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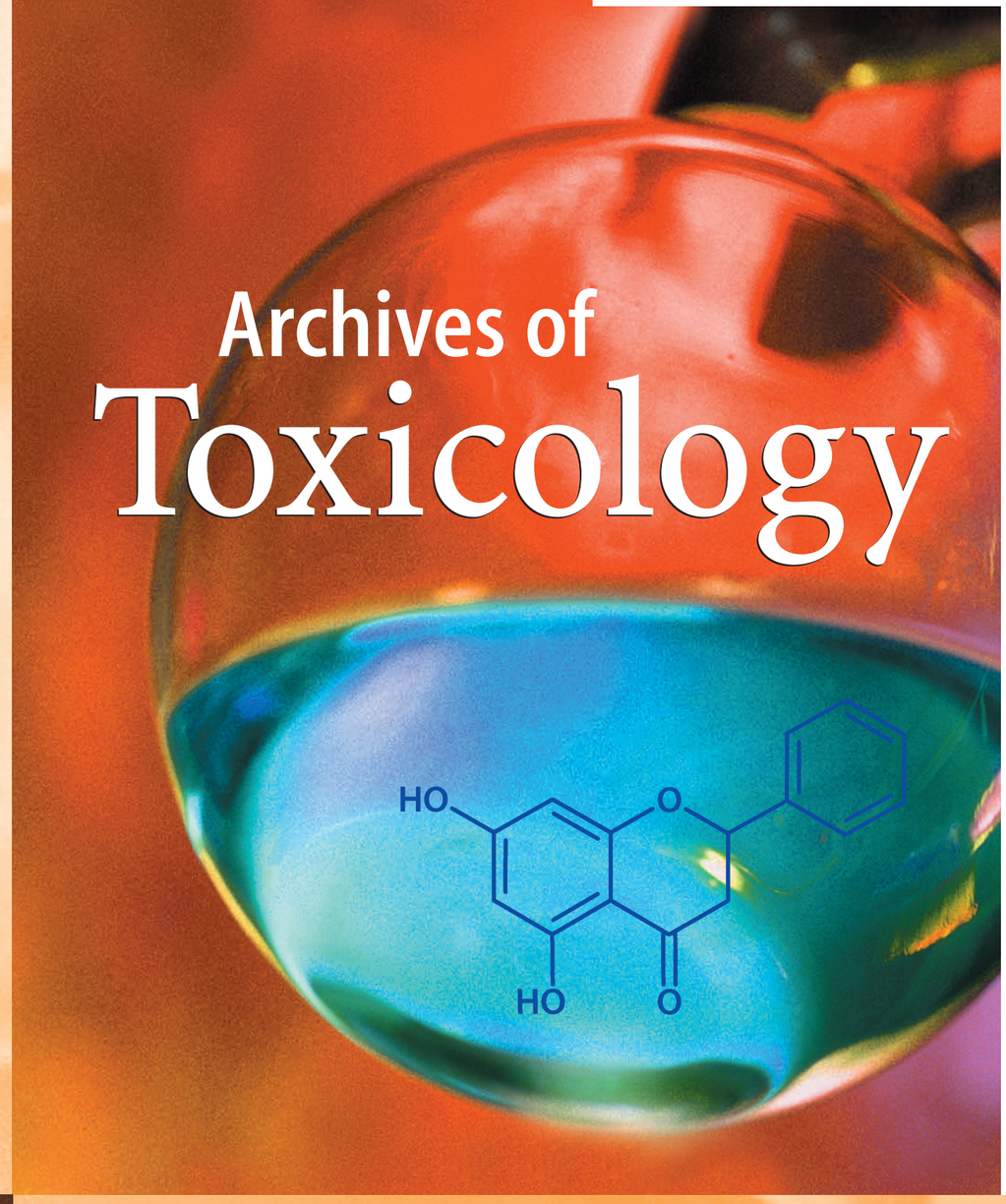

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REPRODUCTIVE TOXICOLOGY

Investigations of putative reproductive toxicity of low-dose exposures to flutamide in Wistar rats

Karma C. Fussell[1] · Steffen Schneider[1] · Roland Buesen[1] · Sibylle Groeters[1] · Volker Strauss[1] · Stephanie Melching-Kollmuss[2] · Bennard van Ravenzwaay[1]

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Abstract The current investigation examines whether the model anti-androgenic substance flutamide is capable of disrupting endocrine homeostasis at very low doses. The data generated clarify whether a non-monotonic dose–response relationship exists to enhance the current debate about the regulation of endocrine disruptors. Moreover, it is part of a series of investigations assessing the dose–response relationship of single and combined administration of anti-androgenic substances. A pre–postnatal in vivo study design was chosen, which was compliant with regulatory testing protocols. The test design was improved by additional endpoints addressing hormone levels, morphology, and histopathological examinations. Doses were chosen to represent a clear effect level (2.5 mg/kg bw/d), a low endocrine effect level (LOAEL, 0.25 mg/kg bw/d), a NOAEL for endocrine effects (0.025 mg/kg bw/d), a further dose at 0.0025 mg/kg bw/d flutamide, as well as an "ADI" (0.00025 mg/kg bw/d or 100-fold below the NOAEL) for the detection of a possible non-monotonic dose–response curve. Anti-androgenic changes were observable at LOAEL and the clear effect dose level but not at lower exposures. Nipple retention appeared to be the most sensitive measure of anti-androgenic effects, followed by age at sexual maturation, anogenital distance/anogenital index and male sex organ weights, as well as gross and histopathological findings. The results of all five doses indicate the absence of evidence for effects at very low dose levels. A non-monotonic dose–response relationship was not evident for the anti-androgenic drug flutamide.

Keywords Flutamide · Low dose · Non-monotonic dose response · Anti-androgenic · Endocrine disruptor

Introduction

There is a concern that current risk assessment methodologies may be insufficient to protect against low-dose exposure to endocrine active chemicals, even when the individual exposures may be several orders below the no observed adverse effect level (NOAEL). This concern is based on a hypothesis that the endocrine system is somehow different and uniquely sensitive to low doses of exogenous hormonally active substances. Under this hypothesis, such substances may pose a risk to human health and wildlife at doses below those currently evaluated in regulatory studies and hazard assessments.

Current risk assessment methodologies are based on single-substance determinations of hazard. It is assumed that the pharmacological or toxicological effects of chemicals, either beneficial or deleterious, are due to the amount of active material at the site(s) of action. When the internal dose is too small to elicit a response, it is said that the "threshold for that effect has not been reached" (Holsapple and Wallace 2008). The threshold is the concentration above which the noticeable effect is triggered. As the experimental basis for the threshold, toxicity studies are usually designed to determine the no observed adverse effect level (NOAEL).

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✉ Bennard van Ravenzwaay
bennard.ravenzwaay@basf.com

[1] Experimental Toxicology and Ecology, BASF SE, 67056 Ludwigshafen am Rhein, Germany

[2] Product Safety, Regulations, Toxicology and Ecology, BASF SE, Ludwigshafen am Rhein, Germany

It has been postulated that endocrine disrupting modes of actions do not follow this threshold concept and that non-monotonic J-shaped or inverted U-shaped dose responses are to be expected. The concept of hormesis was first described for radiation effects (Calabrese and Blain 2005). However, the premise that hormesis is common or important has not been convincingly established (Holsapple and Wallace 2008). It should also be noted that the effect of hormesis may be beneficial for the organism, e.g., induced DNA repair following radiation exposure.

As a result, there exists a current need to clarify the relationship between low doses of endocrine active substances and their disruptive effects. Depending on when (and how) endocrine disruption occurs, this can interfere with development at critical time periods (Toppari and Skakkebaek 1998). For instance, exogenous anti-androgens disrupt sexual differentiation and maturation in rats, resulting in reduced anogenital distances (AGDs), delayed puberty, decreased sex organ weights, and altered sexually dimorphic behavior patterns. At higher exposure levels, a pattern of alterations can be observed in the reproductive tissues and developmental malformations often occur (Gray et al. 1999b, 2001; Hellwig et al. 2000; Laier et al. 2006; Schneider et al. 2011; van Ravenzwaay et al. 2013). Since such high doses significantly alter morphology and development, these malformations must be considered adverse.

However, a question remains as to how minor or even transient endocrine effects arising from low-dose exposures should be judged. As part of a larger project to investigate the potential occurrence of low-dose effects of anti-androgens, we attempted to clarify some of these concerns by comparing the endocrine effects of flutamide, a prototypical androgen receptor antagonist, over five doses with a 10,000-fold range between the highest and lowest dose.

Flutamide is commonly used as a nonsteroidal treatment for hormonally responsive prostate cancer, which principally acts as an androgen receptor antagonist, out-competing testosterone and its metabolite dihydrotestosterone (DHT) for androgen receptor binding without activating receptor-mediated signaling. *In utero* exposure to 2 mg/kg bw/d (mg/kg body weight per day) flutamide has been shown to cause reduced AGDs, increased nipple retention, pubertal delays, decreased sex organ weights, hypospadias, and reduced penile length in male rat offspring (McIntyre et al. 2001; Welsh et al. 2010), all classic indications of an anti-androgenic mode of action. This androgen receptor-mediated toxicity has been well characterized at the high doses tested in regulatory and mechanistic studies; however, whether these parameters are altered at the much lower doses relevant to environmental exposure levels has never been reported.

The present report aims to fill this gap in knowledge by determining whether anti-androgenic substances disrupt endocrine homeostasis at very low doses using flutamide as a prototypical anti-androgen. Importantly, these data should clarify whether the flutamide–dose–endocrine–response relationship is non-monotonic or results in a threshold to enhance the current debate about the regulation of endocrine disruptors. To accomplish these aims, a pre–postnatal in vivo study design has been chosen, which is compliant with regulatory testing protocols for anti-androgens.

The results of the present investigations on a single anti-androgenic compound will also be part of a broader research program to assess the dose–response relationship of single and combined exposure to several anti-androgenic substances.

Materials and methods

These investigations exceeded the requirements of any specific guideline, but reference is made to OECD 414 (OECD 2001) and 443 (Fegert et al. 2012; OECD 2011), as well as OPPTS 870.3700 (US Environmental Protection Agency 1998). Beyond the standard regulatory parameters, the test design was supplemented by additional endpoints examining hormone levels, morphology, and histopathology (Fig. 1; Table 1). The study was performed according to GLP standards (US Congress et al. 1972, 1976), excepting only the analyses of hormone levels which followed good scientific practices, but not formal GLP, principally meeting EPA/FDA requirements.

Test substance

Flutamide, 2-methyl-*N*-(4-nitro-3-[trifluoromethyl]phenyl) propanamide (FLT), CAS number 13311-84-7, was purchased from Sigma-Aldrich (St. Louis, MO, USA) as a yellow powder of purity >99 %. The solid powder was then thoroughly dissolved in corn oil vehicle to prepare each dosing solution. The correctness and the homogeneity of prepared gavage solutions were determined by HPLC–MS analysis of several aliquots sampled from the bottom, middle, and top of the preparation vessels. Furthermore, the stability of flutamide in the corn oil vehicle was proved by testing a sample stored at room temperature at intervals over a period of 7 days. Fresh gavage solutions were prepared weekly.

Parental female animals

For technical reasons, the investigations were performed as three separate experiments of identical study design, resulting in three equivalent groups of concurrent controls called α, β, and γ. For clarity, only the first control (α) is reported in the figures and tables enclosed, but all statistical tests are

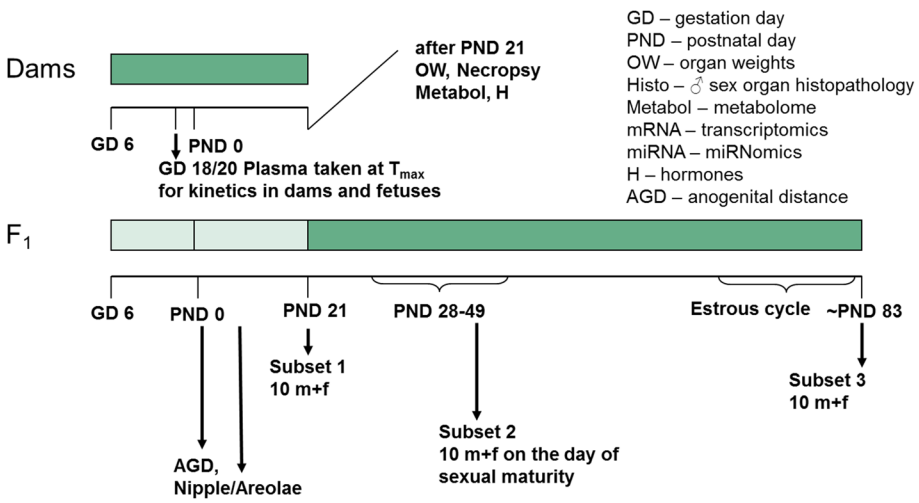


Fig. 1 Overall experimental design. It should be noted that samples were taken for future molecular analyses (metabolome, miRNome, and transcriptome), the results of which are not described in this publication

Table 1 Parameters investigated at killing

Parameter	Dams (~PND 30)	Subset 1 (PND 21)	Subset 2 (puberty)	Subset 3 (PND 83)
Full necropsy	+	+	+	+
Organ weights	+	+	+	+
Histopathology	+	+	+	+
Transcriptome		Males only	Males only	Males only
miRNome		Males only	Males only	Males only
Metabolome	+	+	+	+

based on comparisons between the treatment and its concurrent control group. In addition, all three equivalent concurrent control groups were compared with each other in order to establish a control range for each parameter (Supplementary Figures 1–3 and all Supplementary Tables).

Permission for animal studies was obtained from local regulatory agencies, and all study protocols were in compliance with German and EU animal welfare requirements. The study was performed in an AAALAC-approved laboratory. Time-mated outbred Wistar rats (WI:Han) were obtained from Charles River Laboratories (Sulzfeld, Germany) on gestational day zero (GD 0), defined by the presence of sperm or a vaginal plug in the vaginal canal. Throughout the study, all animals were maintained under standard conditions: one animal (or litter) per Makrolon type MIII cage with LTE E001 bedding (ABEDD, Vienna, Austria) and maintained at 20–24 °C and 30–70 % humidity with 15 air changes per hour and a 12-h light/dark cycle. All animals had free access to food (ground Kliba maintenance diet mouse/rat "GLP" [Provimi-Kliba, Kaiseraugst, Switzerland]), water, and a wooden gnawing block as enrichment. The animals were allowed to acclimatize to the laboratory conditions until GD 6, when the dams were randomized into 4 dose groups of 25 animals per group.

The dams were administered 0, 0.00025, 0.0025, 0.025, 0.25, or 2.5 mg/kg bw/d of flutamide in corn oil (4 mL/kg bw/d) by gavage every morning from gestational day (GD) six until the day of killing (GD 18 or about postnatal day 30 [PND 30]), except during labor. The doses were chosen to represent a clear effect level (2.5 mg/kg bw/d), a low endocrine effect level (LOAEL, 0.25 mg/kg bw/d), a NOAEL for endocrine effects (0.025 mg/kg bw/d), and an "ADI" (0.00025 mg/kg bw/d or 100-fold below the NOAEL). An additional dose group (0.0025 mg/kg bw/d) was also added to the study design to better characterize a possible non-monotonic dose–response curve.

On GD 18, blood (1 mL with 10 μL of 10 % EDTA as an anticoagulant) was collected from five dams in each dose group. Thereafter, these dams were killed by cervical dislocation under isoflurane and carbon dioxide anesthesia and necropsied. The pregnant uteri were dissected and opened, and the fetuses were removed. All implants and fetal weights were recorded, before the fetuses were killed by snap freezing in liquid nitrogen and stored for future kinetic studies. The remaining dams were allowed to deliver and rear their pups until PND 21 (weaning).

During gestation and lactation, each dam was examined daily for clinical signs of morbidity and toxicity, as well as parturition and lactation behavior. The food consumption and body weight of each dam were evaluated on GD 0, 6, 13, 18, and 20. The food consumption was also determined weekly for each litter during lactation (PND 0-21) and

later for every weaned F1 animal in subsets 2 and 3. All females which littered were weighed on PND 0, 7, 14, and 21. Females which did not litter were killed and examined for gross abnormalities. The uteri were removed from these animals and stained with Salewski stain for implantation sites (Salewski 1964).

Offspring

The gender, status (live- or stillborn), and any gross-morphological abnormalities of each delivered pup were recorded as soon as possible after birth. Pup viability, mortality, and any clinical signs of toxicity or morbidity were determined at least daily. The pups were weighed on PND 1, 4, 7, 14, 21, as well as on the day of sexual maturation (vaginal opening or preputial separation). Anogenital measurements were obtained on all living pups on PND 1 using a measuring ocular in a blind, randomized fashion. All living, male pups were examined for the presence or absence of nipple/areola anlagen on PND 12 and re-examined on PND 20.

Before weaning on PND 21, twenty pups of each gender per dose group were selected randomly to be allowed to mature (ten per gender in each of subsets 2 and 3). After blood sampling on PND 21 (at least 1 mL with 10 µL of 10 % EDTA per mL as an anticoagulant and a further 300 µL without anticoagulant from the dams for estradiol measurement), the dams and a further ten male and ten female pups were killed under isoflurane/carbon dioxide anesthesia and dissected. The relevant tissues were obtained from these animals for pathological and molecular analyses. Any surplus pups were also similarly killed on PND 21, but only macroscopically examined for gross abnormalities.

From PND 21 until sexual maturity (subset 2) or young adulthood (subset 3), the maturing F1 pups were exposed to test substance orally by gavage. One day after reaching sexual maturity, blood was sampled from each animal in subset 2 (1 mL with 10 µL of 10 % EDTA as an anticoagulant and another 300 µL without anticoagulant from the females) before it was killed and necropsied. Again, the relevant tissues were harvested from these animals for pathological and molecular (metabolome, miRNome, and transcriptome; not reported in the present paper) analyses. Similarly, each F1 animal of subset 3 was killed as young adult (PND 81-85) after blood sampling with 10 µL of 10 % EDTA per mL as an anticoagulant and a further 300 µL without anticoagulant (females only), and the sexually dimorphic tissues were harvested.

Serum preparation and hormone analysis

The blood samples collected with EDTA after weaning (both parental and filial samples), sexual maturity, and during young adulthood were centrifuged under refrigeration to separate out the plasma. This plasma was aliquoted (>200 µL per aliquot) and stored under N_2 at −80 °C for general hormone analysis. Serum was also similarly prepared and stored from the blood sampled without anticoagulant from all females, with the exception of the PND 21 pups, for the measurement of estradiol levels. The serum estradiol concentration in these samples was determined using a commercially available ELISA kit from DRG Diagnostics (EIA-4399; Marburg, Germany) measured on a Sunrise MTP-reader, (Tecan AG, Mannedorf, Switzerland) and evaluated by the Magellan software of the instrument producer.

Tissue preparation and histopathological analysis

At all time points, the harvested organs were carefully asservated, trimmed of excess fat and tissue, and weighed fresh, unfixed, and without blotting to the nearest 0.1 mg. During the dissection of male subset 1 and 2 animals, the ventral prostate was halved immediately after weighing. One of these halves, as well as the right testis and the right seminal vesicle, was snap frozen in liquid nitrogen and stored at −80 °C for future analyses. In subset 3 male animals, both the ventral prostate and the right testis were halved after weighing. Half of each tissue plus the right seminal vesicle were snap frozen for future transcriptomic analysis. Sperm were sampled from the residual half of the right testis and the complete right cauda epididymis for motility, morphology, and sperm and spermatid head counts.

The remaining organs and tissues from both sexes were then prepared for histopathological analysis. All tissues were fixed in 4 % buffered formaldehyde solution, excepting only the ovaries, left epididymis, and left testis, which were fixed in modified Davidson's solution. Each tissue was then histotechnically processed, stained with hematoxylin–eosin, and examined by light microscopy.

Statistical analysis

Means and standard deviations were calculated for all measured parameters. In addition, a number of statistical analyses were performed to compare the treatment and control groups. These are summarized in detail for each parameter in Supplementary Table 1. Generally, most clinical data were subjected to a two-sided Dunnett test, although those relating to parental fertility indices, pup mortality, developmental landmarks, and sperm morphology used Fisher's exact test instead. However, any findings evaluated on a per litter basis were evaluated by a one-sided Wilcoxon test. All weights at necropsy were subjected to Kruskal–Wallis analysis with a Wilcoxon post hoc test, except those of the

male sex organs, for which a one-sided Dunnett test was used.

Results

All concentration control analyses showed that the achieved flutamide concentrations were within acceptable limits, and prepared dosing suspensions were stable and homogeneous. Contaminants in the feed or water or changes in the environmental conditions, which might have the potential to influence the outcome of the studies, were not observed (data not shown).

Gestation and littering

Except for only one single animal, all dams in this study were pregnant and survived until scheduled termination. No treatment-related clinical signs were observed in the dams during either gestation or lactation. Food consumption and body weight parameters were comparable to the concurrent control group and within historical control ranges throughout the entire dosing period. No alterations to reproductive performance were observed. Litter size and offspring weights were also as expected, and there was no intergroup difference in sex ratio. As pup development continued postpartum, a slight decrease in pup viability was observed in the 0.25 mg/kg bw/d dose group during the lactation period due to poor maternal postpartum care of 1 litter. This finding was deemed unrelated to treatment as poor maternal care sometimes occurs in this rat strain and a dose–response relationship was not detected. Moreover, the pup viability of the 0.25 mg/kg bw/d dose group was normal after a recalculation was performed, which excluded the single litter (data not shown).

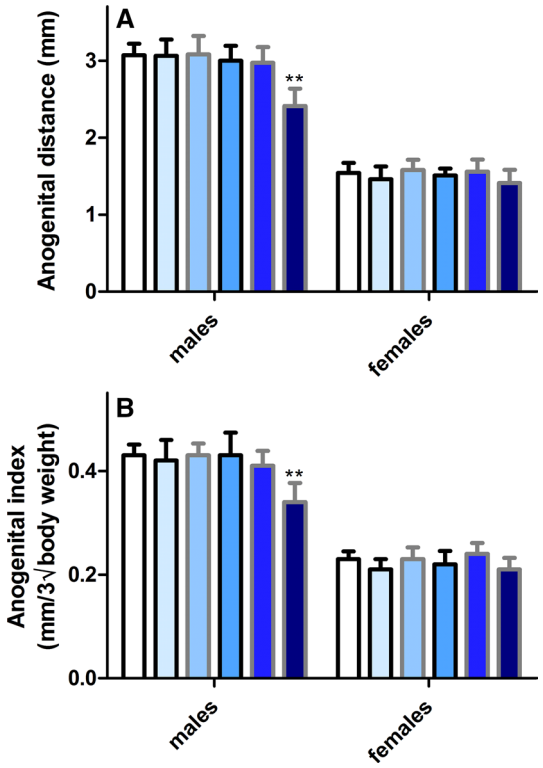


Fig. 2 Effect of increasing dose of flutamide on anogenital distance and anogenital index. On PND 1, the anogenital distance (AGD) of all live-born pups exposed in utero to vehicle control, 0.00025, 0.0025, 0.025, 0.25, and 2.5 mg/kg bw/d flutamide was measured (**a**). The anogenital index (AGI), a parameter which accounts for any differences in animal size, was then calculated from these data (**b**). Prenatal exposure to 2.5, but not 0.25, 0.025, 0.0025, or 0.00025 mg/kg bw/d flutamide, significantly reduced male, but not female AGDs and AGIs. *Note:* These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent control of the first study (α) is presented here. A comparison of the three control datasets can be found in **a** and **b** of Supplementary Figure 1

Offspring development

No systemic toxic effects were observed in the offspring during lactation. However, statistically significant decreases of 22 % in anogenital distance and 21 % in anogenital index (a calculated parameter that takes into account the individual animal weight) were observed on PND 1 in the males exposed in utero to 2.5 mg/kg bw/d of flutamide (Supplementary Table 2; Fig. 2). The similarity between these reductions suggests that these effects were due to an endocrine-mediated effect rather than as a result of any body weight differences between the control and high-dose groups. In contrast, neither the anogenital distance nor the anogenital index was altered in females.

The male pups were also examined for the presence of nipples or areolae on PND 12 and again on PND 20 (Supplementary Table 2; Fig. 3). On PND 12, a statistically significant increase was noted in the number of offspring in the 0.25 and 2.5 mg/kg bw/d dose groups, which had nipples/areolas, defined as at least one nipple or hairless pigmented spot identified as an areola, when compared with controls (Fig. 3a). The number of areolas was also similarly elevated in these dose groups (Fig. 3b). To determine whether the areolas persisted (retained nipples), a reexamination was performed on PND 20 (Fig. 3a). By this age, the areolas of the males of the 0.25 mg/kg bw/d group had completely regressed; only in the 2.5 mg/kg bw/d group, some animals were found to still have nipples/areolas (Fig. 3c). Regardless, the average number of nipples per animal in this dose group was substantially reduced when compared to the means on PND 12.

