased testosterone concentrations triggered an increase of estradiol levels (O'Connor et al. 1999). These changes were detected reliably at 100 mg/kg, while detection was achieved in only one of both studies at 10 mg/kg (Table 2). Corresponding histological alterations were the increased number of LH- and FSH-secreting (PAS-positive) cells and Leydig-cell hypertrophy, which were both detected reliably at ≥10 mg/kg (Table 5).

Although there were substantial endocrine-mediated effects on the testes and the accessory male sex organs, only doubtful alterations in spermatology (reduction in sperm motility) resulted from treatment of male rats at 100 mg/kg. In contrast to a previous report (Toyoda et al. 2000), there were no changes in oestrus cyclicity, therefore suggesting that rat strains may differ in their sensitivity to flutamide.

In line with the histopathological findings, flutamide at 100 mg/kg generally induced cytochrome P-450-dependent monooxygenases, epoxide hydrolase, and conjugating enzymes in the liver in both genders. The induction of UDP-glucuronyltransferase should result in increased metabolic disposition of thyroid hormones, thus explaining the moderately lowered T4 levels in male rats (Curran and DeGroot 1991). A possibly peripheral endocrine-mediated effect of flutamide has been detected in liver tissue when looking at the activity of the sex-specific, testosterone-dependent liver enzyme CYP2C11 (represented by ALD, 16α-, and 2α-testosterone hydroxylation). This enzyme was strongly decreased in male rats at 100 mg/kg, obviously reflecting antiandrogenicity of flutamide at the liver enzyme level (Fig. 1). Comparable changes in testosterone hydroxylation were also observed at postnatal day 21 following in utero exposure to flutamide (You et al. 1999).

In male rats of both studies, 100 mg/kg was the MTD resulting in reduced body-weight gain, but no or little other effects on clinical, hematological, clinico-chemical, or behavioral parameters, whereas 1 mg/kg represented the NOAEL. Thus, doses had been selected as required by OECD test guideline no. 407. In female rats, a NOAEL of 10 mg/kg was determined, indicating that females rats were less susceptible in both studies.

In the course of this study, 83 parameters were determined quantitatively in the three treatment groups, each comprising five male and five female animals, thus $83 \times 3 \times 2 = 498$ group mean values were obtained per study. In 32 of these 498 pairs of individual results (or 6.4%), differences occurred between studies, while 93.6% of the quantitative measurements were in agreement, thus attesting to the general reliability of the methods employed. This rate of 6.4% disparate results is close to the 5% false-positive results expected to result from significance testing at the 95% confidence level. Doubling the animal number from five to ten per sex and dose did not increase the sensitivity of detection of endocrine-mediated effects above the level already obtained by histopathological examination of groups of five animals (Table 5).

Only some of the enhancements to OECD test guideline no. 407 evaluated in this pre-validation study (additional organs weights and additional histopathological investigations) were helpful in detecting the endocrine-mediated effects of flutamide. Other proposed enhancements did not contribute towards this aim: spermatology resulted in doubtful effects at the high-dose level only, female cyclicity was not affected, and hormone determinations provided mechanistic information, but did not increase sensitivity.

Care must be taken that only such end points will be included in the future version of OECD test guideline no. 407 that are proven to contribute to a reliable and sensitive detection of potential endocrine-mediated effects of an unknown test substance. The scope of hormone determinations, spermatological examinations, and the time period for which oestrus-cycle observations are required has already been reduced for the current validation phase 2 of the enhanced protocol (OECD 2000). This phase-2 investigation is currently underway in 13 laboratories in different countries with ten compounds known or suspected to interact with the endocrine system through different mechanisms. Evaluation of the results of these studies will provide a basis for the identification of the most appropriate enhancements.

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