Ten males and females per dose group per subset were selected to be reared; the age and weight at sexual maturation (preputial splitting in males, vaginal opening in

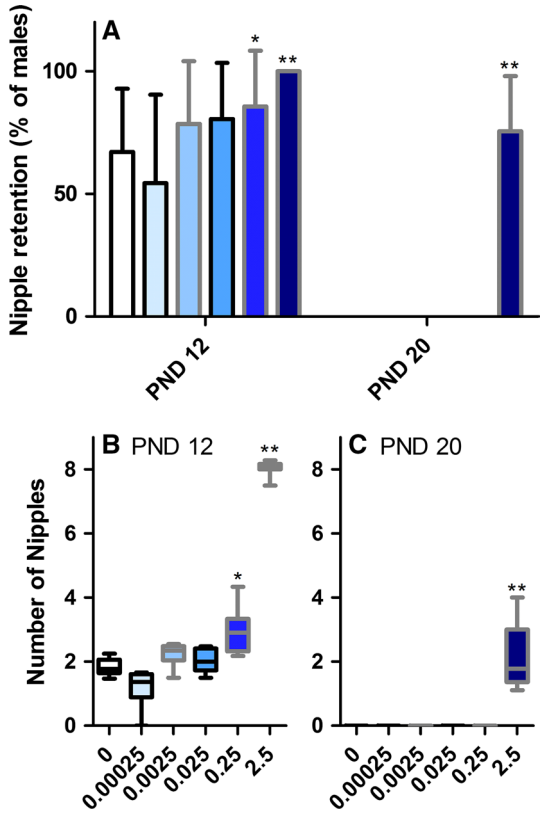


Fig. 3 Effect of increasing flutamide dose on nipple retention. On PND 12, the number of male pups exposed to vehicle control, 0.00025, 0.0025, 0.025, 0.25, and 2.5 mg/kg bw/d flutamide which had retained nipples or areolas was counted. These animals were then recounted on PND 20 (**a**). Similarly, the number of nipples/areolas retained by each male pup was also recorded on PND 12 (**b**) and PND 20 **(c)**. Despite the relatively high background rate of nipple retention in control animals, prenatal and/or lactational exposure to increasing doses of flutamide dose-dependently raised both the number of males with nipple/areola retention and the average number of nipples/areolas retained by each male pup on PND 12. This effect was largely transient; by PND 20, only pups from the 2.5 mg/kg bw/d dose group had a significantly elevated percentage of affected males or nipple/areola count. *Note*: These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent control of the first study (α) is presented here. A comparison of the three control datasets can be found in Panels C and D of Supplementary Figure 1

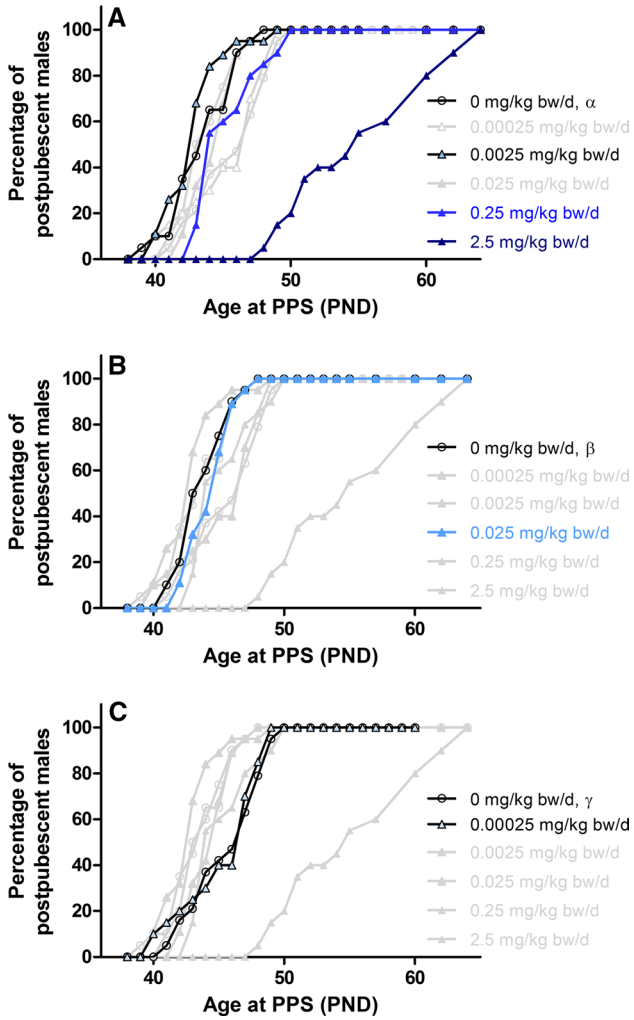


Fig. 4 Kaplan–Meier plot of the sexual maturation of pubescent male rats exposed to flutamide since GD 6. Twenty male offspring which had been exposed to either vehicle only (*open circles*), or increasing concentrations of flutamide (blue triangles), were examined for preputial separation daily from PND 38 to 64. These data were collected in the course of three different experiments (α, β, and γ) with similar study design. So that each dose group can be compared with its concurrent study control, as well as for visual clarity, these data were subdivided into panels by study, with the results of the other two studies shown in gray. **a** The pubertal development in study α. While both the 0.25 and 2.5 mg/kg bw/d groups were delayed beyond the historical control range, only the delay resulting from treatment with 2.5 mg/kg bw/d flutamide was statistically significant ($p \leq 0.01$). Delays were not evident after treatment with either 0.025 (study β, **b**) or 0.00025 (study γ, **c**) mg/kg bw/d flutamide

females) were then determined in about 20 animals of each gender. To better evaluate the lags in average male age at puberty, the individual ages at preputial separation were plotted as Kaplan–Meier curves (Fig. 4). Across all dose groups, no change in the course of sexual development was observed in the female offspring; however, male sexual development was delayed in animals exposed to the two top doses of flutamide (Supplementary Table 2; Fig. 4). In the case of the 0.25 mg/kg bw/d dose group, this represents a statistically insignificant delay of about 1 day beyond the historical control range (PND 39.7–44.8). Treatment with 2.5 mg/kg bw/d flutamide caused a much larger, statistically significant delay in male sexual development of nearly 2 weeks. Being older, these animals also were significantly heavier on the day of preputial separation.

Role of hormone disruption in anti-androgen delayed sexual maturation

Sexual development is controlled by several factors, including the general growth progression and health of the animal, as well as hormonal signaling. We and others have previously observed that delays in preputial separation can result from impaired growth, as measured by body weight development (Carney et al. 2004; Chernoff et al. 2009; Delemarre-Van de Waal et al. 2002; Dunger et al. 2006; Lau et al. 1996; Laws et al. 2007; Marty et al. 2003; Melching-Kollmuss et al. 2014; van Weissenbruch et al. 2005; Warren 1983). Furthermore, as impaired growth is a common sign of the general toxicity required at the uppermost dose by regulatory authorities, we observed that often the delays in preputial separation observed during regulatory studies are the secondary effects of general toxicity instead of specific indicators of endocrine modulation (Delemarre-Van de Waal et al. 2002; Melching-Kollmuss et al. 2014). These primary and secondary effects can be differentiated using graphical analysis.

Briefly, the ages and weights of the individual animals on the day of sexual maturation (Fig. 5, scatterplots) were compared to the normal body weight progression of the control animals being reared to early adulthood, which were weighed at weekly intervals (gray diamonds, regression line). Most individuals in the 0, 0.00025, 0.0025, and 0.025 mg/kg bw/d groups are clustered with maturation days between PND 40 and PND 46 and distributed both above and below the regression line, signifying generally normal sexual development.

Figure 5 also demonstrates the previously observed pubertal delay at doses of 0.25 and 2.5 mg/kg bw/d flutamide; however, the graphical analysis also reveals that these animals were proportionally larger at the time of puberty as a result of their increased age. Since the growth progression of the individuals in these dose groups was normal, the delay in preputial separation cannot be explained by lags in overall maturation due to any general toxicity of the test substance. Therefore, these results suggest that the male pubertal delay resulting from flutamide administration was an effect of endocrine modulation.

Pathology

Sex organ weights

Overall, though flutamide treatment reduced male sex organ weights, female sex organ weights remained unaffected. When compared to the control group 0, no other mean organ weight parameters, whether absolute or relative, showed significant, treatment-related differences (a complete dataset can be found in Supplementary Tables). In contrast, treatment-related decreases in male offspring sex organ weights were noted. These reductions were most distinct at the 2.5 mg/kg bw/d flutamide dose (Fig. 6).

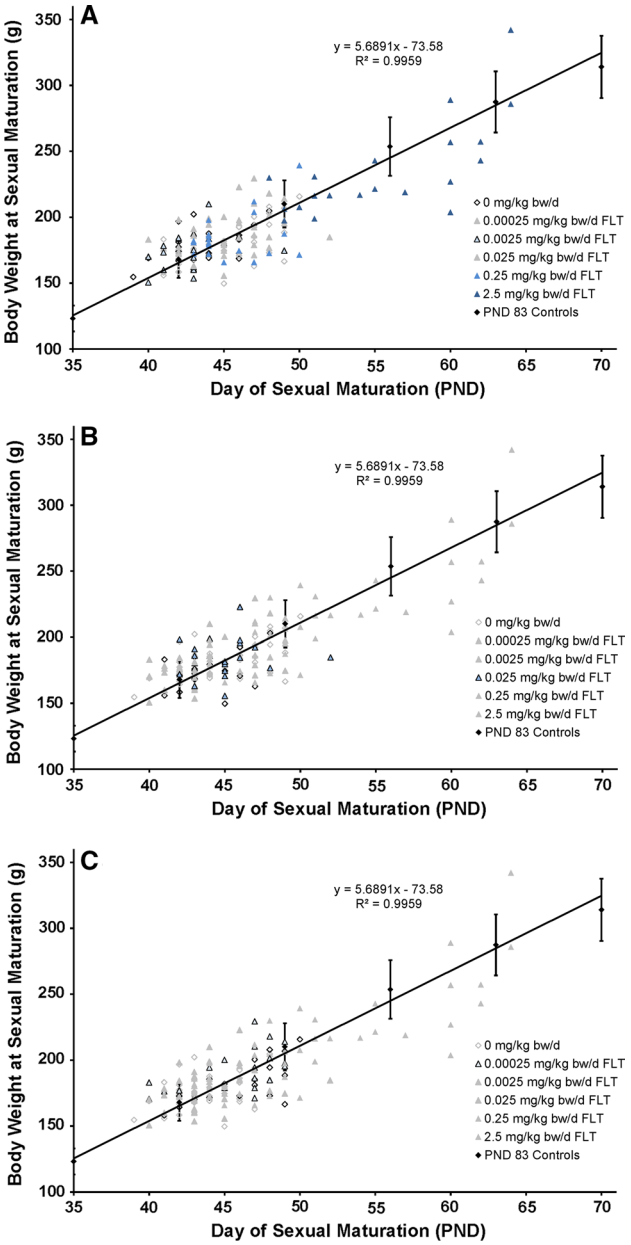
Statistically significant decreases in the mean absolute and relative organ weights of the cauda epididymis, epididymides, bulbocavernosus and levator ani muscles, total prostate and ventral prostate were observed at weaning (PND 21) in the male offspring, which had been exposed to 2.5 mg/kg bw/d flutamide (Supplementary Table 6 and 8, Supplementary Figure 5; Fig. 6). A statistically significant decrease in the absolute organ weights of the bulbourethral gland, cauda epididymis, epididymides, glans penis, bulbocavernosus and levator ani muscles, total and ventral prostate as well as the seminal vesicles was also observed in the offspring of this dose group on PND 83 $\pm$ 2. This also corresponded to a reduction in the relative organ weights in all of these organs except the epididymides (Supplementary Table 18 and 20, Supplementary Figure 5; Fig. 6).

In contrast, the absolute organ weights of the animals at puberty exposed to 2.5 mg/kg bw/d flutamide were generally heavier than those of the controls. This increase is related to the significantly delayed time at which puberty was noted in this group and hence a clearly higher absolute terminal body weight at necropsy (Supplementary Table 12). As was noted above, the animals of this dose group are larger in size in proportion to their more advanced age. Thus, the heavier absolute weights of kidneys, liver, and pituitary gland observed are a not unexpected secondary effect of the delay in preputial separation at this dose.

However, caution should be exercised in the interpretation of the sex organ weights on the day of puberty. An overall increase in the absolute sex organ weights at this dose does not mean that no hormonal effects can be observed. While it is true that absolute sex organ weights at 2.5 mg/kg bw/d were increased due to its relationship with body weight, the relative sex organ weights can still be used to evaluate possible endocrine-related effects. The mean relative weights of total and ventral prostates in males at sexual maturity exposed to 0.25 and 2.5 mg/kg bw/d flutamide were dose-dependently and statistically significantly reduced (Supplementary Table 14 and Supplementary Figure 5); a weight decrease is considered to be an anti-androgenic effect of flutamide.

Gross and histopathology

The severe delay in male puberty and decreased sex organ weights in subset 2 animals administered 2.5 mg/kg bw/d flutamide was accompanied by an increased incidence of gross developmental defects in the sexual organs. Before killing at puberty, a single hypospadia and two occurrences



◀ **Fig. 5** Comparison of age and body weight of individual male offspring remaining at sexual maturation (subsets 2 and 3). The comparison between age and weight on the day of preputial separation for all individual males administered either vehicle (*open diamonds*) or increasing doses of flutamide (*blue triangles*) was made with the mean body weight development of the subset 3 controls (shown as closed diamonds ±SD with a least-squares regression line). These data were collected in the course of three different experiments (α, β, and γ) with similar study design. So that each dose group can be compared with its concurrent study control, as well as for visual clarity, these data were subdivided into panels by study, with the results of the other two studies shown in gray. **a** The relationship between body weight and pubertal development in study α. While preputial separation was delayed in both the 0.25 and 2.5 mg/kg bw/d groups, the individual body weights were generally proportional to the age at sexual maturation. However, treatment with 2.5 mg/kg bw/d flutamide did cause a small decrease in individual body weight, which was associated with the delay in preputial separation. Delays were not evident after treatment with either 0.025 (study β, **b**) or 0.00025 (study γ, **c**) mg/kg bw/d flutamide

of small penis were observed clinically among the ten high-dose males. These findings do not occur in the historical control data at BASF's toxicology facility.

These developmental defects persisted until PND 83 ± 2; two of ten animals from the 2.5 mg/kg bw/d group had hypospadias, two had small penises, and the testes of one male could not be palpated. This last observation corresponded to gross pathological findings of cryptorchism at necropsy. A severe bilateral reduction in the size of the testes and epididymides, as well as a moderate reduction in prostate size and a severe reduction in the size of the left seminal vesicle, was also observed. No right seminal vesicle could be detected in this animal at killing. These latter findings of poor sexual development in a single animal are probably related to flutamide dosing, as they fit the expected pattern of an anti-androgen. No treatment-related gross developmental defects were observed in any PND 21 animals.

The male and female sex organs of all three subsets were also examined histopathologically. No differences related to treatment were detected in the females of any dose group. In males, however, the primary pathological effects of flutamide treatment were in the 2.5 mg/kg bw/d dose group: reduced organ size and/or function on PND 83 ± 2 and a more advanced developmental stage at delayed onset of puberty.

Both the seminal vesicles and the prostates of animals treated with 2.5 mg/kg bw/d were found to be reduced in size on PND 83 ± 2 with decreased secretion in nine and five of the ten animals, respectively. The testes of the animals of this dose group also exhibited tubular degeneration: a minimal multifocal defect in the left testis of one animal and a marked diffuse degeneration in a second. This second animal also had a moderate Leydig cell hyperplasia and aspermia in the corresponding epididymis. With the exception of this single case of aspermia, histopathological analysis of the left epididymis was comparable across all dose groups, with no findings in any other control or treated animal. It cannot be determined whether any of these effects are bilateral or unilateral, as only half the left testis, epididymis, seminal vesicle, and the distal portion of the prostate were fixed and examined. The right testis, epididymis, seminal vesicle, and half the ventral prostate were frozen and saved for future molecular analyses. All other findings occurred individually and were considered to be incidental.

Fewer differences between control and treated animals were detected at puberty. The seminal vesicles, coagulating glands, and prostate of all killed control and treated animals

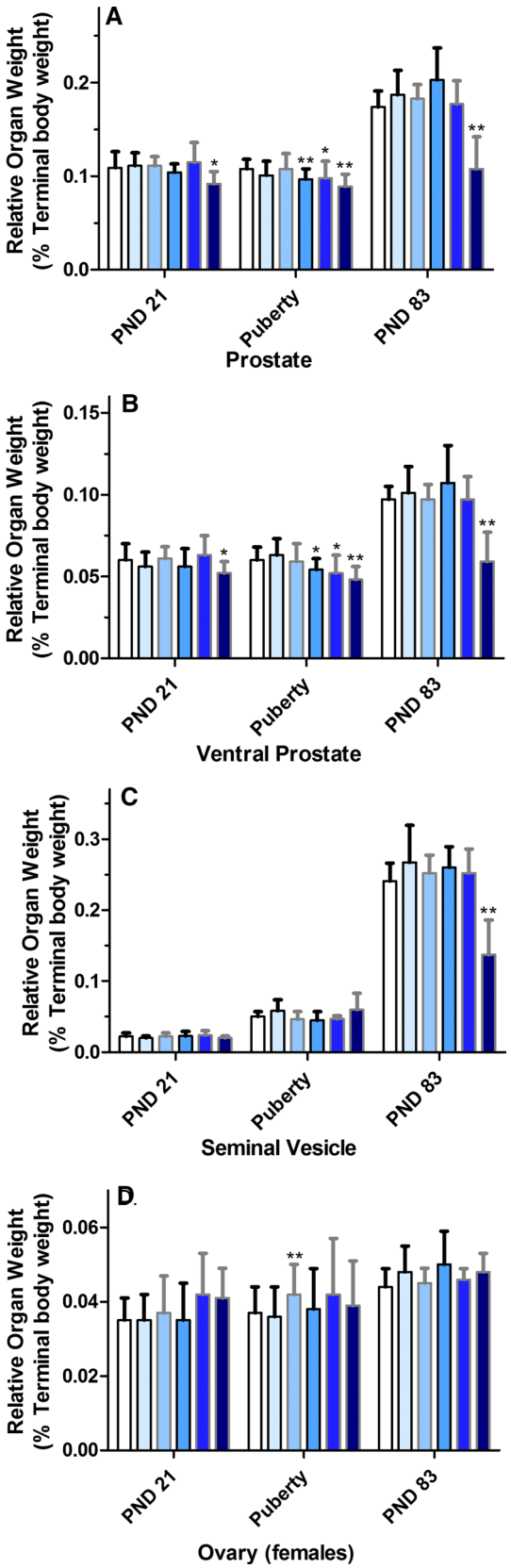


Fig. 6 Weights of selected sexual organs. On PND 21 (subset 1), the ▶ day of preputial separation or vaginal opening (puberty, subset 2) and PND 83 ± 2 (subset 3), the sex organs from each of ten male and ten female rats were asservated, weighed, and reported as relative organ weights. These data are graphed as vehicle control, 0.00025, 0.0025, 0.025, 0.25, and 2.5 mg/kg bw/d at each time point. In general, increasing anti-androgen exposures reduced male, but not female, sex organ weights dose-dependently. Three of the most sensitive male sex organs are shown (**a**–**c**) in comparison with a female sex organ (**d**). *Note:* These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent control of the first study (α) is presented here. A comparison of the three control datasets can be found in Supplementary Figure 4

indicated a comparable juvenile developmental stage with a moderate amount of secretion. (Again, only the left male sex organs were examined, as the right ones were set aside for future molecular experiments.) Similarly, the left testis of the control and treated animals exhibited a comparable juvenile developmental stage, with a fully developed spermatogenic cycle in all dose groups. However, the left testes of the male offspring of the 2.5 mg/kg bw/d flutamide group seemed to have an increased number of Leydig cells. The corresponding epididymis exhibited a juveno-adult transitional developmental stage characterized by a thickened epithelium and many sperm in all compartments, whereas those of the control and all other dose groups were at a juvenile stage without any sperm or some sperm only in the region of the head. The more advanced developmental stage in these organs reflects the more advanced age of the animals at killing due to the severely delayed puberty in the 2.5 mg/kg bw/d dose group. Consequentially, these effects are probably only a secondary effect of treatment.

Hormone analysis

Steroid hormone levels were determined in serum prepared from blood collected from the parental females post-weaning and all offspring selected for study (subsets 1, 2, and 3). These data were graphed as boxplots, with each plot representing a different steroid hormone at a specific developmental stage and sex. While selected changes are shown in Fig. 7, a complete set can be found in the supplementary data (Supplementary Figures 6–10, Supplementary Tables 23–36).

Figure 7a–d demonstrates the effects of flutamide on circulating androgen levels in pubertal and PND 83 males. During puberty (Fig. 7a, c), a statistically significant increase in both testosterone and androstenedione levels was observed at a dose of 2.5 mg/kg bw/d. These changes appear to be a clear treatment-related effect and consistent

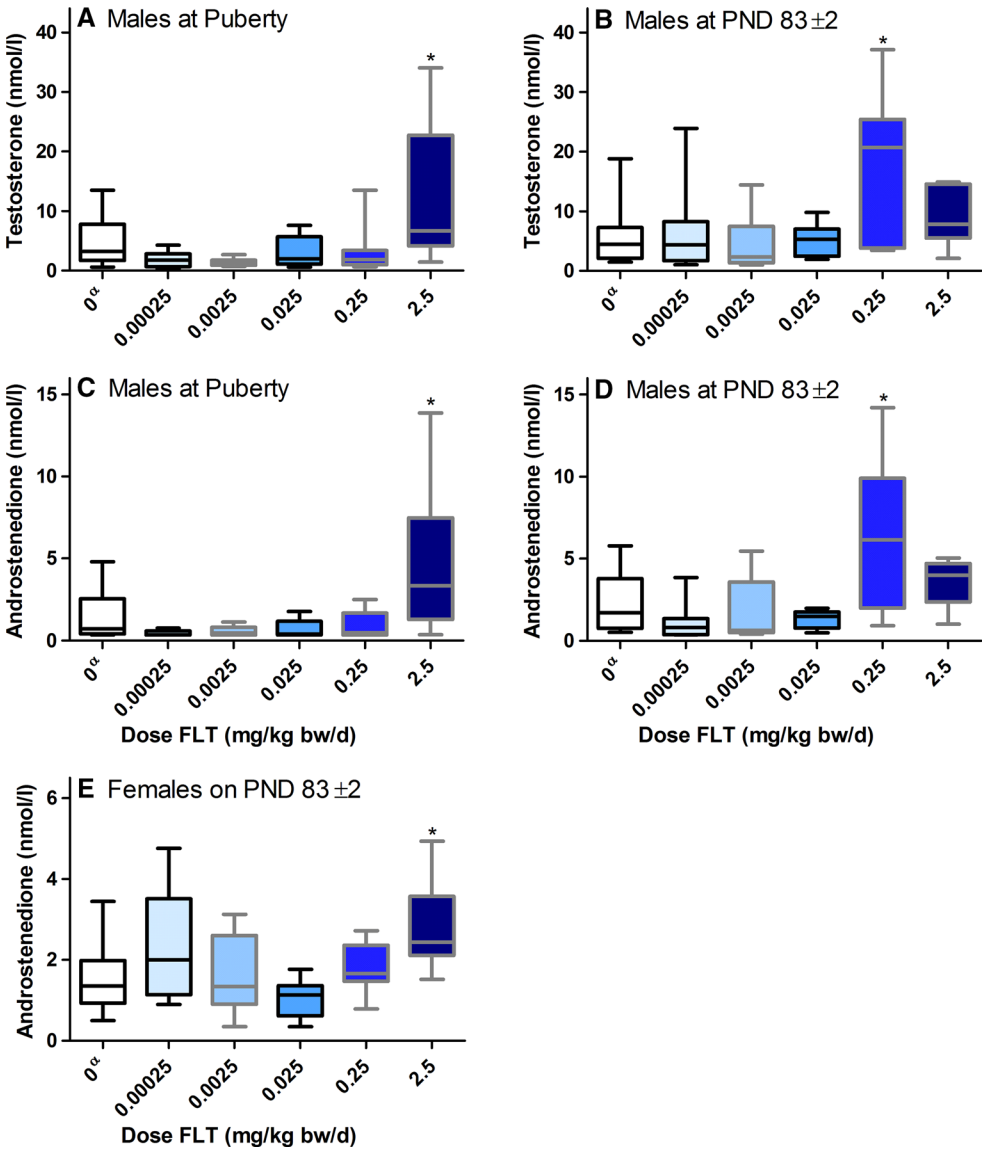


Fig. 7 Circulating concentrations of selected steroid hormones. Circulating steroid hormone concentrations were measured in the serum of the dams in proestrus, as well as the offspring on PND 21 (subset 1), the day of vaginal opening or preputial separation (puberty, subset 2), and PND 83 ± 2 (Subset 3). Steroid hormone concentrations were determined by LC–tandem mass spectrometry or EIA. The data are graphed as *box plots* representing the median (*bar*), interquartile range (*box*) and range (*whiskers*) for each treatment group, which is color-coded according to dose: vehicle control, 0.00025, 0.0025, 0.025, 0.25, and 2.5 mg/kg bw/d. Each graph represents a different steroid hormone and time point/sex, but only those changes judged a consequence of treatment are shown here (A complete set may be found in the supplementary data)

with the expected changes from an anti-androgen mode of action. On PND 83 (Fig. 7b, d), a statistically significant increase in the androgen levels was observed at the 0.25 mg/kg bw/d dose, but not at 2.5 mg/kg bw/d. The decrease in testosterone in the 0.0025 mg/kg bw/d flutamide group at puberty was judged incidental. And while the variation between individual females on PND 83 was high, a statistically significant increase in serum androstenedione concentration was noted in the 2.5 mg/kg bw/d dose group. No effects were noted in female testosterone levels at this time point, nor were any changes noted in the concentration of either androgen at puberty (see Supplementary Figures 4 and 5).

Elevated 11-deoxycorticosterone and corticosterone levels were observed in the parental and PND 83 females dosed with 0.0025, 0.25, and 2.5 mg/kg bw/d flutamide. Similar increases in serum progesterone were also revealed in these dose groups on PND 83 only. In all cases, no dose-dependency was evident. Suspiciously, all the affected dose groups were part of the same experiment (α). Furthermore, no effects were noted in dose groups, which were run as part of the other studies, nor were any dose groups from experiment α found to be unaffected. Elevated progesterone, 11-deoxycorticosterone, and corticosterone levels observed are often taken as a sign of general increase in stress in the animals (Kalil et al. 2013). However, there was no relation to flutamide administration. The statistically significant decrease in cortisol in the PND 21 females treated with 0.00025 mg/kg bw/d was likewise regarded as incidental and not treatment-related, due to the lack of a dose-dependent effect.

Table 2 Analysis of sperm and spermatids of male offspring (subset 3, PND 83 ± 2)

Dose (mg/kg bw/d)	0[α]	0[β]	0[γ]	0.00025[γ]	0.0025	0.025[β]	0.25	2.5
Motile sperm	88 ± 4 % (10)	89 ± 4 % (10)	91 ± 6 % (9)	92 ± 6 % (10)	85 ± 6 % (10)	90 ± 5 % (10)	86 ± 5 % (10)	**82*** ± 6 % (9)
Abnormal sperm morphology	3 ± 3 % (10)	6 ± 0 % (10)	2 ± 1 % (9)	2 ± 1 % (10)	4 ± 2 % (10)	6 ± 0 % (10)	3 ± 2 % (10)	6 ± 6 % (9)
Spermatid count ($\times 10^6$/g testes)	190.89 ± 31.37 (10)	132 ± 29 (10)	199 ± 23 (9)	233 ± 49 (10)	192.18 ± 42.57 (10)	118 ± 14 (10)	188.01 ± 28.95 (10)	186.39 ± 76.09 (10)
Spermatozoa count ($\times 10^6$/g Cauda epididymis)	566.28 ± 114.13 (10)	515 ± 103 (10)	643 ± 100 (9)	677 ± 125 (10)	587.60 ± 150.89 (10)	488 ± 84 (10)	616.74 ± 138.71 (10)	493.97 ± 185.88 (10)

Data are presented as mean ± SD (N = number of animals)

* $p \leq 0.05$

Sperm analysis

Sperm were sampled from half of the right testis and the complete right epididymis of subset 3 offspring and analyzed. A statistically significant reduction in sperm motility was noted at the 2.5 mg/kg bw/d dose; however, this change was marginal and still fell well within the historical control range for the test facility (Table 2). Thus, the reduction in sperm motility at this dose is not considered an anti-androgen effect. But when considered as part of the overall weight of evidence, this effect on sperm motility might be a borderline effect of the 2.5 mg/kg bw/d flutamide treatment.

Discussion

It has been suggested that the endocrine system is uniquely sensitive to exogenous modulation during critical developmental periods, even at doses of hormonally active substances significantly below previously described no observed adverse effect levels (Kortenkamp et al. 2011; Vandenberg et al. 2012; WHO/IPCS 1998). As a result, there is concern that current risk assessment methodologies do not adequately assess the hazard associated with potentially endocrine active substances. To test this hypothesis, we compared the effects of five sub-therapeutic doses for humans of the anti-androgen flutamide in a peri-postnatal regulatory developmental and reproductive toxicity study. Flutamide was chosen for this investigation because it is a pharmaceutical, which was synthesized to be a specific androgen receptor antagonist. It is an archetypical anti-androgen, ideal for examining the low-dose effects of such endocrine active substances.

The study design drew from the methods laid down in OECD TG 414, 416, and 443, as well as the corresponding OPPTS/OCSPP guidance. The resulting study closely resembled the peri-postnatal study design recommended by ICH (2005) and was enhanced by a number of endocrine-sensitive pathological and molecular endpoints. Doses were chosen to represent a significant endocrine effect level at the uppermost dose, a low endocrine effect level, a no endocrine effect level, and then two additional doses which were tenfold and 100-fold below the no endocrine effect level to test for a non-monotonic dose–response relationship. These five doses represent five orders of magnitude; thus, this study is designed to adequately describe not only the expected endocrine toxicity at the high doses, but also examine the relationship between low doses of endocrine active substances and their effects in an apical study.

Dose levels above the NOAEL

As expected, flutamide treatment at doses above the NOAEL resulted in classic dose-dependent signs of

anti-androgenicity in the developing offspring. Males exposed to 2.5 and 0.25 mg/kg bw/d flutamide had decreased anogenital distance and indices, as well as reduced nipple regression on PND 12. Interestingly, by PND 21 those of the 0.25 mg/kg bw/d dose group had completely receded, while those at the 2.5 mg/kg bw/d dose were largely maintained. These data indicate that the disruptive effects of flutamide may in fact be transitory, at least at low doses, in contrast to what was observed by Foster and McIntyre (2002). As the development of sexually dimorphic external genitalia and mammary function is controlled by androgens, together these findings lend support to the previously characterized role of flutamide as an androgen receptor antagonist (Viguiermartinez et al. 1983).

Delays in sexual maturation were only observed in male, not female offspring treated with 2.5 and 0.25 mg/kg bw/d. The delay in the age of preputial cleavage observed in the 0.25 mg/kg bw/d dose group was mild, but significant, and represents a delay of about 1 day beyond the historical control range (PND 39.7–44.8, mean of means 42.1). However, 2.5 mg/kg bw/d flutamide caused a dramatic delay of nearly 2 weeks. This severe delay in male sexual development was accompanied by cases of hypospadias, small penises, and undescended testes. These findings closely match those of Welsh et al. (2010), in which a similar oral dose of 2 mg/kg bw/d flutamide, the lowest dose tested, caused almost a 1-mm average reduction in penile length and a 10 % incidence in hypospadias. These data indicate that doses of flutamide as low as 2.5 mg/kg bw/d substantially disrupt antiandrogen signaling during pubescence.

Additionally, delayed sexual maturation due to continued receptor block could explain the presence of reduced body weight in the pubescent 2.5 mg/kg bw/d animals. Since puberty is progressing at half the rate at which it would otherwise, it is extremely likely that some of the late pubertal development, including the sexually dimorphic increase in muscle mass, may not yet be fully completed. This explanation would suggest that the decreased body weights in this dose group may not be permanent; this dose group may eventually "catch up" to the others. This hypothesis is corroborated by the lack of a decrease in the terminal body weights of the subset 3 (PND 83 ± 2) males from this dose group. However, whether this alteration is truly permanent or merely temporary does remain an important open question for further study.

This does not mean that sexual maturation did not occur in these offspring. Despite the slowed puberty at the highest dose, the expected developmental changes in hormone levels were clearly notable in the serum of both 2.5 and the 0.25 mg/kg bw/d offspring. Increased testosterone and androstenedione levels were also observed as a result of 2.5 mg/kg bw/d flutamide treatment, indicating that the androgen receptor was at least partially antagonized at this dose (the negative feedback loop was activated), but these effects were only observed in the pubescent male offspring and not the immature offspring on PND 21 or the young adult males on PND 83 ± 2. Nor were increases in serum androgen hormones observed in the 0.25 mg/kg bw/d dose group, despite the apparent receptor antagonism. Thus, although a specific influence of flutamide on hormone levels cannot be ruled out, it seems probable that the higher androgen levels observed at the 2.5 mg/kg bw/d dose may be a function of the delay in sexual maturation, since these animals are almost 2 weeks older than those of the concurrent control group. This is somewhat corroborated by similar serum concentrations when testosterone and androstenedione levels in the pubescent 2.5 mg/kg bw/d dose group are compared with those from control males in early adulthood (PND 83 ± 2).

Higher serum androgen concentrations were also noted in early adulthood female offspring (PND 83 ± 2), which had increased androstenedione, but not testosterone levels. These effects are generally consistent with activation of the negative feedback loop by antagonism of the androgen receptor; however, the biological relevance of these observations in female animals is much less clear than in the males. Whether acting as an androgen receptor antagonist or via some other mode of action, there are reports that flutamide can disrupt steroid biosynthesis, albeit at doses much higher than the doses tested in this study (Adamsson et al. 2008; Mikkila et al. 2006). The results in our investigation were also quite variable, resulting in large standard deviations within each group. This wide variation increases the likelihood of statistically significant changes, which are the product of random chance rather than a true result of flutamide treatment. As the measured hormone concentrations are quite small, often at or near the lower limit of quantitation at some time points, this means that both the detection and the interpretation of small perturbations in sex steroid hormone biosynthesis can be quite difficult. Previous reports do suggest that any flutamide-induced alterations in serum sex steroid hormone levels at doses near or below the LOAEL are too minute to be significant.

Across all time points, no biologically relevant differences were observed between the sex organ weights of treated female animals and their age-matched controls. In contrast, across all time points a number of male reproductive organ weights were significantly reduced in animals exposed to 2.5 mg/kg bw/d and to a lesser extent at 0.25 mg/kg bw/d flutamide. In general, these weight data describe treatment effects most likely linked to the antiandrogenic properties of flutamide. More specifically, it was noted that the test groups which demonstrated an effect in one organ generally displayed similar effects in the other male sex organs. Furthermore, a comparison of the organ weights at weaning (PND 21) or in young adults (PND

83 ± 2) revealed few differences between the absolute and relative organ weights.

However, the pubescent offspring have to be assessed even more carefully, because each subset 2 offspring was killed at a different age (i.e., on the day of puberty of each individual). This is particularly important at the 2.5 mg/kg bw/d dose, because the significant delay in sexual maturation prevents comparison with age-matched controls. As the male animals of this group, and therefore their organ weights, are larger at the time of puberty, the absolute body and organ weights of these offspring vary as widely as the ages of the animals. Thus, only the relative organ weights are considered toxicologically relevant at this time point. For the purposes of this analysis, we will focus on the relative weights of the three male sex organs we observed to be most sensitive to anti-androgen treatment: the prostate, ventral prostate, and seminal vesicles.

The secretory activity of the accessory sex glands is extremely sensitive to androgen exposure; thus, weight change is often related to altered secretory activity in the prostate and seminal vesicle and can be used as a sensitive, and relatively rapid, integrated indicator of altered androgen signaling (OECD 2009). Direct comparison of the organ weights over time shows the general increase in relative sex organ weight as a result of sexual maturation. The exception to this trend was in the 2.5 mg/kg bw/d dose group, in which the organs either did not seem to increase in weight (in the case of the prostate and ventral prostate), or increased only very little (the seminal vesicles). In effect, the prostate, including the ventral portion, of the animals in this dose group does not appear to have adequately developed with the sexual maturation of the animal. Administration of 2.5 mg/kg bw/d flutamide resulted in a nearly 50 % reduction in relative organ weights by early adulthood. In contrast, the statistically significant reduction at the 0.25 mg/kg bw/d was much milder (9–12 %) and was observed only in the prostate at sexual maturation.

Similarly, as the secretions of the coagulating gland, prostate, and seminal vesicle are also androgen dependent, particularly those of the seminal vesicle, they can be used as a marker of androgen effects (OECD 2009). No alterations to secretion were noted in any of the young adult animals treated with 0.25 mg/kg bw/d flutamide; however, a number of changes were observed at 2.5 mg/kg bw/d. The seminal vesicles of the young adult offspring were reduced in size with decreased secretion in nine of ten males, and the prostate was also smaller, with decreased secretion in five of ten animals. These findings correlate very well to the described organ weight reduction in the respective organs and are similar to those found in previous reports at higher doses (Imperato-McGinley et al. 1992; O'Connor et al. 1998).

Macroscopic lesions were also observed in one young adult male animal of this dose group, which exhibited a severe bilateral size reduction in testes and epididymides combined with cryptorchism, as well as a moderate reduction in the size of the prostate. This same animal was found to have impalpable testes in clinical examinations during the in-life phase of the study. In addition to its small size, the left testis exhibited marked diffuse tubular degeneration accompanied by a moderate multifocal Leydig cell hyperplasia during histopathological analysis, as well as aspermia in the corresponding epididymis. The left seminal vesicle was extremely small (severe size reduction) in this animal, and the right seminal vesicle was either missing or not detectable. Histopathologically, the left testis of another animal treated with 2.5 mg/kg bw/d also exhibited a minimal multifocal tubular degeneration, though no other findings were observed. Taken together, these results are typical of the effects previously reported for flutamide exposure, though these studies used higher doses, and therefore, the findings were more frequent (Imperato-Mcginley et al. 1992).

Therefore, a weight of evidence analysis suggests that these findings are probably related to the anti-androgenic properties of flutamide; however, this is not definitive because of the singular nature of each of these findings. Moreover, the spermatid counts in the epididymides and spermatozoa counts in the testis of these animals were comparable to those of concurrent and historical controls, suggesting that spermatogenesis proceeds normally. These results indicate that the local androgen level in the testes is still sufficient for testicular maturation and spermatogenesis, even if sperm motility is slightly reduced.

Despite these findings in adult offspring, very little was observed during development after exposure to 2.5 or 0.25 mg/kg bw/d flutamide. The reproductive organs of PND 21 males (weaning), i.e., left testis, left epididymis, seminal vesicles, coagulating glands, and prostate, all exhibited an immaturity, which was normal for their age and comparable to controls. By puberty, the seminal vesicles, coagulating glands, and prostates of all control and treated offspring had matured to a comparably juvenile stage with a moderate amount of secretion, with no observable differences between the vehicle and treatment groups.

Similarly, the left testis was also at a juvenile stage with a fully developed spermatogenic cycle in both control and treated animals. However, the number of Leydig cells seemed to be increased in pubescent offspring treated with 2.5, but not 0.25 mg/kg bw/d flutamide. This is almost certainly treatment-related, but not necessarily toxicologically relevant, as the increase might be a consequence of the more advanced age of these animals (killed later due to delayed preputial separation). As the entire testis was also larger in these older animals, this is likely not a true

finding. The delay in the date of killing was also evident in the epididymides. While the controls and most of the treated animals were juvenile, all males treated with 2.5 mg/kg bw/d exhibited a more advanced development called the juveno-adult transition stage, which correlates very well with the age of the animals on the day of preputial separation. Therefore, none of these findings is considered a primary indicator of systemic toxicity.

When all these clinical, clinical chemistry, and pathological findings are taken together, they provide clear evidence of anti-androgenicity of flutamide at 2.5 mg/kg bw/d and a milder endocrine effect at 0.25 mg/kg bw/d. As expected for an androgen receptor antagonist, effects were observed in males; treated females generally exhibited no obvious sign of an endocrine-dependent effect. The effects observed were all classic downstream consequences of interferences in androgen hormone signaling. This was true even at doses where findings were evident in only the most sensitive parameters (nipple retention, age at puberty, and relative sex organ weights); no alterations to anogenital distance/anogenital index were observed, nor were there any pathological findings. In addition, the anti-androgenic findings were often transient, e.g., exhibited at puberty, but not by the time the animals were adults. Thus, these data were sometimes inconsistent and difficult to interpret (e.g., transient nipple retention and unaltered anogenital distance) and represented the gray area between doses, which were clearly anti-androgenic and a true NOAEL, with only the minor effects indicative of a true LOAEL.

Dose levels at or below the NOAEL

In contrast to what was observed at 2.5 and 0.25 mg/kg bw/d, flutamide treatment at doses at the NOAEL (0.025 mg/kg bw/d) or below (0.0025 or 0.00025 mg/kg bw/d) resulted in no clinical or histopathological signs of anti-androgenicity. A few statistically significant alterations to steroid hormone levels were noted, but these changes were scattered across the dose groups/time points and generally reflected altered stress hormone levels rather than the expected hormone pattern for anti-androgenic effects. Several organ weights were also changed (prostate, ventral prostate, and ovary), but again, these effects were very borderline and scattered randomly among the dose levels and time points. All findings were observed singly and in the absence of any other corroborating observations at the same dose groups or time points. Therefore, any assumption of a treatment-related effect stretches credulity. Thus, despite our extensive attention to a wide variety of anti-androgenic parameters, we were unable to find an anti-androgenic effect in the dose groups at or below the NOAEL despite the marked effects at doses above the NOAEL level.

While these observations are not novel to such a well-characterized member of this compound class, they do provide clear positive indications of the validity of the study. Regardless of the lack of findings at doses at or below the NOAEL, the present investigation is important because it conforms to current guidance for the evaluation of chemicals for endocrine disruption (OECD 2002, 2012). Moreover, the study also conforms to the criteria established by proponents of the non-monotonic dose–response curve (NMDRC) hypothesis. In their review of over 800 studies for low-dose effects on the endocrine system, Vandenberg et al. (2012) established three criteria for the acceptance of studies: The experimental system, species, or animal strain chosen must be responsive to endocrine effects, a negative control group must confirm the experiment is "free of background contamination," and a positive effect group must indicate that the experimental system is capable of responding to a low-dose acting on the same pathway. An outbred Wistar rat experimental model was chosen for its genetic diversity and its documented sensitivity to endocrine effects (Diel et al. 2004). No endocrine effects were observed in the three negative control groups; however, a dose-dependent modulation of a variety of endocrine-sensitive parameters was observed at the top two doses. Thus, any lack of findings at the other three doses can be interpreted as a lack of endocrine effects at these lower dose levels. This is important because the results of all five doses when considered together indicate the absence of evidence for a NMDRC at any endpoint measured.

Non-monotonic dose response

As noted in Vandenberg et al. (2013), "[NMDRCs] can manifest in apical endpoints due to the interaction of two monotonic curves at lower levels of biological organization…" The same authors also cite the expression of cell- and tissue-specific receptors and cofactors, receptor selectivity and competing interactions of multiple receptors, receptor down-regulation and desensitization, receptor competition, and endocrine negative feedback loops as mechanisms responsible for NMDRCs (Vandenberg et al. 2012, 2013). However, the principal feature of all these is the interaction of multiple mechanisms. This point is central to any discussion of non-monotonicity.

Proponents of NMDRCs tend to define the term mathematically, as a dose–response relationship where the slope changes sign, and argue that for several reasons endocrine active substances are unique in their ability to produce such response curves (Vandenberg et al. 2012, 2013). Such a definition, however, ignores the critical role that an understanding of the mechanism(s) involved play(s) in the practice of descriptive toxicology.

Although mechanism and mode of action are terms, which are commonly synonymous in the toxicological vernacular, for the purpose of this argumentation a mode of action is a broad categorical description of the observed biological effects of a substance (e.g., anti-androgen). A mechanism is defined to be a qualitative explanation of an action or chain of actions by an individual substance within a test system (e.g., androgen receptor antagonism, resulting in reduced androgen signaling during late gestation and feminization of the external male sex organs). This explanation must be specific enough that when taken together with any other mechanisms, all the effects of the substance can be explained scientifically. It is worthy to note that a single substance can have more than one mechanism and that there may be more than one mechanism within a categorical mode of action (i.e., disrupted steroidogenesis and androgen receptor antagonism are both forms of anti-androgenicity).

Under current practices, it is impossible to divorce this mechanistic understanding from the practice of descriptive and regulatory toxicology. Dose, model system, and endpoint selection are never random; a great deal of expert toxicological judgment and prior experience with a substance is relied on to achieve the appropriate study design. This knowledge base is relied on again in assessing the results, in order to understand the mechanisms underlying the toxicity observed. Each mechanism must be individually appraised to determine whether the changes observed are treatment dependent (versus normal biological variation within the experimental model), adaptive or adverse (restoring homeostasis versus pathological effects) and relevant to human hazard assessment.

Thus, an apparent NMDRC will always be broken down into its multiple monotonic mechanistic components during a thorough expert assessment, even if the overall dose response might be measured using the same endpoint(s).

Under such an appraisal, it is clear that at the doses studied, flutamide has only one mode of action, androgen receptor antagonism, which hinders androgen signaling and ultimately results in decreased anogenital distances and indices, increased nipple retention, delayed puberty, decreased sex organ weights, and the observed developmental pathology. When considered separately, each of these endpoints is altered beyond the historical control range (a marker of the biological variation of this species and strain) at the 2.5 mg/kg bw/d dose. The changes also appear to be dose dependent, with some weaker effects already apparent at 0.25 mg/kg bw/d. Taken together, these results suggest that the observed effects are indeed both biologically relevant and caused by the flutamide treatment. Qualitatively, many of these alterations are significant, permanent, and irreversible, going beyond the transient, mild, and reversible effects which indicate an adaptive biological response. Therefore, these data indicate an adverse change in the homeostasis of the animals, at least in the 0.25 and 2.5 mg/kg bw/d dose groups.

But it is also important to note that the dose-dependent changes to anogenital distances and indices, areola retention, age at puberty, sex organ weights, and incidences of developmental pathology do not occur in isolation from each other. Indeed, the mechanism of toxicity is such that all these effects combined represent an apical pattern of consequences of the endocrine mechanism of flutamide. The whole anti-androgenic pattern has to fit; as antagonism of the androgen receptor is the upstream event responsible for each of these findings in the various tissues and no interference from another mechanism of toxicity is likely, the same downstream pattern of observations is to be expected from each case of androgen receptor antagonism.

While an understanding of the mechanism(s) of toxicity can assist greatly with data interpretation, the corollary is also true: the data should inform the understanding of the mechanism(s) involved. If only one of a number of androgen-signaling-mediated endpoints is affected, it is extremely unlikely that a mechanistic series of events begun by androgen receptor antagonism led to the measured effect, suggesting an alternative mechanism of substance toxicity. We and others have shown that endpoints affected by endocrine modulation can also be independently altered via non-endocrine mechanisms (Carney et al. 2004; Marty et al. 2003; Melching-Kollmuss et al. 2014). It is important, however, that this idea is never completely divorced from the realities of endpoint sensitivity (Borgert et al. 2014). Certainly, some endpoints were sensitive to flutamide at the 0.25 mg/kg bw/d LOAEL dose (e.g., areolas and age at puberty), while others not until 2.5 mg/kg bw/d (e.g., the incidence of developmental abnormalities). Crucially though, the entire anti-androgenic pattern of changes did occur within this tenfold dose range, which is compatible with the idea that these differences are due to endpoint sensitivity rather than an alternative mechanism or mechanisms.

Moreover, having a fundamental understanding of the mechanism(s) of toxicity should assist in the decision of whether there is a need to test doses substantially below the NOAEL. As competitive androgen receptor antagonism is the well-characterized initial step in the pathway of the only mechanism of flutamide toxicity, it makes no sense to systematically test doses, which are not high enough to out-compete the endogenous androgen hormones at the site of action. Nor would any long-term effects be logically expected with continued exposure to doses lower than the measured threshold of flutamide activity at the site of action.

Taken together, all these observations form a logical argument in favor of the current regulatory paradigm.

With an understanding of the mechanism(s) of toxicology involved, determining when one mechanism ends and the next begins, becomes relatively straightforward. Attention can be focused on the mechanism of toxicity, which occurs at the lowest doses. Regulatory studies can then be designed to determine where the threshold begins for this mechanism, setting a NOAEL for the substance. Because knowledge of the mechanism(s) was taken into account when planning the study, we can be sure that this experimentally derived NOAEL represents a true threshold for the substance below which no adverse effects occur.

Conclusion

While theory precludes effects at doses below the NOAEL, this has never been conclusively verified. The current investigation was therefore designed to test threshold theory using flutamide as a prototypical anti-androgenic endocrine disruptor. This study tested 5 flutamide dose levels, two above the NOAEL (2.5 and 0.25 mg/kg bw/d), the NOAEL dose (0.025 mg/kg bw/d), and two further doses below the NOAEL (0.0025 and 0.00025 mg/kg bw/d).

In general, we observed anti-androgenic changes at the 2.5 mg/kg bw/d (chosen to represent a clear effect level) and 0.25 mg/kg bw/d (chosen as a LOAEL) doses, but not at lower exposures. Nipple/areola counts appeared to be the most sensitive measure of effect, closely followed by age at sexual maturation, then anogenital distance/anogenital index and male sex organ weights, and finally gross and histopathological findings. This order generally coincides with the order of sensitivities seen in the literature (Borgert et al. 2014). The quantification of hormone levels was found to be inconclusive, even at 2.5 mg/kg bw/d; these results may require further specialized analysis, which is beyond the scope of this body of work. While these endpoints for anti-androgenicity had varying sensitivities, when taken together these data reveal a very important observation: monotonicity.

Regulatory toxicity studies are based on the paradigm of an activity threshold; when the internal concentration of a single substance at the site(s) of action is too low to stimulate a noticeable adverse response, the corresponding dose is determined to be at or below the NOAEL (Holsapple and Wallace 2008). However, some recent scientific work has suggested the potential for endocrine disrupting modes of action at very low doses, i.e., doses below the no observed adverse effect level (NOAEL) or doses which are relevant to human exposures, postulating the existence of non-monotonic J- or inverted U-shaped dose curves (Calabrese and Blain 2005; Vandenberg et al. 2012). This hypothesis implies that no threshold for adverse effects can be characterized and any exposure poses increased hazard to humans.

However, the endocrine system is specifically designed to respond to environmental fluctuations and still maintain stable hormonal signaling. Such regulations generally are considered normal, adaptive, and necessary as long as they are transient and within the normal homeostatic range (Goodman et al. 2010; Rhomberg and Goodman 2012). It seems logical that the same adaptive processes, which allow humans to reassert hormonal homeostasis in a changing natural environment, might also compensate for exposure to endocrine active substances at low doses. As long as these processes remain truly adaptive, then they do not necessarily pose an increased hazard to humans. Therefore, it is important to determine not only whether effects are observed at human relevant exposures, but also whether any effects observed are adverse.

Importantly, no anti-androgenic effects were noted in the present study at doses below 0.25 mg/kg bw/d; however, anti-androgenic changes were observed at the 0.25 mg/kg bw/d and 2.5 mg/kg bw/d doses, thereby demonstrating the capability of the test system to fulfill all Klimisch criteria (Klimisch et al. 1997). Despite testing five logarithmically spaced doses, which stretched up to 1000-fold below the 0.25 mg/kg bw/d LOAEL, no evidence for non-monotonic endocrine disruption by flutamide was observed. Notwithstanding the fact that few studies have been performed measuring parallel exposures to comparably low doses of anti-androgens, missing evidence for a non-monotonic dose response closely matches observations of several other low-dose exposures to endocrine disruptors (Christiansen et al. 2010; Gray et al. 1999a, b; Holsapple and Wallace 2008; Howdeshell et al. 2008).

Supported by the data in the current investigation, the authors suggest that caution should be exercised when assumptions and conclusions are drawn about non-monotonic dose–response curves and low-dose effects. Evaluation of this hypothesis should only be based on evidence from valid animal data with an understanding of the underlying mechanisms of toxicity. Therefore, we submit that the current tendency toward over-extrapolation of any endocrine modulation as being of concern without considering the magnitude of the effect and its potential adversity is inappropriate.

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REGULATORY TOXICOLOGY

Comparing effect levels of regulatory studies with endpoints derived in targeted anti-androgenic studies: example prochloraz

Stephanie Melching-Kollmuss[1] · Karma C. Fussell[2] · Steffen Schneider[3] · Roland Buesen[3] · Sibylle Groeters[3] · Volker Strauss[3] · Bennard van Ravenzwaay[3]

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Abstract Prochloraz is an imidazole fungicide, and its regulatory toxicological data package has been primarily generated in the 1990s. More recently, studies have been published demonstrating an interaction with hormone receptors/steroidogenesis and effects with an endocrine mode of action. In the present study, prochloraz has been investigated in a comprehensive in vivo study including relevant elements of current regulatory reproduction toxicity studies and additional mechanistic parameters. Prochloraz was administered per gavage in oil from GD 6 to PND 83 to pregnant and lactating Wistar rats and their respective offspring, at doses of 0.01 mg/kg bw/day (acceptable daily intake of prochloraz), 5 mg/kg bw/day [expected no-observed-effect-level (NOEL)] and 30 mg/kg bw/day. At 30 mg/kg bw/day maternal and offspring effects (decreased viability, lower number of live offspring) were seen including a delayed entry into male puberty (+1 day) accompanied by lower male offspring body weights, increased anogenital distance/index in females and transiently retained nipples in males at PND 12 (not seen at PND 20). The only finding at the "expected NOEL" was increased incidences of transiently retained nipples in males which are not considered adverse. No effects were seen in the low-dose group. There was no evidence for a non-monotonic dose–response curve or effects at low levels.

Keywords Reproductive toxicology · Anti-androgenicity · Reference value · Low dose · Prochloraz

Introduction

Current risk assessment for pesticides are conducted based on the lowest no-observed-adverse-effect-level (NOAEL) identified in a comprehensive set of regulatory toxicological studies conducted in several animal species covering all relevant endpoints (repeated dose toxicity in rats, mice and dogs, chronic toxicity and carcinogenicity in rats and mice, reproduction toxicity in rats and developmental toxicity in rats and rabbits). Based on this lowest NOAEL a—considered-to-be-safe—reference value for human lifelong exposure (ADI—acceptable daily intake) is derived usually using an extrapolation factor of 100. Thus, pesticides are a class of chemical substances, which are extensively studied, before they can be registered. The European toxicological data requirements are summarized in detail in the relevant EU Directives (EC 1107/2009; Commission Regulation No 283/2013). It is recommended that toxicological testing is conducted according to the most recent OECD or European or US Environmental Protection Agency OPPTS/OCSPP Guidelines for the respective toxicological study under assessment. Depending on when the respective pesticide active ingredient was developed, the testing protocol valid at the time may differ significantly from the current standard.

Taking into account the differences between new and earlier technical guidance standards for reproduction toxicity testing, hazard identification clearly has improved

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✉ Bennard van Ravenzwaay
bennard.ravenzwaay@basf.com

[1] Product Safety, Regulations, Toxicology and Ecology, BASF SE, Ludwigshafen am Rhein, Germany

[2] Chemical Food Safety, Nestlé, Lausanne, Switzerland

[3] Experimental Toxicology and Ecology, BASF SE, 67056 Ludwigshafen am Rhein, Germany

recently, particularly when anti-androgenic modes of action are considered. In the intervening 18 years between the previous and current OECD 416 technical guidance (OECD, TG 416 1983), parameters evaluating sperm quality, anogenital distance, nipple/areola retention and entry into puberty have been added important and sensitive markers for the detection of endocrine modulation.

There is a concern that the regulatory studies conducted might not be able to identify sensitive effects and would systematically overlook some effects caused by an endocrine mode of action (Kortenkamp et al. 2011; Hass et al. 2013). Further, there is a concern expressed at an international level that current risk assessment methodologies may be insufficient to protect against (low-dose) exposure to endocrine active chemicals. This concern is based on a hypothesis that the endocrine system is somehow different that hormones are acting in mammalian bodies at very low concentrations and that the likelihood for non-monotonic dose responses to occur is higher for substances with an endocrine mode of action (Vandenberg et al. 2012; Kemi 2013; UNEP and WHO 2013). This hypothesis is heavily debated in the scientific and regulatory community (Rhomberg and Goodman 2012; Lamb et al. 2014; Dekant and Colnot 2013), and scientific and regulatory bodies have come to the conclusion that risk assessment is an important element in the full toxicological assessment of compounds with an endocrine mode of action (Bars et al. 2012; EFSA 2013; Marx-Stoelting et al. 2011, 2014).

Prochloraz has been tested in a full set of regulatory toxicological studies including two multi-generation reproductive toxicity studies, which was performed according or comparable to the US EPA OPPTS 870-3380, OECD TG 416 (1983) (EFSA conclusion 2011). These guidelines, however, precede both OPPTS and OECD harmonization and lack specific parameter to identify anti-androgenicity (e.g., sperm parameter, onset of puberty). Besides these regulatory studies, prochloraz has been extensively studied in mode of action studies during the last 15 years.

Prochloraz is a fungicide belonging to the imidazole group and acts as an inhibitor of ergosterol biosynthesis in fungi. Imidazole fungicides inhibit the activity of lanosterol 14α-demethylase, which is a fungus-specific cytochrome P450 enzyme. The lowest NOAELs from long-term studies were 0.9 mg/kg body weight/day (mg/kg bw/day) in dogs, 5.1 mg/kg bw/day in rats and 7.5 mg/kg bw/day in mice (EFSA conclusion 2011). The chronic reference value (ADI—acceptable daily intake) of prochloraz is 0.01 mg/kg bw and is based on the lowest NOAEL of 0.9 mg/kg bw, derived in dogs based on liver weight increases seen in the next higher dose level of 2.5 mg/kg bw/day (EFSA conclusion 2011).

In the first two-generation toxicity study, prochloraz showed impaired female fertility in rats: Reproductive outcome was impaired as indicated by extended gestation lengths, dystocia and reduced live birth and viability indices seen at a dietary dose of 625 ppm (corresponding to 57–81 mg/kg bw) in the two-generation toxicity study in rats. The only effect seen at the next lower dietary concentration of 150 ppm (corresponding to around 15 mg/kg bw/day) was slightly increased gestation lengths. Prochloraz did not impair male fertility in rat studies and was not teratogenic in rats and rabbits (studies conducted according to OECD TG 414 1981). During the EU peer review process, a second multi-generation study was evaluated and the overall NOAEL for reproduction was considered to be 50 ppm (EFSA conclusion 2011). The doses corresponding to this dietary concentration range from 2.26 to 7.76 mg/kg bw/day in the male rats, depending on the actual food consumption and body weights during the different phases of the study.

As can be retrieved from published papers, rats displayed typical signs of anti-androgenicity after treatment with prochloraz, like transiently retained nipples, reduced testosterone, increased progesterone levels, reduced male reproductive organ weights and delayed entries into male puberty (Blystone et al. 2007a; Laier et al. 2006; Vinggaard et al. 2002). A NOEL—identified for the most sensitive endpoint retained nipples—of 5 mg/kg bw/day in rats was determined by (Christiansen et al. 2009). A summary of the findings seen in regulatory studies and in in vivo studies published in peer-reviewed journals is provided in Tables 1 and 2.

The results of several in vitro assays, which have been published over the last 15 years, indicate that prochloraz has endocrine mode(s) of action. These in vitro screening studies were carried out in various test systems, different concentrations or concentration ranges and comprised different endpoints or assays like cell proliferation, estrogen receptor (ER) transactivation assays (ERTA), androgen receptor (AR) transactivation assays (ARTA), aromatase assays and steroid hormone synthesis assay [e.g., OECD TG 456 (2011)]. Prochloraz was found to be able to interact with estrogen and/or androgen receptors, with aromatase and with the steroid hormones (Andersen et al. 2002; Birkhoj et al. 2004; Grünfeld and Bonefeld-Jorgensen 2004; Hecker et al. 2006; Kojima et al. 2004; Laier et al. 2006; Sanderson et al. 2002; Trösken et al. 2004; Vinggaard et al. 2000, 2002, 2005). More recent studies suggest that prochloraz interferes with steroidogenesis in in vitro systems by inhibition of P450c17 (17α-hydroxylase and 17,20-lyase) (Nielsen et al. 2012). Cortisol and corticosterone levels were shown to decrease after exposure of H295R cells to prochloraz (Winther et al. 2013; Ohlsson et al. 2010). In some of these studies also gene expression of relevant steroidogenesis genes were investigated: H295R cells exposed to 0.03 µM prochloraz showed decreased expressions of some of the

Table 1 Reproductive toxicity studies with prochloraz in rats

Study design	Doses tested	NOAEL	Relevant findings	References
Two-generation rat study (diet)	0; 37.5; 150 and 625 ppm corresponds to 3.1–4.5, 13–18 and 57–81 mg/kg bw/day	$NOAEL_{parental}$: 150 ppm $NOAEL_{reproduction}$: 37.50 ppm $NOAEL_{offspring}$: 150 ppm	Parental toxicity 625 ppm: mortality (in females only) ↑ clinical symptoms including aggressive behavior, hunched posture, walking on toes, piloerection, pallor; body weight and body weight gain↓, liver weight↑ Reproductive toxicity and litter parameters ≥150 ppm: gestation length↑(not statistically significant); 625 ppm: dystocia, parturition difficulties, total litter loss↑, mean litter size↓, weight from birth to weaning↓, live birth and viability index↓	Cozens et al. (1982) summarized in draft assessment report of prochloraz
Two-generation rat study (diet)	0; 50; 150 and 450 ppm corresponds to 0, 2.26–8.09, 6.58–23.71 and 21.10–73.9 mg/kg bw/day*	$NOAEL_{parental}$: 50 ppm $NOAEL_{reproduction}$: 50 ppm $NOAEL_{offspring}$: 150 ppm	Parental toxicity ≥50 ppm: body weight gain in females during gestation ↓ ≥150 ppm: impairment body weight gain and body weight, relative liver weight↑ Reproductive toxicity and litter parameters ≥50 ppm: increased gestation length (not statistically significant at 50 ppm) ≥150 ppm dystocia, parturition difficulties, 450 ppm: male reproduction organ weights ↑ body weight gain of pups↓	Reader et al. (1993) summarized in draft assessment report of prochloraz
Teratogenicity study in rats (gavage)	0; 6; 25; 100 mg/kg bw/day in 10 % acacia solution	For maternal and developmental toxicity: 25 mg/kg bw/day Not teratogenic. No increased incidences of early or late resorptions	Maternal toxicity reduced food consumption, decreased body weight gain, increased liver weights Embryotoxicity slightly reduced litter size, reduced implantation and viability index, increased number of dead fetuses related to maternal toxicity, decreased fetal weights, calcification of sternebrae and arches retarded	Beswick (1980) summarized in draft assessment report of prochloraz

* Food intake ranges for different phases of the study in males and females; no dose adjustment has been done during lactation to account for increased food intake of lactating dams

Table 2 Studies with prochloraz during postnatal development

Study design	Exposure window	Doses of prochloraz and positive controls	NOAEL	Relevant findings	References
Male pubertal rat study (gavage)	PND 23–42/43 or 23–50/51	0 (corn oil), 31.3, 62.5, 125 mg/kg bw/day	<31.3 mg/kg bw/day	Serum/testis progesterone↑, serum/testis hydroxyprogesterone↑, serum/testis testosterone↓, testis androstenedione↓	Blystone et al. (2007b)
			31.3 mg/kg bw/day	Body weight↓, liver↓, kidney↓, epididymis↓, levator ani plus bulbocavernosus muscle↓	
Male pubertal rat study (gavage)	PND 23–42/43	0 (corn oil), 3.9, 7.8, 15.6, 31.3, 62.5 mg/kg bw/day	7.8 mg/kg bw/day	Testis hydroxyprogesterone↑, serum/testis testosterone↓, testis androstenedione↓	Blystone et al. (2007b)
Hershberger (gavage + subcutaneous)	PND 49–58/59	0 (corn oil), 15.6, 31.3, 62.5, 125 mg/kg bw/day, ±100 µM testosterone propionate, sc	15.6 mg/kg bw/day	Liver wt↑	Blystone et al. (2007b)
			62.5 mg/kg bw/day	Seminal vesicle↓, levator ani plus bulbocavernosus muscle↓, luteinizing hormone↓	
Hershberger (gavage + subcutaneous)*	At week 6 of age, daily for 7 consecutive days	0 (untreated), 25 mg/kg bw/day, positive control: 20 mg Flutamide/kg bw/day, sc; ± 0.5 mg testosterone propionate, sc	<25 mg/kg bw/day	Liver↑	Birkhoj et al. (2004)
Hershberger (gavage + subcutaneous)	At week 6 (castrated) or week 10 of age, daily for 7 consecutive days	0 (peanut oil), 250 mg/kg bw/day, +0.5 mg testosterone propionate, sc	<250 mg/kg bw/day	Liver↑ (intact/castrated), seminal vesicle↓ (intact/castrated), ventral prostate/levator ani bulbocavernosus muscle/bulbourethalis gland↓ (castrated only), LH↑ (intact/castrated)	Vinggaard et al. (2002)
Hershberger (gavage + subcutaneous)	At week 6 (castrated) or week 10 of age, daily for 7 consecutive days	0 (peanut oil), 50, 100, 200 mg/kg bw/day ±0.5 mg testosterone propionate	<50 mg/kg bw/day	ventral prostate/seminal vesicle/levator ani bulbocavernosus muscle/bulbourethalis gland↑, TSH↓	Vinggaard et al. (2002)
Screening for anti-androgenic effects after pre- and postnatal exposure (gavage)	GD 7–PND 16, daily administration	0 (peanut oil), 30 mg/kg bw/day (alone) or 20 mg/kg bw/day (mixture of 5 pesticides including 15 mg/kg bw/day prochloraz)	Dams: <30 mg/kg bw/day	Transient body weight gain↓, delay of parturition	Vinggaard et al. (2005)
			Offspring: 30 mg/kg bw/day (transient hormone changes and nipple retention not seen as adverse)	Areola/nipple retention (PND 13), plasma/testicular testosterone↓ (GD 21, normal by PND 16), testicular progesterone↑ (GD 21, normal by PND 16), ex vivo progesterone synthesis↑, EROD/BROD↑, body weight↓ (females PND 224)**	

Table 2 continued

Study design	Exposure window	Doses of prochloraz and positive controls	NOAEL	Relevant findings	References
Screening for anti-androgenic effects after pre- and postnatal exposure (gavage)	GD 7–PND 16, daily administration interim kill of 8 dams/group at GD 21	0 (peanut oil), 50, 150 mg/kg bw/day	Dams: 50 mg/kg bw/day Offspring: 150 mg/kg bw/day <50 mg/kg bw/day	Body weight gain↓ Anogenital distance: males↓, females↑, males fetuses (GD21): transient testis testosterone/ex vivo production ↓, testis progesterone↑, mild dysgenesis of male external genitalia (scores 1.25 at 50 mg/kg bw/day and score 1.28 at 150 mg/kg bw/day), weight of seminal vesicles/ bulbourethal glands↓, P450c17↑	Laier et al. (2006)
Screening for anti-androgenic effects after prenatal exposure (gavage)	GD 14–18 daily administration	0 (corn oil), 31.25, 62.5, 125, 250 mg/kg bw/day	Dams: 62.5 mg/kg bw/day Offspring: 31.25 mg/kg bw/day	Delay of parturition Transient body weight↓ testicular histopathological findings↑ (that included atrophy and vacuolization of seminiferous epithelium	Noriega et al. (2005)
Screening on fetal testicular steroidogenesis fetal prenatal exposure (gavage)	GD 14–18, daily administration	0 (corn oil), 7.8, 15.6, 31.2, 62.5, 125 mg/kg bw/day	Dams: 31.2 mg/kg bw/day Ex vivo fetal testes: <7.8 mg/kg bw/day	body weight↓ Testis progesterone + 17α progesterone↑, in vitro CYP17 hydroxylase activity↓	Blystone et al. (2007a)

* The study was designed to investigate the combined anti-androgenic effect of a mixture of five pesticides including prochloraz

** In this study no effects on sperm counts or entry into male/female puberty were seen

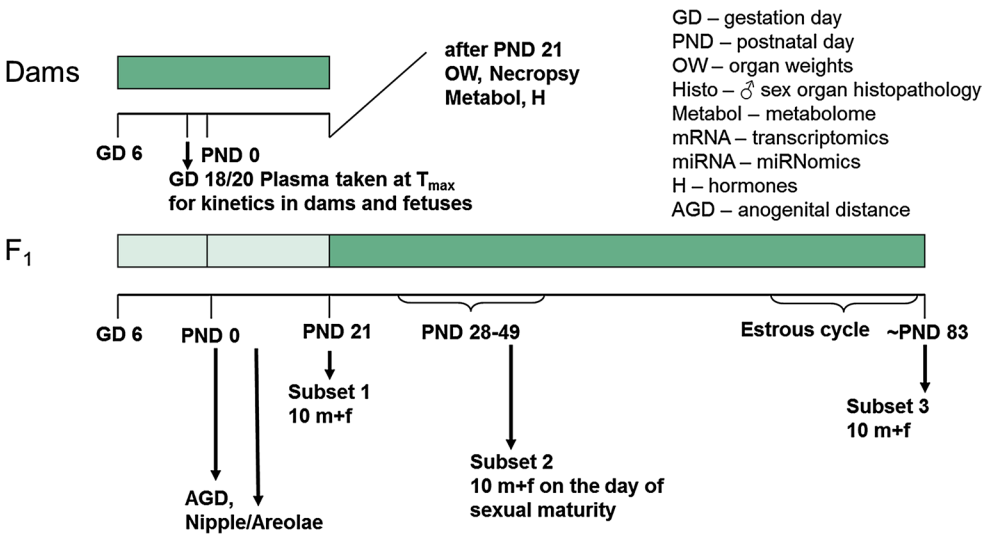


Fig. 1 Experimental design of the study. *Note* it should be noted that samples were taken for future molecular analyses (metabolome, miRNome and transcriptome), the results of which are not described in this publication. Also the plasma kinetics of dams and fetuses taken from an additional number of 5 animals killed on GD 18 are not described in this publication

genes involved in steroidogenesis were seen (Ohlsson et al. (2009); Ohlsson et al. 2010). With regard to the results of the steroidogenesis assay, it is to be mentioned that the OECD TG 456 is validated only to detect testosterone and estradiol, not for other steroid hormones.

Overall, this battery of published in vitro screening studies indicates that prochloraz is able to induce hormonal imbalance by affecting the androgen and estrogen receptor, to inhibit the aromatase activity and to interfere with steroidogenesis probably at the level of P450c17. A review of the endocrine-related data available for prochloraz has been conducted by OECD which led to the conclusion that "The combined dataset provides sufficient evidence of endocrine activity" (OECD 2012).

The present report aims to fill this gap in knowledge by determining whether anti-androgenic substances disrupt endocrine homeostasis at very low doses using prochloraz as a compound with an anti-androgen mode of action. Importantly then, these data should clarify whether the prochloraz-dose–endocrine-response relationship is non-monotonic or results in a threshold to enhance the current debate about the regulation of endocrine disruptors, in order to be able to confirm, that the reference value can be considered to be a safe threshold. To accomplish these aims, a pre- and postnatal in vivo study design has been chosen which is closely resembling the ICH Guideline on peri- and postnatal toxicity study (ICH S5 R2 4.1.2) and has been found to be able to identify endpoints caused by an endocrine mode of action in vivo (e.g., DeSesso et al. 2014; Vinggaard et al. 2005; Laier et al. 2006). Consequently, the present study has several objectives: (1) to identify effects with an endocrine mode of action, (2) to identify a NOAEL for anti-androgenicity and (3) to investigate the shape of the dose–response relationship. Beyond the standard regulatory parameters (OECD TG 416 2001), the test design was expanded by the investigation of several endpoints in additional subsets (offspring at weaning, puberty, young adulthood) and additional endpoints examining hormone levels (11-desoxycorticosterone, 11-desoxycortisol, aldosterone, androstendione, corticosterone, cortisol, dihydrotestosterone, progesterone, testosterone), morphology and histopathology (Fig. 1).

Materials and methods

The study was performed according to the German Animal Welfare legislation and with the permission of the local authority (permission number LRI-EMSG56-BASF/G11-3-013). The laboratory is Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) certified.

Twenty pregnant dams per dose group were treated with 0.01, 5 and 30 mg prochloraz/kg bw/day. The doses were chosen based on the results of regulatory and published studies and were selected to represent the regulatory relevant ADI (EFSA conclusion 2011), the NOEL for nipple retention (Christiansen et al. 2009) and an effect dose (EFSA conclusion 2011). The gavage dosing to the pregnant dams starts at gestation day (GD 6) and is continued until weaning. From postnatal day 21 (PND 21) onwards, the offspring was dosed once daily with the same doses until PND 83 (see Fig. 1).

The investigations exceeded the requirements of any specific guideline, but reference is made to OECD 414 (OECD TG 414 2001), 416 (OECD TG 416 2001) and 443 (OECD TG 443 2011) as well as OPPTS 870.3700 and 870.3800 (US Environmental Protection Agency 1998a, b), which principally meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 160 (FIFRA) (US Congress and US Environmental

Protection Agency 1972) and Part 792 (TSCA) (US Congress and US Environmental Protection Agency 1976)], excepting only the analyses of hormone levels.

Test substance

Prochloraz, *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide (PRO), CAS number 67747-09-5, was synthesized and fully characterized at BASF (Ludwigshafen, Germany) as a brownish solid of purity 98 ± 1 %. The solid was then thoroughly dissolved in the corn oil vehicle to prepare each dosing solution. The correctness and the homogeneity of prepared gavage solutions were determined by HPLC–MS analysis of several aliquots sampled from the bottom, middle and top of the preparation vessels. Furthermore, the stability of prochloraz in the corn oil vehicle was proven by testing a sample stored at room temperature at intervals over a period of 7 days. Fresh gavage solutions were prepared weekly.

Parental female animals

Time-mated outbred Wistar rats (WI:Han) were obtained from Charles River Laboratories (Sulzfeld, Germany) on gestational day zero (GD 0) (defined by the presence of sperm or a vaginal plug in the vaginal canal). Throughout the study, all animals were maintained under standard conditions: one animal (or litter) per Makrolon type MIII cage with LTE E001 bedding (ABEDD, Vienna, Austria) and maintained at 20–24 °C and 30–70 % humidity with 15 air changes per hour and a 12-h light/dark cycle. All animals had free access to food [ground Kliba maintenance diet mouse/rat "GLP" (Provimi-Kliba, Kaiseraugst, Switzerland)], drinking water and a wooden gnawing block. The animals were allowed to acclimatize to the laboratory conditions until GD 6, when the dams were randomized into 4 dose groups of 20 animals per group.

Each 20 dams were administered 0, 0.01, 5 or 30 mg/kg bw/day prochloraz in corn oil (4 mL/kg bw) by gavage every morning from GD 6 until the day of killing (about PND 30), except during the day of labor. The dams were killed by cervical dislocation under isoflurane anesthesia and necropsied. The dams were allowed to deliver and rear their pups until PND 21 (weaning).

During gestation and lactation, each dam was examined daily for clinical signs of morbidity and toxicity, as well as parturition and lactation behavior. The food consumption and body weight of each dam were evaluated on GD 0, 6, 13, 18 and 20. The food consumption was also determined weekly for each litter during lactation (PND 0–21) and later for every weaned F1 animal in subsets 2 and 3. All females which littered were weighed on PND 0, 7, 14 and 21. Females which did not litter were killed and examined for gross abnormalities. The uteri were removed from these animals and stained with Salewski (1964) stain for implantation sites. Blood was collected from all dams in the first proestrus after weaning (determined by vaginal cytology) by puncturing the retrobulbar venous plexus under isoflurane anesthesia.

Offspring

The gender, status (live- or stillborn) and any gross-morphological abnormalities of each delivered pup were recorded as soon as possible after birth. Pup viability, mortality and any clinical signs of toxicity or morbidity were determined at least daily. The pups were individually weighed on PND 1, 4, 7, 14, 21 as well as on the day of sexual maturation (vaginal opening or preputial separation). The anogenital distance (AGD) is defined as the distance from the center of the anal opening to the base of the genital tubercle. Anogenital measurements were conducted on all living pups on PND 1 using a measuring ocular in a blind, randomized fashion. In order to standardize for the individual pup weights, the anogenital index (AGI) was calculated by dividing the anogenital distance (mm) by the cubic root of pup weight (g). All living, male pups were examined for the presence or absence of nipple/areola anlagen on PND 12 and re-examined on PND 20.

Before weaning on PND 21, twenty pups of each gender per dose group were selected randomly to be allowed to mature (ten per gender in each of subsets 2 and 3). After blood sampling on PND 21, the dams and a further ten male and ten female pups (subset 1) were killed under isoflurane/carbon dioxide anesthesia and dissected. The relevant tissues (Table 3) were harvested from these animals for pathological analyses. Any surplus pups were also similarly killed on PND 21, but only macroscopically examined for gross abnormalities.

From PND 21 until sexual maturity (subset 2) or young adulthood (subset 3), the maturing F1 pups were further exposed to test substance orally by gavage. One day after reaching sexual maturity, blood was sampled from each animal in subset 2 before it was killed and necropsied. Again, the relevant tissues (see Table 3) were harvested from these animals for pathological analyses. Similarly, each F1 animal of subset 3 was killed as young adult (PND 81–85) after blood sampling in the proestrus (determined by vaginal cytology) and the sexually dimorphic tissues were harvested.

Serum preparation and hormone analysis

Blood samples for hormone measurements were collected under isoflurane anesthesia by puncturing the retroorbital venous plexus or after decapitation:

Table 3 Harvested tissues for further examinations

Tissues	Parental females	Subset 1	Subset 2	Subset 3
All gross lesions	Fixation	Fixation	fixation	Fixation
Adrenal glands	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Brain	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Bulbourethral gland		Weights	Weights	Weights
Cauda epididymis		Weights	Weights	Weights
Cervix uteri	Fixation	Fixation	Fixation	Fixation
Coagulating gland		Weight and fixation	Weight and fixation	Weight and fixation
Epididymides		Weights	Weights	Weights
Glans penis			Weights	Weights
Kidneys	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Left epididymis		Fixation	Fixation	Fixation
Left testis		Fixation	Fixation	Fixation
Liver	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Musc. levator ani together with musc. bulbocavernosus		Weights	Weights	Weights
Ovaries	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Oviducts	Fixation	Fixation	Fixation	Fixation
Pituitary gland	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Prostate, ventral prostate		Weights and fixation	Weights and fixation	Weights and fixation
Seminal vesicles		Weights and fixation	Weights and fixation	Weights and fixation
Spleen	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Thyroid glands (with parathyroid glands)	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Uterus	Weights and fixation	Weights and fixation	Weights and fixation	Weights and fixation
Vagina	Fixation	Fixation	Fixation	Fixation

- Dams in the first proestrus after weaning,
- Subset 3 offspring at about PND 83 (females in proestrus),
- Subset 1 offspring at PND 21,
- Subset 2 rats at onset of puberty.

- Blood aliquots were collected with and without K-EDTA supplement and centrifuged under refrigeration. The separated plasma or serum was aliquoted (>200 µL per aliquot) and stored under N_2 at −80 °C for hormone analysis.

Steroid hormones (androstenedione, testosterone, progesterone and corticosterone) were measured in plasma by a proprietary online solid-phase extraction-LC–MS/MS (SPE-LC–MS/MS) (Yamada et al. 2002; Zhang et al. 2011). Absolute quantification was performed by means of stable isotope-labeled standards.

Estradiol concentration in serum samples was determined using a commercially available ELISA kit from DRG Diagnostics (EIA-4399; Marburg, Germany) measured on a Sunrise MTP Reader (Tecan AG, Maennedorf, Switzerland) and evaluated by the Magellan software of the instrument producer. Estradiol was not measured in subset 1 females or in males at any age, as estradiol concentrations in these animals are known to be below the technical lower limit of quantitation for this determination.

Tissue preparation and histopathological analysis

At all time points, the harvested organs were carefully asservated, trimmed of excess fat and tissue, and weighed fresh, unfixed, and without blotting to the nearest 0.1 mg. Parts of the right testis and the complete right epididymis were sampled for cauda epididymis sperm motility, sperm morphology, spermatid head count in the testes and sperm head count in the cauda epididymis.

The remaining organs and tissues from both sexes were then prepared for histopathological analysis. All tissues were fixed in 4 % buffered formaldehyde solution, excepting only the ovaries, left epididymis and left testis, which were fixed in modified Davidson's solution. Each tissue was then histotechnically processed, stained with hematoxylin-eosin and examined by light microscopy.

Table 4 Statistical analyses used in the assessment of measured parameters

Parameter	Statistical test
Food consumption, body weight and body weight change (parental animals and pups); estrous cycle length; duration of gestation; number of delivered pups per litter; developmental landmarks (days up to preputial separation or opening of the vagina); anogenital distance and index; implantation sites; postimplantation loss, weight of the fetuses, implantations, pre- and postimplantation losses, resorptions and live fetuses	Dunnett's test (two-sided)
Number of live and dead pups and different indices (e.g., mating index, fertility index and gestation index) and number of litters with necropsy findings in pups; developmental landmarks (preputial separation or opening of the vagina), sperm morphology, incidence of males with a specific amount of abnormal sperm [cutoff value: 0.9 quantile (90 %) of control groups]	Fisher's exact test
Proportion of pups with necropsy findings per litter, presence of areolas/nipples, sperm evaluation (with Bonferroni–Holms correction	Wilcoxon test (one-sided)
Weight of the anesthetized animals and absolute and relative organ weights (all organs excl. organs listed below); hormones	Kruskal–Wallis and Wilcoxon- or Mann–Whitney *U* test (latter for hormones)
Weight parameters of ventral prostate (VP), seminal vesicles with coagulating gland (SVCG), musc. levator ani together with musc. bulbocavernosus (LABC), Cowpers gland (bulbourethral gland) (COW), glans penis (GP)	Dunnett's test (one-sided)

Comparison of age and body weight at sexual maturation

Preputial separation is a commonly used developmental marker for the onset of male puberty in laboratory rats (Korenbrot et al. 1977; OECD TG 416 2001). While treatment-related delays may be indicative of specifically slowed sexual development, impaired general growth can also alter the onset of puberty. To differentiate between these specific and non-specific effects, an analysis was performed to graphically compare the ages and weights at puberty of the subset 2 and 3 individual animals with the average growth progression of all subset 3 control animals, using the change in body weight as a marker for general animal development.

In the resulting scatterplot, the body weights (*y* axis) of the individual animals from the treatment groups (filled) and concurrent controls (open circles) are compared with the age of the animal at pubertal onset (day of preputial separation, *x* axis). These individual results were then judged in comparison with the curve formed by the mean body weights of the control animals (dk. gray diamonds ± SD). To generate this growth curve, the ages and body weights from the control animals in both generations were obtained from both the pup body weights and weekly weighing postweaning.

If the delay in the onset of puberty corresponds to a body weight which lies along this normal relationship between age and body weight, the pubertal delay in treated animals is considered to be a specific effect, unrelated to any general toxicological effects on growth. If, however, the treated animals are clustered such that they are older than the controls without the expected increase in body weight (below the curve set by the study average), this represents a delay in pubertal onset as an unspecific secondary result of general growth retardation. This analysis is able to differentiate between any specific effects on sexual maturation and unspecific indirect changes resulting from effects of on pup body weight development (Melching-Kollmuss et al. 2014).

Statistical analysis

Means and SD were calculated. In addition, the following statistical analyses were carried out (Table 4).

Results

All analyses showed that the achieved prochloraz concentrations were within acceptable limits (defined as within ±10 % of the nominal concentration), prepared

Table 5 Food consumption and body weight gain in pregnant rats after treatment with prochloraz

Dose PRO (mg/kg bw/day)	0	0.01	5	30
Number of pregnant dams	20	20	19	19
Food consumption (g)				
GD 18–20 ($n = 1$)[a]	21.0 ± 1.84	20.2 ± 2.38	20.9 ± 2.58	**18.7* ± 1.84**
GD 6–20 ($n = 3$)[a]	20.1 ± 1.59	19.4 ± 1.43	19.9 ± 1.44	18.6 ± 1.28
Body weight gain GD 6–20 (g)	104.4 ± 11.50	101.0 ± 10.26	105.9 ± 11.12	99.4 ± 11.25

Data are presented as mean ± SD

In all cases: * $p \leq 0.05$

[a] These food consumption data represent the weighted average value of several time intervals, and as such are reported as mean of mean ± SD. The number of intervals used in this calculation is indicated as *n*

Statistically significant value is in bold

dosing suspensions were stable and homogeneous, and there were no contaminants in the feed or water or changes in the environmental conditions, which might have influenced the outcome of the study (data not shown).

Maternal toxicity

Except for one animal at the high dose (30 mg/kg bw/day), all dams in this study were pregnant. A single parental female in the mid-dose group (5 mg/kg bw/day) was found dead on gestation day 8. This death was not considered test substance related. Clinical signs of systemic maternal toxicity were not observed in dams receiving the low- or mid-doses of prochloraz, but were noted in dams receiving 30 mg/kg bw/day. These findings were general clinical signs of stress and gastrointestinal irritation, including piloerection (2/20 dams during gestation and 4/20 during lactation) and maternal salivation 1–3 h after treatment at several days of gestation and lactation, and extended gestation lengths. The dams of the high-dose group also consumed smaller quantities of feed, when compared to control animals, and consequently had slightly reduced body weights by comparison, as summarized in Table 5.

All dams of the top dose group showed salivation directly after treatment.

Gestation and littering

Alterations in measured reproductive parameters were noted as a result of 30 mg/kg bw/day prochloraz treatment (Table 7). At this dose, 5 dams delivered on GD 21, 11 dams on GD 22 and 3 dams on GD 23, leading overall to the increased mean gestation lengths of 22.9 exceeding slightly the historical control range of 21.5–22.5 days (see Table 7). One animal was not pregnant. Table 6 gives an individual overview on the maternal findings observed in the top dose of this study.

While the number of litters was similar to those of the other groups, the number of pups per litter was reduced; 2 1 consisted entirely of stillborn pups. Consequently, the total number of live pups was decreased, and the number of stillborn pups was increased. In the first 4 postnatal days, a statistically significant increase in pup death was observed in animals exposed to the high dose of prochloraz, which when combined with the statistically significant increase in cannibalization contributed to reduced pup viability in this group. Table 7 indicates a correlation between the duration of gestation, assumed parturition difficulties (e.g., piloerection) and the number of stillborn pups. No difference in sex ratio was observed.

Table 6 Individual maternal clinical observations during gestation and lactation of dams dosed with 30 mg prochloraz/kg bw via gavage (high dose)

Animal number	Clinical observation	Gestation length* (days)
231	Piloerection No more pups alive (at LD 2) Pups not properly nursed	22
232	No more pups alive (at LD 1)	22
233	Piloerection All pups stillborn	23
234	Piloerection	21
244	Piloerection All pups stillborn	23

* Normal gestation days for this rat strain (based on historical control data) are 21.5–22.5 days

Offspring development

No systemic toxic effects were observed in the offspring during lactation. However, 10 % increases in ano-genital distance and ano-genital index (a calculated parameter which takes into account the individual animal weight) were observed on PND 1 in the females exposed in utero to 30 mg/kg bw/day prochloraz (Supplementary Table 1; Fig. 2). No increases in these parameters were observed in females at the low- and

Table 7 Reproductive parameters after treatment with prochloraz

Dose PRO (mg/kg bw/day)	0	0.01	5	30
Number of pregnant dams	20	20	19	19
Duration of gestation (days)[a]	22.1 ± 0.31	22.0 ± 0.39	21.9 ± 0.40	**22.9** ** **± 0.66**
Number of litters	20	20	19	19
Litters with live-born pups	20	20	19	17
Pups per litter	10.4 ± 0.94	10.1 ± 1.84	10.9 ± 1.29	9.9 ± 1.29
Live pups	208 (100 %)	201 (99 %)	205 (99 %)	**146** ** **(78 %)**
Stillborn pups	1 (0.5 %)	2 (1.0 %)	2 (1.0 %)	**42** ** **(22 %)**
Pup death	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	**8** ** **(4.3 %)**
Cannibalization	0 (0.0 %)	1 (0.5 %)	0 (0.0 %)	**7** ** **(3.7 %)**
Pup survival PND 0–4	208 (100 %)	200 (100 %)	205 (100 %)	**131** ** **(90 %)**

Data are presented as mean ± SD

** $p \leq 0.01$

[a] Historical control data indicate a 21.5–22.5 days normal gestation length in this rat strain

Statistically significant values are in bold

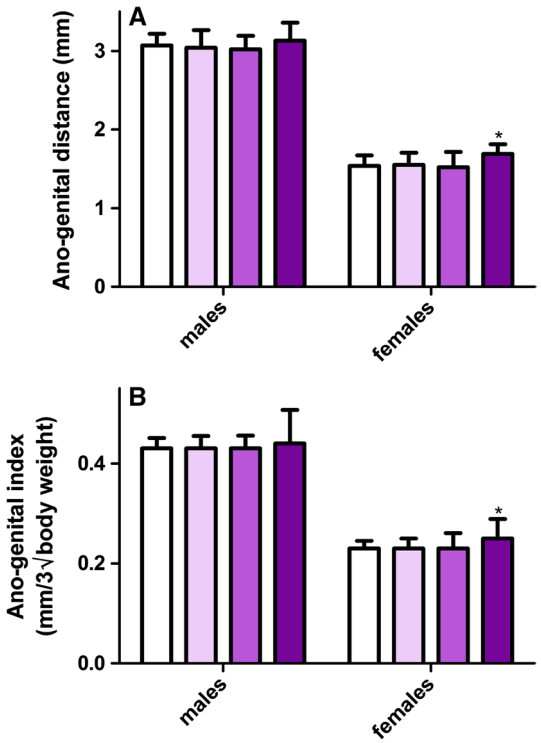


Fig. 2 Effect of increasing dose of prochloraz on anogenital distance and anogenital index. On PND 1, the ano-genital distance (AGD) of all live-born pups exposed in utero to 0 □, 0.01 ▭, 5 ▭ and 30 ▭ mg/kg bw/day prochloraz was measured and is given as AGD and AGI in (mm ± SD) (Panel **a**). The ano-genital index (AGI), a parameter which accounts for any differences in animal size, was then calculated from these data (Panel **b**). Prenatal exposure to 30, but not 5 or 0.01 mg/kg bw/day prochloraz, significantly increased female, but did not alter male AGDs and AGIs (color figure online)

mid-doses. Furthermore, neither the ano-genital distance nor the ano-genital index was altered at any dose in males.

The male pups were also examined for the presence of nipples or areolae on PND 12 and again on PND 20 (Supplementary Table 2; Fig. 3). On PND 12, a statistically significant increase was noted in the number of offspring in the 5 and 30 mg/kg bw/day dose groups which had nipple presence, defined as at least one nipple or hairless pigmented spot identified as a possible areola, when compared to controls (Panel A). The number of nipples was also dose-dependently elevated at these doses (Panel B). This increase in nipple presence was transient; by the time a second analysis was performed on PND 20, there were no retained nipples in any dose group (Panel C). The historical background incidence of retained nipples/areola is high at the observation time point PND 12 and is observed in percentages up to 70 % in untreated controls, raising some doubts on the treatment relationship of this finding.

Ten males and 10 females per dose group per subset were selected to be reared further; the age and weight at sexual maturation (preputial separation in males, vaginal opening in females) were then determined in 20 animals of each gender. Across all dose groups, no change in sexual development was observed in the female offspring; however, male entry into puberty appeared to be delayed in animals exposed to the highest prochloraz dose (Supplementary Table 2; Fig. 4). In this dose group, this represents a statistically insignificant, but potentially biologically relevant delay of about 1 day beyond the historical control range (PND 39.7–44.8). To better evaluate the lags in average male age at puberty, the individual ages at preputial separation were also plotted

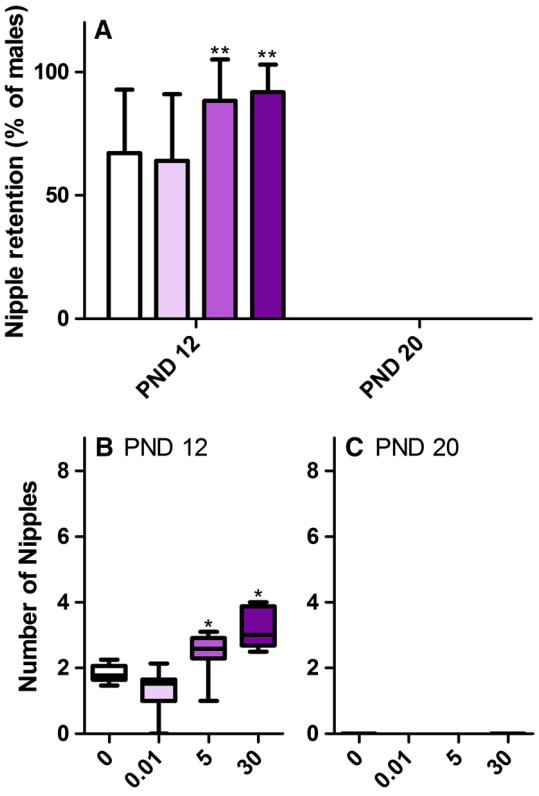


Fig. 3 Effect of increasing prochloraz dose on nipple retention. On PND 12, the number of male pups exposed to 0 □, 0.01 ▭, 5 ▮ and 30 ▬ mg/kg bw/day prochloraz which had retained nipples or areolas was counted. These animals were then recounted on PND 20; given in % males ± SD (Panel **a**). Similarly, the number of nipples/areolas retained by each male pup was also recorded on PND 12 (Panel **b**) and PND 20 (Panel **c**). Data in Panel **b** are graphed as *box plots* representing the median (*bar*), interquartile range (*box*) and range, (*whiskers*) for each treatment group. Despite the relatively high background rate of nipple retention in control animals, prenatal and/or lactational exposure to increasing doses of prochloraz raised both the number of males with nipple/areola retention and the average number of nipples/areolas retained by each male pup on PND 12. This effect was transient; by PND 20, all of the retained nipples had receded (color figure online)

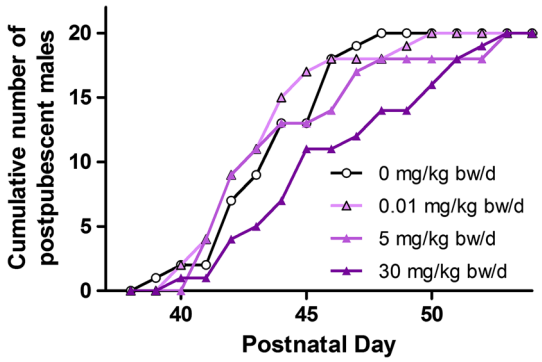


Fig. 4 Kaplan–Meier plot of the sexual maturation of pubescent male rats exposed to prochloraz since GD 6. Twenty male offspring which had been exposed to either vehicle only (*open circles*) or increasing concentrations of prochloraz (*purple triangles*) were examined for preputial separation daily from PND 38–64. The 30 mg/kg bw/day prochloraz-dose group showed a delayed preputial separation (delay in puberty onset) beyond the historical control range. This delay, however, was not statistically significant (color figure online)

as Kaplan–Meier curves (Fig. 4). Under this type of analysis, not only the delay in the onset of puberty is evident, but is also the attenuation of maturation (ca. 30 %) in animals treated with 30 mg/kg bw/day prochloraz.

Role of body weight in anti-androgen delayed sexual maturation

Sexual development is a multifactorial process which is influenced by general body development as well as hormonal signaling. We and others have previously described delays in preputial separation resulting from reduced body weight, as a consequence of both restricted feeding (Carney et al. 2004; Marty et al. 2003; Chernoff et al. 2009) and toxicologic retardation of general animal development (Melching-Kollmuss et al. 2014). Since prochloraz causes both anti-androgenic and general forms of toxicity, a question arises as to whether the delay in sexual maturation is due to endocrine disruption or alterations in general development (as measured by slowed body weight gain). To answer this question, we compared the ages and the weights of pubescent male individuals on the day of preputial separation. Figure 5 shows a scatterplot of the individual age/weight pairs of all the living male offspring (subsets 2 and 3). These individuals were compared with the general trend in the average body weight of the control animals being reared to early adulthood (subset 3 only), which were weighed at weekly intervals (gray diamonds, regression line). The individuals in the control, 0.01 and 5 mg/kg bw/day groups are clustered with maturation days between PND 40 and PND 46 and distributed around the regression line, signifying normal sexual development.

Closer examination of animals treated with 30 mg/kg bw/day prochloraz again reveals the previously observed pubertal delay. While the individuals in this group also generally fell along the regression line of age-matched controls, indicating that male pubertal delay was at least partly a result of an endocrine mode of action, it is evident that many of these individuals have body weights which are lower than those of the age-matched controls. Furthermore, the individuals in this dose group with the greatest decrease in body weight also have the greatest delay in maturation. These data suggest that at 30 mg/kg bw/day, prochloraz caused a small, but important decrease in individual body weight, which was also correlated with delays in sexual maturation. Taken together with the overall effect pattern, the delay in sexual maturation was likely a combined

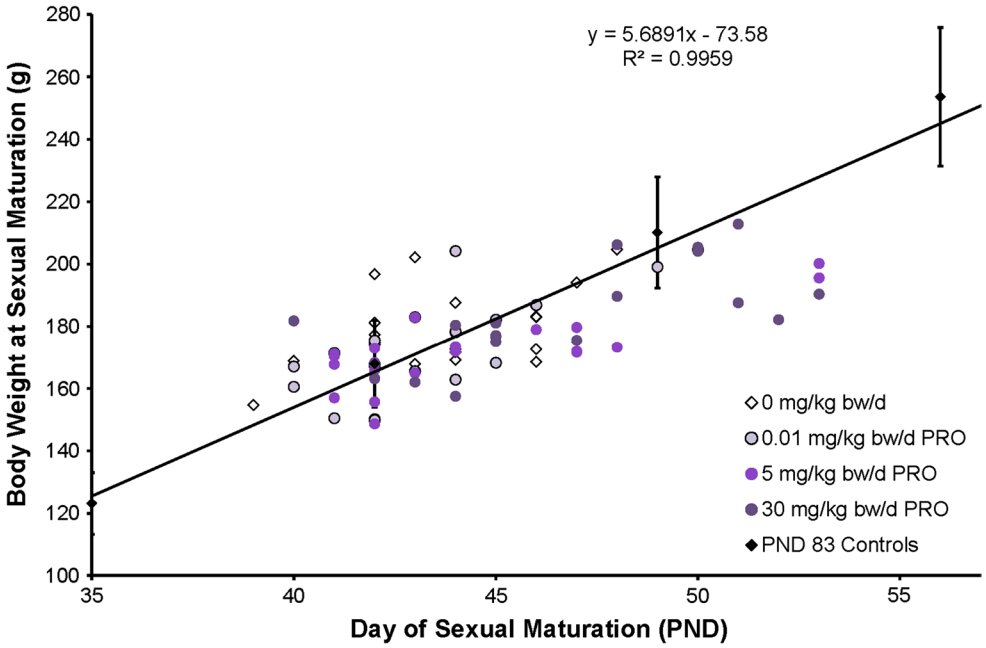


Fig. 5 Comparison of age and body weight of remaining individual male offspring at sexual maturation (subsets 2 and 3). The comparison between age and weight on the day of preputial separation for all individual males administered either vehicle (*blue diamonds*) or increasing doses of prochloraz (*purple circles*) was compared with the mean body weight development of the subset 3 control males (shown as *gray diamonds* ± SD with a *least-squares* regression line). In general, the animals' body weights were proportional to their ages at sexual maturation. However, treatment with 30 mg/kg bw/day prochloraz caused a small decrease in individual body weight which was associated with the delay in sexual maturation (color figure online)

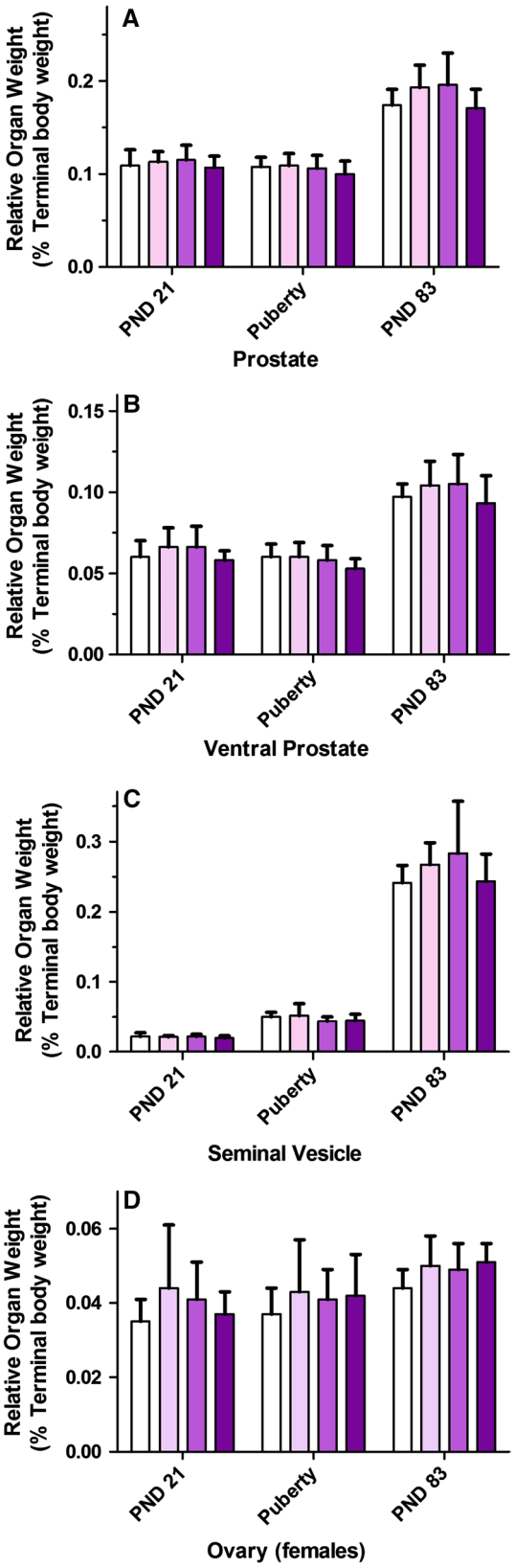
downstream consequence of both general toxicity and specific endocrine-mediated effects.

Sex organ weights and histopathology

When compared to the control group 0, (set to 100 %) the mean reproduction organ weight parameters in parental females, whether absolute or relative, did not show significant, treatment-related differences. In contrast, in males, a statistically significant decrease in the absolute weights of the cauda epididymis and bulb. lev. ani muscles was observed in prochloraz-treated subset 3 offspring (PND 83 ± 2) (Supplementary Table 3). However, these changes corresponded to reductions in terminal body weight; therefore, statistically significant decreases in the corresponding relative organ weights were not observed. These changes in male sex organ weights were without a pathological correlate; no difference between the dose groups was detected during histopathological examination (Fig. 6).

Hormone analysis

Blood samples were collected from the parental females and all reared offspring for determination of the concentrations of steroid hormones (see supplementary Tables 4–10). There were no changes in any of the hormones (androstenedione, testosterone, progesterone, corticosterone and estradiol) measured in the offspring at PND 21 and at the time of puberty in both males and females. The same is true for males at PND 83. In parental females at the time of weaning, there were no changes in androstenedione, progesterone and estradiol. In these animals, there was a statistically significant increase in corticosterone at all dose levels. However, no dose–response relationship was evident and the controls in this study seemed to have quite low corticosterone levels (mean ± SD = 435.8 ± 485.6 nmol/L). Therefore, we evaluated the values of the treatment groups against two concurrent control groups from two similar studies which were performed at the same time in the same laboratory following the same procedures. The corticosterone values in parental females of the three dose groups were not statistically significantly different when compared to the control group of the first comparison study (mean ± SD = 852 ± 516.5 nmol/L). When compared against the control group of the second comparison study (mean ± SD = 731.6 ± 373.8 nmol/L), corticosterone values of the low- and mid-dose groups were higher, but corticosterone in the high-dose group was not statistically significantly different. Thus, it seems likely that the statistically significant increase in corticosterone within the study groups is an incidental result of low serum concentrations in the study control group, rather than an effect of prochloraz treatment.



◀**Fig. 6** Weights of selected sexual organs. On PND 21 (subset 1), the day of preputial separation or vaginal opening (puberty, subset 2) and PND 83 ± 2 (subset 3), the sex organs from each of 10 male and 10 female rats were asservated, weighed and reported as relative organ weights in (% ± SD). These data are graphed as vehicle □, 0.01 ▭, 5 ▭ and 30 ▭ mg/kg bw/day at each time point. In general, exposure to 30 mg/kg bw/day prochloraz reduced male, but not female, sex organ weights, albeit insignificantly. Three of the most sensitive male sex organs and one female sex organ are shown for comparison (color figure online)

There was a statistically significant increase in testosterone in the mid-dose group (5 mg/kg bw/day) and a similar but not statistically significant increase in the high-dose group (30 mg/kg bw/day). In dams of the high-dose group, estradiol levels were biologically relevantly but not statistically significantly decreased (regarding medians about 85 % reduced compared to study control; Fig. 7). This probably reflects the aromatase inhibitory activity—shown in vitro—of prochloraz (Andersen et al. 2002; Vinggaard et al. 2000). In the adult females (PND 83), there was statistically significant increase in testosterone levels at 5 and 30 mg/kg bw/day. In the high-dose group, androstenedione values were also increased whereas estradiol levels were biologically relevantly decreased (median about 46 % compared to control) although both hormones were not statistically significantly altered (Fig. 7). In these individuals, the hormone changes probably reflect also the aromatase inhibitory activity of prochloraz. A distinct increase in serum progesterone was also noted in these animals, at all dose groups. However, when compared to the controls of the parallel studies almost all values in the treated groups were within the control range and are therefore assessed to be biologically not relevant (Fig. 8). Corticosterone levels were statistically significantly increased in the low- and mid-dose level, but not in the high-dose group. This alteration is obviously not dose-dependent and was considered to be not treatment-related.

Overall, it can be concluded that there were no hormonal changes in male animals and that the hormonal changes in females were restricted to dams after weaning in proestrus and adult females at PND 83 in the mid- and high-dose groups (5 and 30 mg/kg bw/day). These hormone changes (testosterone and androstenedione increase and estradiol decrease) reflect the aromatase inhibitor activity of prochloraz. The increase in corticosterone values in female dams and adult females (PND 83) is difficult to assess because of a lack of dose–response relationship and a lack of statistical significance when compared to other control values. Overall, there is no convincing evidence that the detected

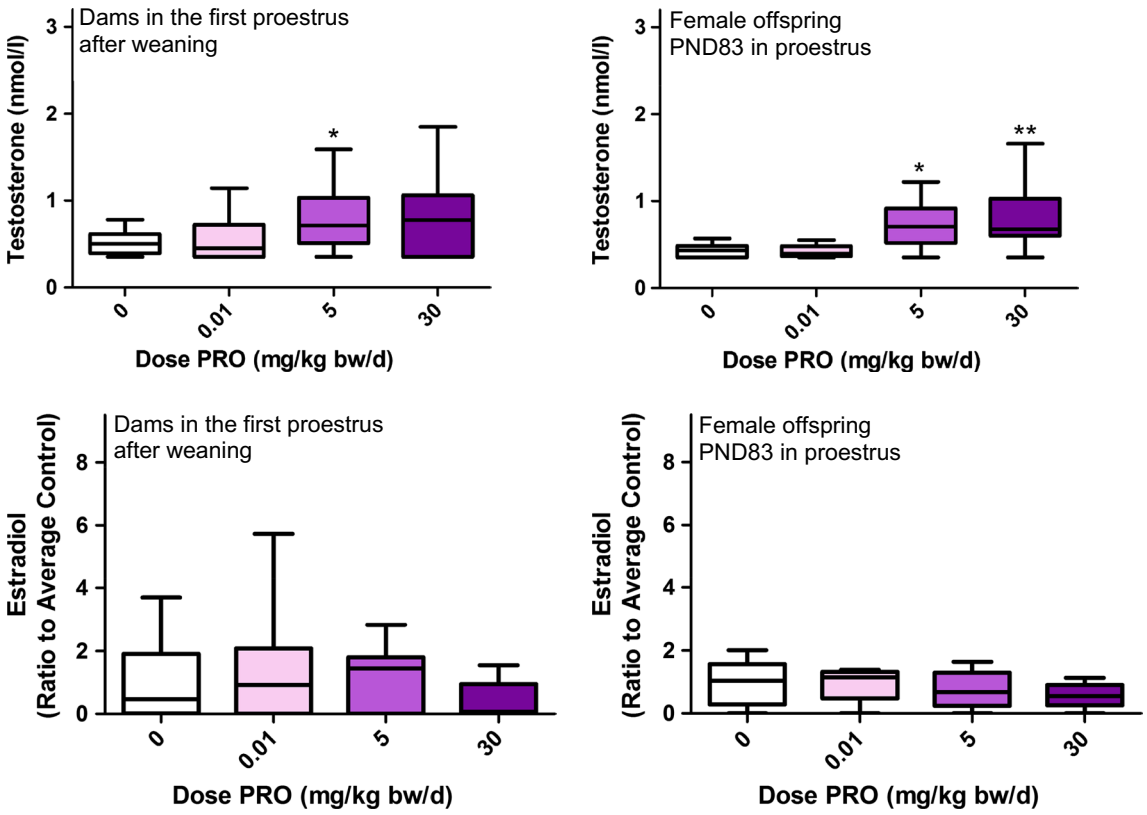


Fig. 7 Circulating concentrations of prochloraz-related changes of testosterone and estradiol in dams in the first proestrus after weaning and in female offspring at about PND 83 in proestrus. These data are graphed as *box plots* representing the median (*bar*), interquartile range (*box*) and range, (*whiskers*) for each treatment group. Each *box plot* is color-coded according to treatment: control □, 0.01 ▭, 5 ▭ and 30 ▭ mg/kg bw/day prochloraz. No statistically significant changes were observed in corresponding males (color figure online)

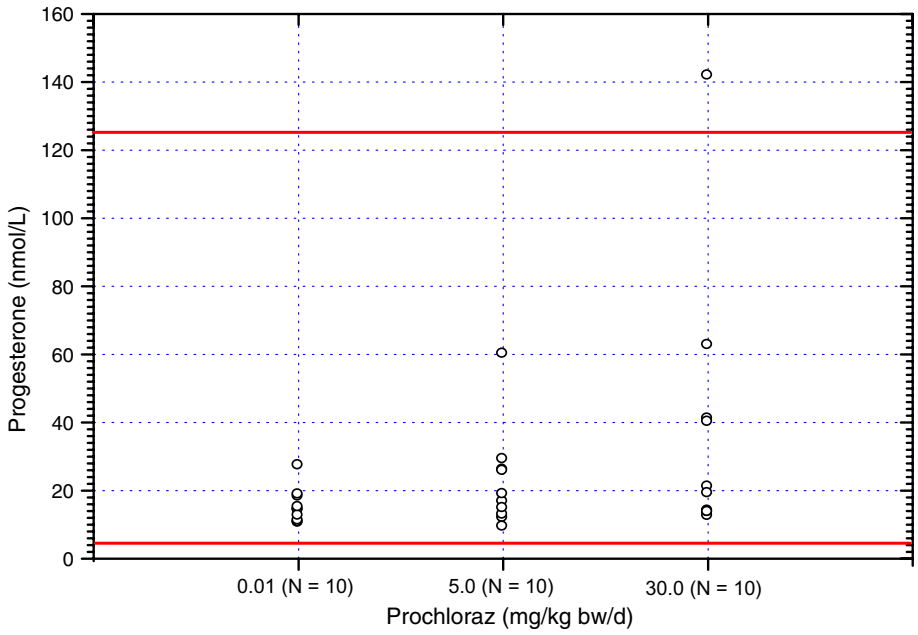


Fig. 8 Distribution of progesterone values in female rats around PND 83 in proestrus dosed with prochloraz. Reference range between *red lines* were established between 5 and 95 percentiles of 29 control values (color figure online)

Table 8 Analysis of sperm and spermatids of male offspring (PND 83 ± 2)

Dose (mg/kg bw/day)	0	0.01	5	30
Motile sperm[a]	88 ± 4 % (10)	85 ± 5 % (10)	86 ± 4 % (10)	**83**** ± 3 % (10)
Abnormal sperm morphology	3 ± 3 % (10)	2 ± 1 % (10)	3 ± 2 % (10)	4 ± 3 %(10)
Spermatid count ($\times 10^6$/g testes)	190.89 ± 31.37 (10)	190.53 ± 19.02 (10)	191.44 ± 31.74 (10)	197.76 ± 20.37 (10)
Spermatozoa count ($\times 10^6$/g cauda epididymis)	566.28 ± 114.13 (10)	640.51 ± 156.03 (10)	535.26 ± 127.12 (10)	590.07 ± 128.89 (10)

Data are presented as mean ± SD (N)

** $p \leq 0.01$

[a] Historical control range for sperm motility is 69–93 % in this rat strain determined in the same laboratory

Statistically significant value is in bold

increases in plasma corticosterone levels in female dams and in the young adult females (PND 83) are treatment-related. In in vitro steroidogenesis assays, using a human H295R adrenocarcinoma cell line, which expresses genes that encode for a number of key enzymes for steroidogenesis (see OECD TG 456), decreases in corticosterone were observed (Winther et al. 2013; Ohlsson et al. 2010).

Sperm analysis

Sperm were sampled from half of the right testis and the complete right epididymis of subset 3 offspring and analyzed. A statistically significant reduction in sperm motility was noted at the 30 mg/kg bw/day prochloraz dose; however, this change was marginal and still fell well within the historical control range (69–93 %) for the test facility (Table 8). Thus, the reduction in sperm motility at this dose is not considered an anti-androgen effect. Also no effects on sperm numbers, numbers of motile sperm cells or sperm velocities have been found in male Wistar rats at PND 224, after perinatal prochloraz gavage dosing with 30 mg/kg bw/day (Vinggaard et al. 2005).

Discussion

It has been suggested that the endocrine system is uniquely sensitive to exogenous modulation during critical developmental periods, even at doses below previously described no-observed-adverse-effect-levels (Vandenberg at al. 2012; Hass et al. 2013). Further, there are doubts expressed that standard regulatory testing, which requires high and toxic doses irrelevant to the expected human exposure situation, is a good basis to derive safe thresholds for humans. As a result, there is concern that current risk assessment methodologies do not adequately assess the hazard associated with potentially endocrine active substances. To test this hypothesis, we studied the effects of prochloraz given at three doses in a peri-postnatal developmental and reproductive toxicity study. Prochloraz was chosen for this investigation because it has some anti-androgenic properties and the focus was on the comparison of the results of standard regulatory generation toxicity studies conducted according to the old TG 416 (1981) with results including all (and more) elements of the more recent required test guidelines.

The study design drew from the methods laid down in OECD TG 414, 416 and 443, as well as the corresponding OPPTS/OCSPP guidance. The resulting study closely resembled the peri-postnatal study design recommended by ICH (ICH S5 R2 4.1.2) and was enhanced by a number of endocrine-sensitive pathological and molecular endpoints. Doses were chosen to represent a significant endocrine effect level, the suspected NOEL for anti-androgenicity and a dose, which is representing the European ADI. These three doses represent a 3000-fold spacing (from 0.01 to 30 mg/kg bw/day); thus, this study is designed to adequately describe not only the expected endocrine toxicity at the high doses, but also examine the relationship between low doses of endocrine active substances and their effects in an apical study. This study was a gavage study in order to guarantee an accurate daily dosing—especially at the low-dose levels, where dietary analytics might have become a challenge. It is, however, acknowledged that the more relevant dosing procedure—with respect to the most relevant oral consumer exposure—is a test substance administration via the diet, which was used in the regulatory generation toxicity studies. An administration of test substance per gavage in oil might enhance the test compound bioavailability and may lead to "artificial" lower effect and no-effect dose levels. Risk assessment should therefore ideally be based on a NOAEL derived in a more relevant dietary study, however, provided that all relevant parameter and endpoints have been investigated and a real NOAEL has been established.

The effects seen at the 30 mg/kg bw/day gavage dose of prochloraz included impairment of body weight gain, body weight and clinical symptoms. At this dose level, reproductive toxicity (lower mean litter size and weight from birth to weaning, decreased live birth index and viability index) and an increased incidence of females with extended

gestation length were observed. In this study (at 30 mg/kg bw/day) and also in the regulatory two-generation toxicity study (at 625 ppm) (Cozens et al. 1981, cited in the draft assessment report: EFSA—http://dar.efsa.europe.eu/dar-web/provision), a clear correlation was seen between those dams having longer gestations and respective total litter losses. In these dams of the two-generation toxicity study (Cozens et al. 1981, cited in the draft assessment report: EFSA http://dar.efsa.europe.eu/dar-web/provision), poor general state of health was observed. These effects in rats are considered to occur secondary to aromatase inhibition, which leads in turn to lower estrogen levels, maintaining a high progesterone level. In the rat, parturition is normally preceded by an increase in estrogen and a decline of progesterone in maternal serum (Fang et al. 1996). If these serum levels are altered, parturition difficulties may be the consequence in rats. The principal source of progesterone in mice, rats and rabbits is the corpus luteum. The progesterone withdrawal shortly before the onset of parturition is due to the death of the corpus luteum mediated by prostaglandin. In humans up to the seventh week of pregnancy, the corpus luteum is also the main source of progesterone and the role of progesterone in early human pregnancy is not dissimilar to that of rabbits and laboratory rodents. However, after the "luteo-placental shift," when the developing placenta becomes the principal site of progesterone synthesis, the regulatory pathways diverge (Zakar and Hertelendy 2007). In monkeys for example, it has been shown that progesterone withdrawal induced preterm labor and delivery (which can be blocked by progesterone substitution) but exogenous progesterone, even in substantial quantities, does not prevent parturition at term in monkeys (Haluska et al. 1997). This has also been reviewed by Mitchell and Taggart (2009) who concluded that parturition in humans has significantly different regulators and mediators from those in most animal models. While mice and rats display abrupt withdrawals of progesterone from the maternal circulation before parturition, humans maintain a high and increased concentration throughout parturition. It is therefore concluded that the observed parturition difficulties seen in rodents after prochloraz administration have low human relevance.

As detected in the present study, in adult females (PND 83) there was a statistically significant increase in testosterone. This change may be associated with treatment as it can be linked to an increase in androstenedione and a decrease in estradiol levels, albeit not statistically significant. It could explain the increases in AGD in high-dose female offspring and is probably related to the known aromatase-inhibiting properties of prochloraz in vitro. The effects on the anogenital distance/index in females described by Laier et al. (2006) are potentially an appropriate biomarker for an anti-estrogenic effect on female rats. This, however, is not leading to developmental or reproduction effects in females, as no changes in the onset of female puberty were seen and also no changes in conception rates or time to pregnancy were observed.

In general, exposure to 30 mg/kg bw/day prochloraz reduced male, but not female, sex organ weights, albeit insignificantly. The male reproductive organ weight decreases were statistically significant only for absolute cauda epididymides and levator ani and bulbocavernosus muscles, which was related to lower body weights. This may also be associated with treatment, as it was also observed in the regulatory two-generation toxicity study at 450 ppm (corresponding to 21.10–73.9 mg/kg bw/day) (Reader et al. 1993, cited in the draft assessment report: EFSA http://dar.efsa.europe.eu/dar-web/provision). No treatment-related changes at the plasma hormone levels were seen in males in any of the treatment group in this study set up; however, the observed marginal effects on male reproductive organ weights might be correlated with the in vitro observed inhibitory effects on the 17, 20 lyase, as shown by Laier et al. (2006) and Nielsen et al. (2012). The data on male sexual maturation suggest that at the high dose, prochloraz caused a delay in preputial separation which was also correlated with a decrease in individual body weight. Taken together with the overall effect, the delay in sexual maturation was likely a combined downstream consequence of both general toxicity and specific endocrine-mediated effects, not caused via in utero prochloraz exposure. This is indicated by the Vinggaard et al.'s (2005) study, which did not observe an effect on entry into male or female puberty at a maternal prochloraz gavage dose of 30 mg/kg bw. In this study, only the dams were dosed with prochloraz, not the offspring, as it was the case here.

At dose levels at or below the NOAEL, there were no adverse effects on any apical endpoints measured in males and females at four different points in time (parental males and females, offspring at PND 21, offspring at puberty, young adults at PND 81–85). While male plasma hormone levels were not affected by treatment, some statistically significant (testosterone and androstenedione increase and estradiol decrease) in hormone values in dams at weaning and adult young females (day 83 PND) at the 5 mg/kg bw/day group were seen. Without any concurrent effect on reproductive parameters or other apical endpoints, these hormone level changes are regarded as suggestive of a start of an effect of the compound on the organisms, but not as adverse in nature yet. At the 5 mg/kg bw/day group at PND 12 also an increased incidence of males displaying nipple/areola were seen. At the second observation time (PND 20), none of the males had retained nipples or areola. It is important to note that the background incidence for retained nipples at PND 12 is up to 70 % of the male

animals showing this feature. PND 13 is probably a better day of investigation, as the distinction between background level and treatment relationship is more accurate. At PND13 Christiansen et al. (2009) determined the (gavage) dose of 5 mg prochloraz/kg bw/day (using a similar dosing regime) to be the no-effect level for retained nipples in Wistar rats. The parameter of nipple/areola retention (at PND 12/13) is considered rather a biomarker than a parameter indicating adversity as it is a transient finding, it is not associated by any changes in the tissue, and there is strain differences in the physiological androgen peak (Woodman 1997).

The results of the present study do not indicate the existence of a non-monotonic dose response. At the high-dose level, several changes in apical endpoints were observed, such as reduced body weight and body weight gain, clinical symptoms and indications of reproductive toxicity (lower mean litter size and weight from birth to weaning, decreased live birth index and viability index). Moreover, there was a delayed sexual maturation of the males and an increased AGD in female offspring at this high-dose level. None of these parameters were affected at the mid (NOEL)- or low (ADI)-dose level. At the NOEL dose, there is an indication of a start of an effect of the compound on the organism, however, not yet resulting in an adverse effect. Thus, the absence of a non-monotonic dose response has been conclusively shown. This is in line with more general evidence that thresholds do exist for endocrine-related effects (Borgert et al. 2013; EFSA Scientific Opinion 2013; Borgert et al. 2012; Caldwell et al. 2012). The existence of threshold doses was recently also shown for the anti-androgenic drug flutamide in a pre- and postnatal in vivo study in Wistar rats (Fussell et al. 2015). Underlying uncertainties in an appropriate hazard assessment of substances with an endocrine mode of action are probably more the result of inappropriate study designs and the omission of relevant parameter.

Supported by the data in the current investigation, the authors suggest that caution should be exercised when assumptions and conclusions are drawn about non-monotonic dose–response curves and low-dose effects. Evaluation of this hypothesis should only be based on evidence from valid animal data with an understanding of the underlying mechanisms of toxicity.

Conclusions

Prochloraz has been studied in a one-generation reproduction toxicity study. The animals were dosed from GD 6 (dams) until PND 83 (offspring). The investigated parameter included all required parameter from the relevant OECD (416, 443) and OPPTS (870.3800) guidelines and additional mechanistic parameter (e.g., plasma hormones). The doses administered by gavage were 0.01 (low dose = ADI of prochloraz), 5 (suspected NOEL for anti-androgenicity) and 30 mg/kg bw/day (expected effect level). Maternal toxicity, increased gestation lengths, increased number of stillborn pups and lower pup viability were seen at 30 mg/kg bw/day. Also a delayed entry into male puberty (accompanied by lower male offspring bodyweights) was observed and an increased anogenital distance/index in females at birth at this dose. At ≥5 mg/kg bw/day transiently increased incidences of retained nipples in male offspring at PND 12 were seen, which was no longer observable at PND 20. No conclusive treatment-related changes in plasma hormone levels were seen in males, while females had higher testosterone levels and lower estradiol levels in plasma taken from dams after weaning and in female offspring at PND 83. The most prominent and dose-limiting prochloraz-related effect is maternal toxicity and related parturition difficulties with decreased pup viability in rats. No severe endocrine-related adverse effects (like, e.g., reproductive organ failures, effects on sperms) were seen at the prochloraz-treated animals. No adverse other endocrine-related adverse effects were seen at lower-dose levels. The NOAEL was accurately determined in the regulatory studies conducted with prochloraz, and the derived reference value for prochloraz is a safe human health-related threshold. There was no evidence for non-monotonicity or effects at very low human-relevant dose levels.

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REPRODUCTIVE TOXICOLOGY

Investigations of putative reproductive toxicity of low-dose exposures to vinclozolin in Wistar rats

Burkhard Flick[1] · Steffen Schneider[1] · Stephanie Melching-Kollmuss[2] · Karma C. Fussell[3] · Sibylle Gröters[1] · Roland Buesen[1] · Volker Strauss[1] · Bennard van Ravenzwaay[1]

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Abstract The current investigation examines whether the fungicide vinclozolin, which has an anti-androgenic mode of action, is capable of disrupting endocrine homeostasis at very low doses. The data generated clarify whether a non-monotonic dose–response relationship exists to enhance the current debate about the regulation of endocrine disruptors. Moreover, it is part of a series of investigations assessing the dose–response relationship of single and combined administration of anti-androgenic substances. A pre-post-natal in vivo study design was chosen which was compliant with regulatory testing protocols. The test design was improved by additional endpoints addressing hormone levels, morphology and histopathological examinations. Doses were chosen to represent an effect level (20 mg/kg bw/d), the current NOAEL (4 mg/kg bw/d), and a dose close to the "ADI" (0.005 mg/kg bw/d) for the detection of a possible non-monotonic dose–response curve. Anti-androgenic changes were observable at the effect level but not at lower exposures. Nipple/areola counts appeared to be the most sensitive measure of effect, followed by male sex organ weights at sexual maturation, and finally gross and histopathological findings. The results indicate the absence of evidence for effects at low or very low dose levels. A non-monotonic dose–response relationship was not evident.

Keywords Vinclozolin · Low dose · Non-monotonic dose response · Anti-androgenic · Endocrine disruptor · Experimental test guidelines

Introduction

There is a concern expressed that current risk assessment methodologies for endocrine active substances may be insufficient to protect even when the individual exposure may be several orders below the no-observed adverse effect level. Under this hypothesis, particularly when combined exposure to such substances, e.g. endocrine active chemicals discussed in this context, could occur, may pose a risk to human health and wildlife at doses below those currently evaluated in regulatory studies and hazard assessments (Kortenkamp et al. 2012; Hass et al. 2013).

Current risk assessment methodologies are based on the determination of hazard and the assumption of a threshold concentration above which noticeable effects are triggered. Therefore, regulatory experimental toxicology studies are designed to both determine the toxicological profile as well as the no-observed adverse effect level (NOAEL). Toxicological effects of chemicals are caused due to specific amount of active material at the site(s) of action. When the internal dose is too small to elicit a response, it is said that the "threshold for that effect has not been reached" (Holsapple and Wallace 2008).

In contrast to that, it is postulated for endocrine disruptors to have a dose response whereby low doses are stimulating and high doses are inhibiting. Accordingly, one would expect non-monotonic J-shaped or inverted

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✉ Bennard van Ravenzwaay
bennard.ravenzwaay@basf.com

[1] Experimental Toxicology and Ecology, BASF SE, 67056 Ludwigshafen, Germany

[2] Product Safety, Regulations, Toxicology and Ecology, BASF SE, Ludwigshafen, Germany

[3] Chemical Food Safety, Nestlé, Lausanne, Switzerland

U-shaped dose responses if this postulation would be true. However, the premise that hormesis is common or important has not been convincingly established (Holsapple and Wallace 2008; Borgert et al. 2013).

As a consequence of the ongoing discussion, there is a current need if a relationship exists between low doses of endocrine active substances and their disruptive effects. Exogenous anti-androgens could disrupt sexual differentiation and maturation in rats, manifested, e.g., in reduced ano-genital distances (AGDs), delayed puberty and/or decreased sex organ weights at high exposure levels (Gray et al. 1999b, 2001; Hellwig et al. 2000; Laier et al. 2006; Schneider et al. 2011; Van Ravenzwaay et al. 2013).

However, a question remains as to how minor or even transient endocrine effects claimed to arise from low-dose exposures can be verified. Since in regulatory toxicology no experimental data were collected for very low exposure levels, we aimed to fill this gap with the anti-androgenic compound vinclozolin.

Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione, CAS Number 50471-44-8) is a non-systemic dicarboximide fungicide which inhibits spore germination and could be used in grapes, strawberries, vegetables, fruit, ornamental plants, and turf. Vinclozolin and its active metabolites principally bind competitively to the androgen receptor thereby antagonizing the binding of natural androgens to the receptor (Kelce et al. 1994; Kolle et al. 2010). In the F1-extended one generation rat reproduction protocol, vinclozolin caused treatment-related adverse findings (Schneider et al. 2011) indicative for this mode of action. At high dose levels, vinclozolin caused adverse findings such as reduced parental and offspring body weight and decreased prostate, seminal vesicles and epididymides weights, and increased adrenal gland weights/adrenal gland cortical hypertrophy, reduced ano-genital distance and retained areolae in male F1, hypospadias, purulent prostatitis and seminal vesicle inflammation with atrophy, and Leydig cell hyperplasia, and accelerated vaginal opening at 20 and/or 100 mg/kg (Schneider et al. 2011), which are all considered indications of an anti-androgenic mode of action.

To investigate the presence or absence of effects at very low dose levels and non-monotonic dose responses for vinclozolin, the present study has been conducted as part of a larger project to look for the potential occurrence of low-dose effects of three anti-androgens (flutamide, prochloraz, and vinclozolin) after single and combined exposures. With the presented appropriate experimental study design including elements of regulatory toxicological studies performed under good laboratory praxis, we attempted to clarify some of the concerns by comparing the endocrine-related effects of vinclozolin over dose levels covering an effect level (20 mg/kg bw), the no-observed effect level from the current risk assessment approaches for endocrine and reproduction toxicity-related effects (4 mg/kg bw) as well as a very low dose (0.005 mg/kg bw) at approximately the acceptable daily intake (ADI) level (Food and Agriculture Organization/World Health Organization 1998). Thus, the results of the present study should help to clarify whether the vinclozolin-dose–endocrine-response relationship is non-monotonic or results in a threshold to enhance the current debate about the regulation of endocrine disruptors. To accomplish these aims, a pre-postnatal *in vivo* study design was chosen which contains relevant elements (and further mechanistic parameters) of Level 4/Level 5 tests of the OECD Conceptual Framework on endocrine disruptor testing.

The results of the present investigations on a single anti-androgenic compound will also be part of a broader research program to assess the dose–response relationship of single and combined exposure to several anti-androgenic substances.

Materials and methods

The study was performed according to the German Animal Welfare legislation and with the permission of the local authority (permission number LRI-EMSG56-BASF/G11-3-013). The laboratory is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) certified.

The investigations exceeded the requirements of any specific guideline, but reference is made to OECD 414 (OECD TG 414 2001), 416 (OECD TG 416 2001) and 443 (OECD TG 443 2011; Fegert et al. 2012) as well as OPPTS 870.3700 and 870.3800 (US Environmental Protection Agency 1998a, b). The study was performed according to the OECD Principles of Good Laboratory Practice and the GLP Principles of the German "Chemikaliengesetz" (Chemicals Act) which (apart from few recognized differences) meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 160 (FIFRA) and Part 792 (TSCA)], excepting only the analyses of hormone levels, which followed good scientific practice, but not formal GLP, principally meeting EPA/FDA requirements.

An overview of the experimental design is given in Fig. 1.

Test substance

Vinclozolin, (*RS*)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione, (VIN), CAS number 50471-44-8, was obtained from BASF SE (Ludwigshafen, Germany) as a white powder of purity >99 %. The solid powder was

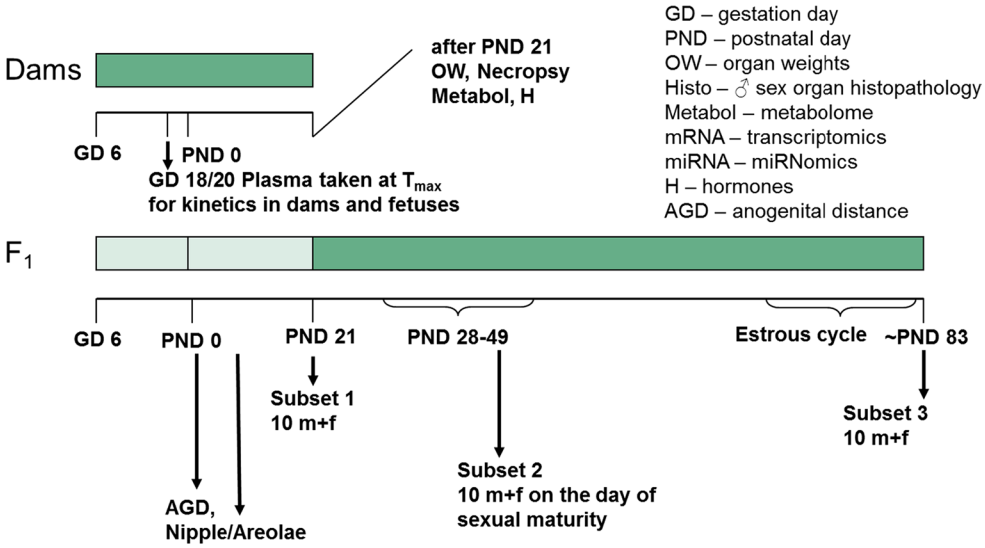

Fig. 1 Experimental design of the study. *Note*: it should be noted that samples were taken for future molecular analyses (metabolome, miRNome, and transcriptome), the results of which are not described in this publication. Also the plasma kinetics of dams and fetuses taken from an additional number of 5 animals killed on GD 18 are not described in this publication

then thoroughly dissolved in corn oil vehicle to prepare each dosing solution. The correctness and the homogeneity of prepared gavage solutions were determined by HPLC-MS analysis of several aliquots sampled from the bottom, middle and top of the preparation vessels. Furthermore, the stability of vinclozolin in the corn oil vehicle was proven by testing a sample stored at room temperature at intervals over a period of 7 days. Fresh gavage solutions were prepared weekly.

Parental female animals

Time-mated outbred Wistar rats (Crl:WI(Han)) were obtained from Charles River Laboratories (Sulzfeld, Germany) on gestational day zero (GD 0 as defined by the presence of sperm or a vaginal plug in the vaginal canal) at an age of about 10–12 weeks. Throughout the study, all animals were maintained under standard conditions: one animal (or litter) per Makrolon type MIII cage with LTE E001 bedding (ABEDD, Vienna, Austria) and maintained at 20–24 °C and 30–70 % humidity with 15 air changes per hour and a 12-h light/dark cycle. All animals had free access to food (ground Kliba maintenance diet mouse/rat 'GLP' [Provimi-Kliba, Kaiseraugst, Switzerland]), water and a wooden gnawing block. The animals were allowed to acclimatize to the laboratory conditions until GD 6, when the dams were randomized into 4 test groups of 20 animals per group.

The dams were administered 0, 0.005, 4, or 20 mg/kg bw/d vinclozolin in corn oil (4 ml/kg) by gavage every morning from GD 6 until the day of killing (GD 18 or about PND 30), except during labor. The doses were chosen to represent a clear effect level (20 mg/kg bw/d), a NOAEL for endocrine effects (4 mg/kg bw/d), and approximately at an ADI level (0.005 mg/kg bw/d). On GD 18, five dams/dose groups were killed by cervical dislocation under isoflurane anesthesia and necropsied. The pregnant uteri were dissected and opened, and the fetuses were removed. All implants and fetal weights were recorded, before the fetuses were killed by snap freezing in liquid nitrogen and stored for future kinetic studies. The remaining dams were allowed to deliver and rear their pups until PND 21 (weaning).

During gestation and lactation, each dam was examined daily for clinical signs of morbidity and toxicity, as well as parturition and lactation behavior. The food consumption and body weight of each dam were evaluated on GD 0, 6, 13, 18, and 20. The food consumption was also determined weekly for each litter during lactation (PND 0–21) and later for every weaned F1 animal in subsets 2 and 3. All females with litters were weighed on PND 0, 7, 14, and 21. Females which did not litter were killed by decapitation under isoflurane anesthesia and examined for gross abnormalities. The uteri were removed from these animals and stained with Salewski stain for implantation sites (Salewski 1964). After blood sampling in the first proestrus after weaning, i.e., PND 21, the dams were killed under deep isoflurane anesthesia and necropsied. The tissues were preserved from these animals for further analyses.

Offspring

The gender, status (live- or stillborn) and any gross-morphological abnormalities of each delivered pup were recorded as soon as possible after birth. Pup viability, mortality and any clinical signs of toxicity or morbidity were determined at least daily. The pups were weighed on PND 1, 4, 7, 14, 21, as well as on the day of sexual maturation (vaginal opening or preputial separation). Anogenital measurements were obtained on all living pups on PND 1 using

a measuring ocular in a blind, randomized fashion. All living male pups were examined for the presence or absence of nipple/areola anlagen on PND 12 and re-examined on PND 20 or 21.

Before weaning on PND 21, twenty pups of each gender per test group were selected randomly to be allowed to mature (ten per gender in each of subsets 2 and 3). After blood sampling on PND 21, the dams and a further ten male and ten female pups were killed by decapitation under deep isoflurane/carbon dioxide anesthesia and necropsied (pups: subset 1). The tissues were preserved from these animals for further analyses. Any surplus pups were similarly killed on PND 21, but only macroscopically examined for gross abnormalities.

From PND 21 until sexual maturity (subset 2) or young adulthood (subset 3), the maturing F1 pups were exposed daily to test substance orally by gavage. One day after reaching sexual maturity, blood was sampled from each animal in SUBSET 2 before it was killed by decapitation under deep isoflurane anesthesia and necropsied. Again, the relevant tissues were sampled and preserved. Similarly, each F1 animal of subset 3 was killed as young adult (PND 81–85; females in proestrus determined by vaginal cytology) after blood sampling and necropsied as given for subset 2, and organs were sampled and preserved.

Serum preparation and hormone analysis

Blood samples for hormone measurements were collected under isoflurane anesthesia by puncturing the retroorbital venous plexus or after decapitation:

- Dams in the first proestrus after weaning.
- Subset 3 offspring at about PND 83 (females in proestrus).
- Subset 1 offspring at PND 21.
- Subset 2 rats at onset of puberty.

Blood aliquots were collected with and without K-EDTA supplement and centrifuged under refrigeration. The separated plasma or serum was aliquoted (>200 µl per aliquot) and stored under N2 at −80 °C for hormone analysis.

Steroid hormones (androstenedione, testosterone, progesterone and corticosterone) were measured in plasma by a proprietary online SPE-LC-MS/MS (Solid phase extraction-LC-MS/MS) (Yamada et al. 2002; Zhang et al. 2011). Absolute quantification was performed by means of stable isotope-labeled standards.

Estradiol concentration in serum samples was determined using a commercially available ELISA kit from DRG Diagnostics (EIA-4399; Marburg, Germany) measured on a Sunrise MTP-reader (Tecan AG, Maennedorf, Switzerland) and evaluated by the Magellan-Software of the instrument producer. Estradiol was not measured in subset 1 females or in males at any age, as estradiol concentrations in these animals are known to be below the technical lower limit of quantitation for this determination.

Tissue preparation and histopathological analysis

At all time points (dams and offspring), the organs were carefully trimmed of excess adhering fat and tissue and were weighed (fresh, unfixed). Adrenal glands, brain, cauda epididymis, epididymides, kidneys, liver, ovaries, spleen, testes and uterus were weighed without blotting to the nearest 0.001 g. Any other tissues (Musc. levator ani together with musc. bulbocavernosus, Cowper's gland, glans penis, pituitary gland, prostate (total, ventral only), seminal vesicles with coagulating glands, thyroid glands) were weighed without blotting to the nearest 0.1 mg. The anesthetized animals were weighed to the nearest 0.1 g. Immediately after weighing, the ventral prostate was cut longitudinally in two halves (subset 1 and 2 males). One of these halves, as well as the right testis and the right seminal vesicle, were snap frozen in liquid nitrogen and stored at −80°C for future analyses. In subset 3 male animals, both the ventral prostate and the right testis were halved after weighing. Half of each tissue plus the right seminal vesicle were snap frozen for possible future transcriptome analyses. In subset 3 males, sperm analysis was performed with the residual half of the right testis and the complete right epididymis: cauda epididymis sperm motility according to the method in Slott et al. (1991), sperm morphology, spermatid head count in the testes, and sperm head count in the cauda epididymis (Feuston et al. 1989, slightly modified).

All weighed tissues were preserved (except of glans penis, Cowper's gland, Musc. levator ani together with musc. bulbocavernosus) and fixed in 10 % neutral-buffered formalin or modified Davidson's solution (ovaries, left epididymis, and left testis). In addition to the organs mentioned above, oviducts and male/female mammary glands were sampled. For light microscopical assessment (all gross lesions, adrenal glands, left coagulating gland, left epididymis, pituitary gland, left prostate, left testis, left seminal vesicle), the fixed tissues were trimmed, paraplast-embedded, cut with a thickness of 2–3 µm, mounted on glass slides and stained with routine hematoxylin–eosin stain. All work was done according to published literature ("Revised guides for organ sampling and trimming in rats and mice" (Ruehl-Fehlert et al. 2003; Kittel et al. 2004; Morawietz et al. 2004; Creasy et al. 2012).

Statistical analysis

Means and standard deviations were calculated. In addition, the following statistical analyses were carried out (Table 1):

Table 1 Statistical analyses used in the assessment of measured parameters

Parameter	Statistical test
Food consumption, body weight and body weight change (parental animals and pups); estrous cycle length; duration of gestation; number of delivered pups per litter; developmental landmarks (days up to preputial separation or opening of the vagina); anogenital distance and index; implantation sites; postimplantation loss, weight of the fetuses, implantations, pre- and postimplantation losses, resorptions and live fetuses	Dunnett's test (two-sided)
Number of live and dead pups and different indices (e.g. mating index, fertility index and gestation index) and number of litters with necropsy findings in pups; developmental landmarks (preputial separation or opening of the vagina), sperm morphology, incidence of males with a specific amount of abnormal sperm (cutoff value: 0.9-quantile [90 %] of control groups)	Fisher's exact test
Proportion of pups with necropsy findings per litter, presence of areolas/nipples, sperm evaluation (with Bonferroni–Holm's correction	Wilcoxon test (one-sided)
Weight of the anesthetized animals and absolute and relative organ weights (all organs excl. organs listed below); hormones	Kruskal–Wallis and Wilcoxon or Mann–Whitney *U* test (latter for hormones)
Weight parameters of ventral prostate (VP), seminal vesicles with coagulating gland (SVCG), musc. levator ani together with musc. bulbocavernosus (LABC), Cowper's gland (Bulbourethral gland) (COW), glans penis (GP)	Dunnett's test (one-sided)

Results

The analyses showed that the achieved concentrations were within acceptable limits in the mid- and high-dose group, the prepared dosing suspensions were stable and homogeneous. The determined concentration differed no more than ±10 % of the nominal concentrations that represents our internal specification limit. However, in the low-dose group, the determined concentrations were in the range of 111–124 % of the nominal concentration. This slight deviation from the nominal concentration range is likely to have resulted in a minor increase in substance intake than intended. However, since this dose did not produce any effect in the respective animals, the validity of the study was not regarded as compromised. There were no contaminations in the feed, water or changes in the environment conditions, which might have the potential to influence the outcome of the study (data not shown).

Gestation and littering

18–20 dams per test group were pregnant in this study and survived until scheduled termination, with the exception of one animal in the high-dose group, which died intercurrently from a gavage error. No treatment-related clinical signs were observed in the dams during either gestation or lactation. Food consumption and body weight parameters were comparable to the concurrent control throughout the entire dosing period. No alterations to reproductive performance were observed. Litter size ranging over all test groups including control from 10.6 to 11.0 implants/dam and 9.8–10.4 delivered pups/dam) and offspring weights ranging over all test groups including control from day 1 7.0–7.1 g, day 4 11.2–10.9 g, day 7 16.8–16.5 g, day 14 30.0–30.2, and day 21 47.0–48.1 g were in the normal expectation for the rat strain used. There was no intergroup difference in sex ratio (observed range over all test groups including control: day 0 male pups 45.6–52.9 %, and day 21 male pups 45.9–52.6 %). As pup development continued post-partum, no decrease in pup viability was observed nor were any lactational difficulties noted at any dose.

There were no test substance-related necropsy findings in dams of any test group (data not shown).

Offspring development

No systemic toxic effects were observed in the offspring during lactation. However, a slight but statistically not significant decrease of 5 % in ano-genital distance and 5 % in ano-genital index (a calculated parameter which takes into account the individual animal weight) were observed on PND 1 in male pups exposed in utero to 20 mg/kg bw/d vinclozolin in comparison with control (Fig. 2 and Supplementary Table 1). These changes represent a well-characterized effect of vinclozolin exposure at higher doses, and the effects noted here, although too small to be statistically significant, may be indicative of approaching the threshold of a biologically relevant effect. Neither the ano-genital distance nor the ano-genital index was altered in females.

The male pups were also examined for the presence of nipples or areolae on PND 12 and again on PND 20 (Fig. 3 and Supplementary Table 1). On PND 12, a statistically significant increase in the number of offspring, which had at least one nipple or hairless pigmented spot identified as a areola, was observed in the 20 mg/kg bw/d dose group (92.5 %) when compared with controls (67.2 %, Fig. 3: Panel A). The number of nipples/areolas per male was also

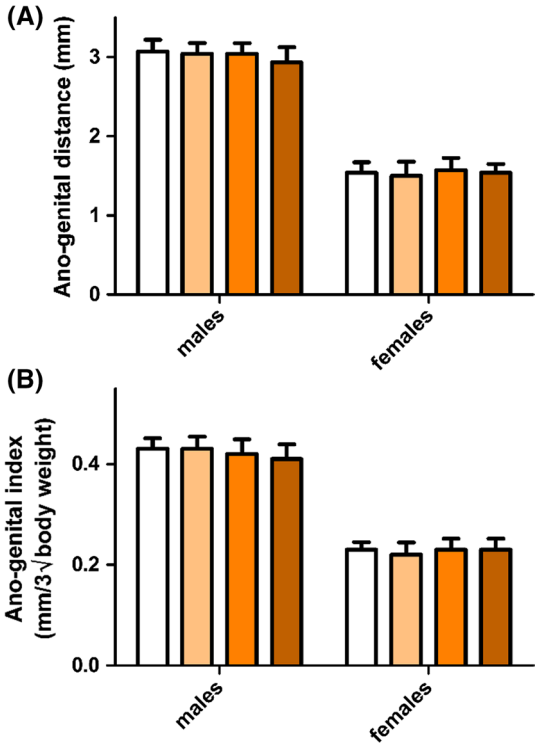

Fig. 2 Low doses of vinclozolin did not affect ano-genital distance or ano-genital index. On PND 1, the ano-genital distance (AGD) of all live-born pups exposed in utero to 0 □, 0.005 ▭, 4 ■ and 20 ■ mg/kg bw/d vinclozolin was measured (*panel A*). The ano-genital index (AGI), a parameter which accounts for any differences in animal size, was then calculated from these data (*panel B*). Prenatal exposure to these doses of vinclozolin did not significantly alter either male or female AGDs and AGIs (color figure online)

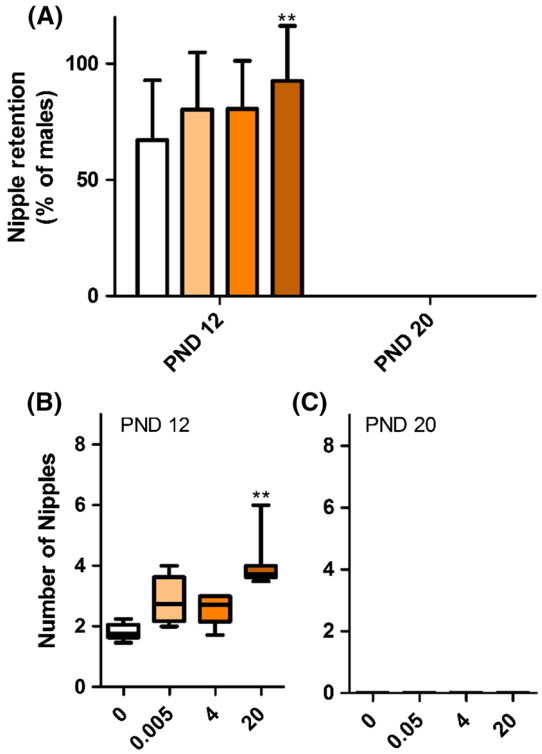

Fig. 3 Effect of increasing vinclozolin dose on nipple/areola presence. On PND 12, the number of male pups which had nipples or areolas was counted in animals exposed to 0 □, 0.005 ▭, 4 ■ and 20 ■ mg/kg bw/d vinclozolin. These animals were then recounted on PND 20 (*panel A*). Similarly, the number of nipples/areolas per male pup was also recorded on PND 12 (*panel B*) and PND 20 (*panel C*). Despite the relatively high background rate of nipple/areolas in control animals, prenatal and/or lactational exposure to 20 mg/kg bw/d vinclozolin raised both the number of males with nipple/areola and the average number of nipples/areolas per male pup on PND 12. This effect was transient; all nipples/areolas had disappeared by PND 20 (color figure online)

elevated in this dose group (3.8 in test group 20 mg/kg bw/d versus 1.8 in control, Fig. 3: Panel B). To determine whether the nipples/areolas persisted (retained nipples), a reexamination was performed on PND 20. By this age, no nipples or areolae were observed at any dose (Fig. 3: Panels A and C).

Ten males and females per dose group per subset were selected to be reared further; the age and weight at sexual maturation (preputial separation in males, vaginal opening in females) was then determined in about 20 animals of each gender. To better evaluate the delayed onset of male puberty, the individual ages at preputial separation were plotted as Kaplan–Meier curves (Fig. 4). Across all dose groups, no change in the course of sexual development was observed in the female offspring. The mean number of days to reach the criterion of vaginal opening in the control and 0.005; 4 and 20 mg/kg bw/d test groups amounted to 31.7, 30.9, 31.4 and 31.8 days. The mean body weight on the day, when vaginal opening was recorded, amounted to 93.0 g, 88.2 g, 93.4 g, and 90.1 g in test groups 0–3. However, male sexual development was delayed in animals exposed to the top dose of vinclozolin (Fig. 4 and Supplementary Table 2). The mean number of days to reach the criterion of preputial separation in the control and 0.005; 4 and 20 mg/kg bw/d test groups amounted to 43.8, 43.8, 44.2 and 45.9 days. The mean body weights on the day when preputial separation was recorded amounted to 178.2 g, 178.3 g, 182.2 g, and 191.2 g in test groups 0–3, respectively. A statistically significant delay of sexual maturation was only determined on two time points in the window of daily observation between postnatal day (PND) 38 and 64. The number of pups with preputial separation were significant lower on PND 41 (1 pup in test group 20 mg/kg bw/d versus 7 pups in control) and PND 48 (15 pups in test group 20 mg/kg bw/d versus 20 pups in control). Overall, in case of the 20 mg/kg bw/d dose group, this represents a statistically insignificant delay. However, taking into account the MoA, this finding may still be biologically relevant, because it is 1 day beyond the historical control range (PND 39.7–44.8).

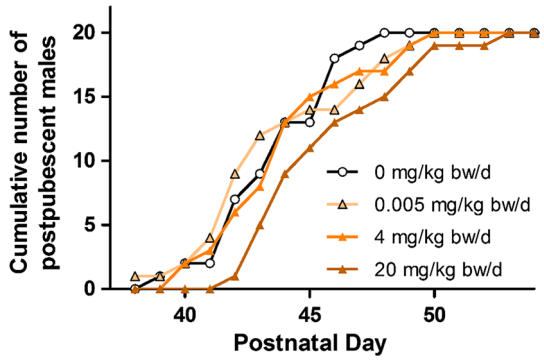

Fig. 4 Kaplan–Meier plot of the sexual maturation of pubescent male rats exposed to vinclozolin since GD 6. Twenty male offspring which had been exposed to either vehicle only (–), or increasing concentrations of vinclozolin (*filled triangles*), were examined for preputial separation daily from PND 38–64. The dose group exposed to 20 mg/kg bw/d was the only group where preputial separation was delayed beyond the historical control range (PND 39.7–44.8), but this lag was not statistically significant

Role of hormone disruption in anti-androgen delayed sexual maturation

Sexual development is controlled by several factors, including the general growth progression and health of the animal, as well as hormonal signaling. We and others have previously observed that delays in preputial separation can result from impaired growth, as measured by body weight development (Lau et al. 1996; Carney 2004; Melching-Kollmuß et al. 2014). Furthermore, as impaired growth is a common sign of the general toxicity required at the uppermost dose by regulatory authorities, we observed that often the delays in preputial separation observed during regulatory studies are the secondary effects of general toxicity instead of specific indicators of endocrine modulation (Delemarre-Van de Waal et al. 2002; Melching-Kollmuß et al. 2014). These primary and secondary effects can be differentiated using graphical analysis.

Briefly, the ages and weights of the individual animals on the day of sexual maturation (Fig. 5, scatterplots) were compared to the normal body weight progression of the control animals being reared to early adulthood, which were weighed at weekly intervals (gray diamonds, regression line). Most individuals in the 0, 0.005, 4 and 20 mg/kg bw/d groups were clustered with maturation days between PND 40 and PND 46 and distributed both above and below the regression line, signifying generally normal sexual development.

In Fig. 5 the statistically insignificant pubertal delay at doses of 20 mg/kg bw/d vinclozolin is difficult to identify. However, the graphical analysis still reveals that all animals were proportionally larger at the time of puberty as a result of their increased age. Since the growth progression of the individuals in these dose groups was normal, the delay in preputial separation cannot be explained by lags in overall maturation due to any general toxicity of the test substance. Therefore, these results suggest that the male pubertal delay resulting from vinclozolin administration was an effect of its known anti-androgenic MoA.

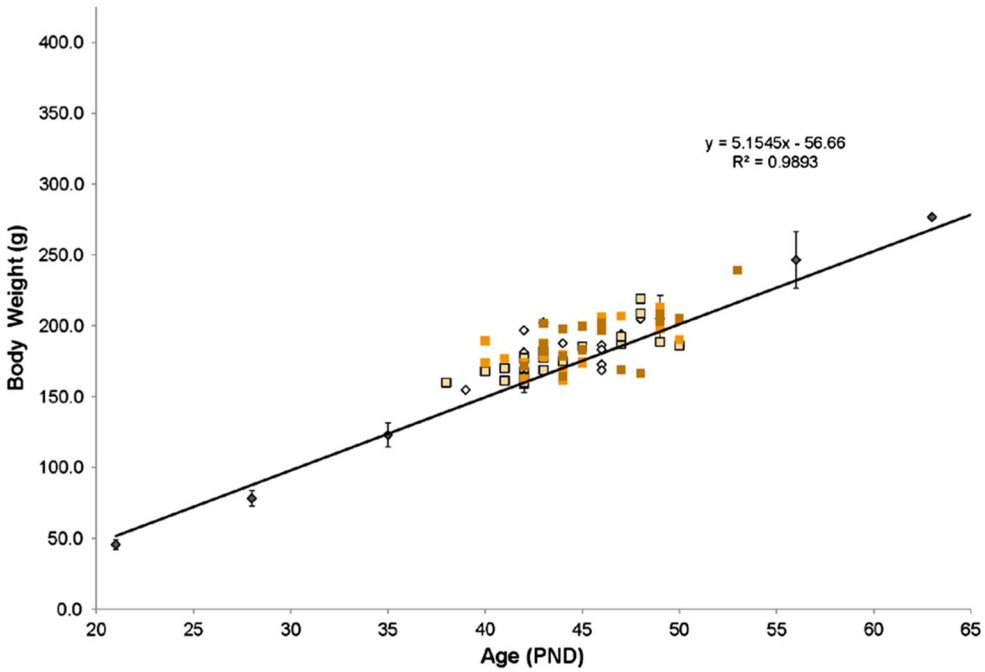

Fig. 5 Comparison of individual ages and weights at preputial separation with normal body weight development, after exposure to vinclozolin. *Scatterplots* of the ages (*x* axis) and body weights (*y* axis) of individual male offspring at sexual maturation, as compared to the body weight development of subset 3 control animals (gray diamonds ± standard deviation)

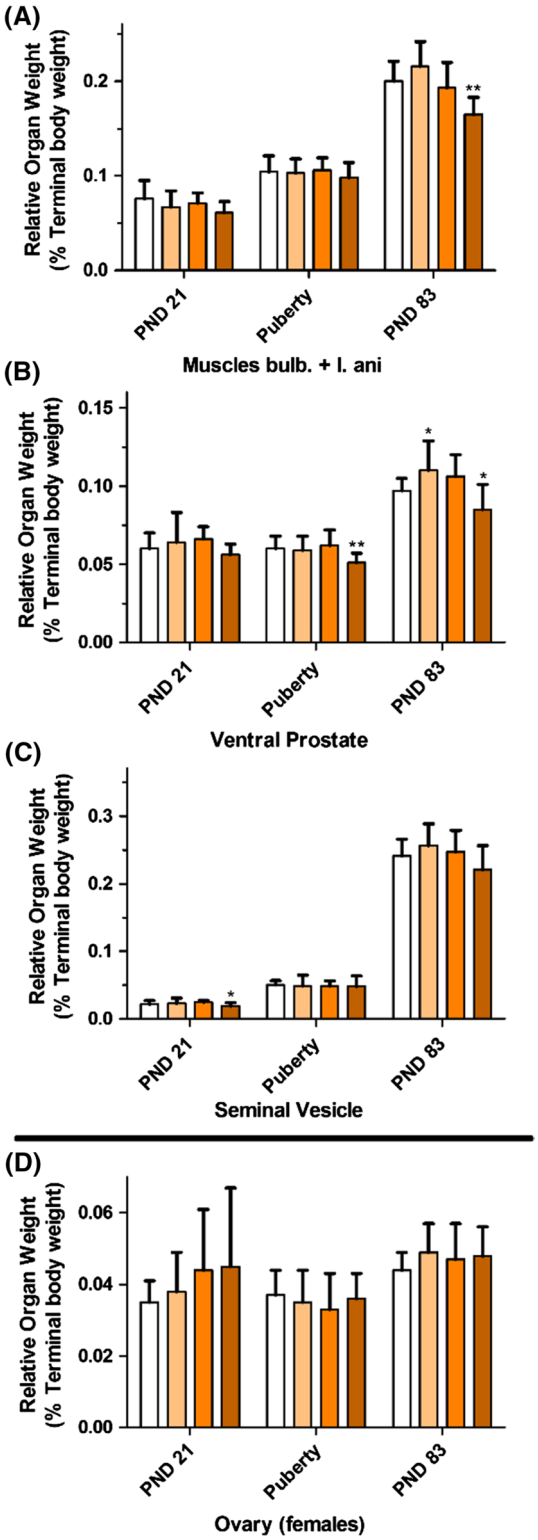

Fig. 6 Weights of selected male sexual organs. On PND 21 (subset 1), the day of preputial separation (Puberty, subset 2) and PND 83 ± 2 (subset 3), the sex organs from each of 10 male rats were preserved, weighed and reported as relative organ weights. These data are graphed as vehicle control, 0.005, 4 and 20 mg/kg bw/d at each time point. In general, increasing anti-androgen exposures reduced male, but not female, sex organ weights dose-dependently. Three of the most sensitive male sex organs are shown (*panels A–C*) in comparison with a female sex organ, exemplarily given by ovary (*panel D*) (color figure online)

Estrous cycle data

Estrous cycle data, generated in all females of subset 3 during the 3 weeks prior to necropsy, revealed regular cycles in the females of all test groups including the control. The mean estrous cycle duration in the different test groups was similar: 4.3 days in control, 4.1 days in low- and high-dose groups and 4.5 days in the mid-dose group.

Sex organ weights

Overall, the high-dose-level vinclozolin treatment (20 mg/kg bw/d) reduced male sex organ weights in the offspring, female sex organ weights, however, remained unaffected (Fig. 6, Panel D). When compared to the control group 0, (set to 100 %) the mean weight parameters in parental females, whether absolute or relative, did not show significant, treatment-related differences (data not shown). In contrast, treatment-related decreases in male offspring sex organ weights were noted at the 20 mg/kg bw/d dose. The most sensitive male sex organ weight changes were noted in total and ventral prostate, seminal vesicles and musculus levator ani together with musculus bulbocavernosus (Fig. 6). The absolute weight changes were more pronounced than the relative orange weights (Table 2, 3, 4 and Supplementary Fig. 1).

In subset 1 male animals (weaning), a statistically significant decrease in the mean absolute and relative organ weights of the seminal vesicles, 81 and 85 % of control, respectively, were observed in male offspring exposed to 20 mg/kg bw/d vinclozolin (Table 2; Fig. 6). In subset 2 animals (puberty), a 15 % reduction in ventral, but not total relative prostate weight, was the only significant effect observed (Table 3; Fig. 6). All other alterations in either subset 1 or subset 2 animals were too minor to be significant. In subset 3 animals (PND 83 ± 2), significant decreases in absolute cauda epididymis, epididymides, muscles bulbocavernosus and levator ani, as well as total and ventral prostate weights were observed (Table 4; Fig. 6). The relative weights are also similarly, though less notably decreased; only the weights of the muscles bulbocavernosus and levator ani and ventral prostate were statistically significant (Table 3; Fig. 6).

Histopathology

No histopathology was performed in dams and female offspring. The male sex organs of all three subsets were examined histopathologically as described above. Generally,

Table 2 Organ weights of subset 1 (PND 21) male offspring exposed to vinclozolin

Test group	1		2		3	
Dose (mg/kg bw/d)	0.005		4		20	
	Absolute (%)	Relative (%)	Absolute (%)	Relative (%)	Absolute (%)	Relative (%)
Seminal vesicles	99	100	110	110	**81***	**85***

Data are presented as percentages relative to control group 0

Values differing statistically significant from control are given in bold numbers

* $p \leq 0.05$

Table 3 Organ weights of subset 2 (puberty) male offspring exposed to vinclozolin

Test group	1		2		3	
Dose (mg/kg bw/d)	0.005		4		20	
	Absolute (%)	Relative (%)	Absolute (%)	Relative (%)	Absolute	Relative (%)
Prostate, ventral	98	98	102	103	90	**85***

Data are presented as percentages relative to control group 0

Values differing statistically significant from control are given in bold numbers

* $p \leq 0.05$

Table 4 Organ weights of subset 3 (PND 83 ± 2) male offspring exposed to vinclozolin

Test group	1		2		3	
Dose (mg/kg bw/d)	0.005		4		20	
	Absolute (%)	Relative (%)	Absolute (%)	Relative (%)	Absolute (%)	Relative (%)
Cauda epididymis	104	**113****	99	104	**86****	92
Epididymides	100	**122****	99	105	**92***	104
Musc. bulb. lev. ani	99	108	92	96	**77****	**82****
Prostate, total	102	**112***	103	108	**86***	93
Prostate, ventral	104	**113***	103	108	**81****	**87***

Data are presented as percentages relative to control group 0

Values differing statistically significant from control are given in bold numbers

* $p \leq 0.05$, ** $p \leq 0.01$

histopathological analysis of the male offspring exhibited signs of normal maturation. The reproductive organs of all subset 1 male animals (left testis, left epididymis, seminal vesicles, coagulating glands, prostate) showed an immature stage, which was comparable between all animals. This stage is developmentally normal for rats of this age. By puberty (subset 2), the seminal vesicles, coagulating glands and prostates of all pubescent males had developed to a comparable juvenile stage with a moderate amount of secretion without any differences between control and treated animals, and the left testis of all control and treated animals was judged to be at a juvenile stage with a fully developed spermatogenic cycle. Only the epididymides exhibited relevant treatment-related differences. While the controls and most of the treated animals showed a juvenile stage, two animals administered 20 mg/kg bw/d vinclozolin revealed a more advanced developmental stage called juveno-adult transition. These findings correlated very well with the older age of the animals on the day of preputial separation (the day of necropsy) and are thought to be a secondary effect of the delay in pubertal onset at this dose. Animals with delayed preputial separation and therefore a later day of necropsy had testes and epididymides which had developed to a more advanced stage. On PND 83 ± 2 (subset 3), the sex organs of most male offspring had matured fully; the organs generally appeared normal. The main findings were seen in the seminal vesicles and the prostate; all other organs were considered normal. The seminal vesicles were reduced in size with decreased secretion in 1/10 animals administered 20 mg/kg bw/d vinclozolin. Similarly, the prostate showed a reduced size with decreased secretion in 2/10 animals at this dose. These findings correlate very well with the described

organ weight reduction in the respective organs and have been previously observed in vinclozolin studies (Schneider et al. 2011). Thus, this finding is considered to be related to the 20 mg/kg bw/d vinclozolin treatment. The seminal vesicles and prostates of the animals of the 0.005 and 4 mg/kg bw/d dose groups were histopathologically normal. All other findings occurred individually and were considered to be incidental.

Hormone analysis

Blood samples were collected from the parental females and all reared offspring selected for study (subset 1, 2, and 3) for determination of blood concentrations of steroid hormones. The complete set can be found in the supplementary data (Supplementary Tables 3–9).

In dams after weaning dosed with 4 and 20 mg/kg bw/d, corticosterone levels were elevated. However, no dose–response relationship was evident and the controls in this study seemed to have quite low corticosterone levels (mean ± SD = 435.8 ± 485.6 nmol/L). Therefore, we evaluated the values of the treatment groups against two concurrent control groups from two similar studies which were performed at the same time in the same laboratory following the same procedures. Both vinclozolin test groups were not statistically significantly changed when compared to the concurrent control groups and the medians of both dose groups were in the same range as the medians in the controls (Supplementary Table 3). Thus, it seems likely that the statistically significant increase of corticosterone within the study groups is an incidental result of low serum concentrations in the study control group, rather than an effect of vinclozolin treatment.

In females from subset 3 (PND 83 ± 2 at proestrus), a dose-dependent increase in serum progesterone levels was observed reaching a statistical significant level at 20 mg/kg bw/d (Fig. 7 and Supplementary Table 9). However, when comparing the individual values of the three vinclozolin dose groups with the controls including both comparison controls (Fig. 8), all progesterone values in the vinclozolin groups were within the established preliminary reference range and a dose-dependency is not apparent. Therefore, the progesterone changes in females at PND 83 ± 2 in the 20 mg/kg bw/d vinclozolin dose group were regarded as not biologically relevant.

In all other subset groups (i.e. PND21 pups and pups at sexual maturity of both sexes as well as males at PND 83 ± 2), no alteration of blood steroid hormone levels was observed.

Sperm analysis

Sperm head counts in the testis and in the cauda epididymis, as well as the incidence of abnormal sperms and sperm motility in the cauda epididymis in all test groups (0.005, 4, and 20 mg/kg bw/d) of male offspring at PND 83 ± 2 (subset 3), were comparable to the concurrent control group (Table 5).

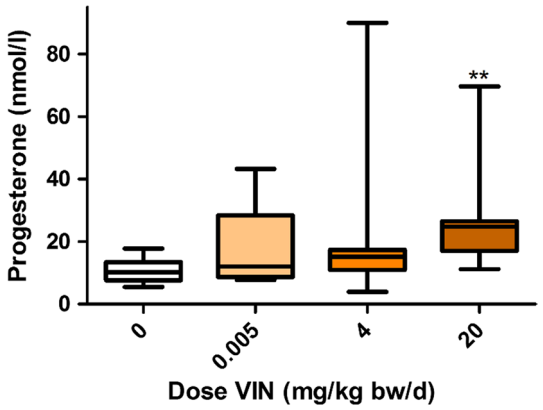

Fig. 7 Circulating concentrations of progesterone in female animals on day PND 83 ± 2. The data are graphed as box plots representing the median (—), interquartile range (*box*) and range (*bars*, two-sided) for each treatment group. Each *box plot* is *color-coded* according to treatment: vehicle control, 0.005, 4 and 20 mg/kg bw/d vinclozolin (color figure online)

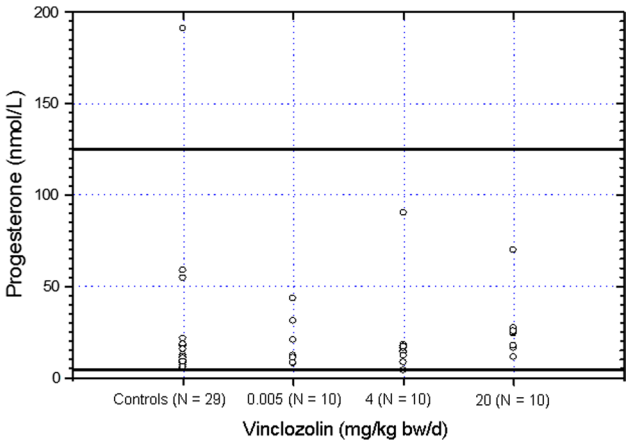

Fig. 8 Distribution of progesterone values in female rats around PND83 in proestrus dosed with vinclozolin. Control values were established with the study controls ($N = 10$) and two comparison control groups ($N = 19$; see *Supplementary data*). Reference range (5.2–124.7 nmol/L) between *horizontal lines* was established between 5 and 95 percentiles of the mentioned 29 control values

Discussion

It has been suggested that the endocrine system is uniquely sensitive to exogenous modulation during critical developmental periods, even at doses of hormonally active substances significantly below previously described no-observed adverse effect levels (Kortenkamp et al. 2012; Vandenberg et al. 2012; WHO/IPCS 1998). As a result,

Table 5 Analysis of sperm and spermatids of male offspring (PND 83 ± 2)

Dose (mg/kg bw/day)	0	0.005	4	20
Motile sperm (%)	88 ± 4 (10)	89 ± 5 (10)	87 ± 6 (10)	84 ± 6 (10)
Abnormal sperm morphology (%)	3 ± 3 (10)	3 ± 1 (10)	4 ± 2 (10)	3 ± 2 (10)
Spermatid count ($\times 10^6$/g testes)	190.9 ± 31.4 (10)	192.7 ± 17.4 (10)	215.4 ± 31.4 (10)	193.4 ± 23.1 (10)
Sperm count ($\times 10^6$/g cauda epididymis)	566.3 ± 114.1 (10)	622.4 ± 101.8 (10)	638.2 ± 144.3 (10)	544.0 ± 178.9 (10)

Data are presented as mean ± SD (*N*)

* $p \leq 0.05$, ** $p \leq 0.01$

there is concern that current risk assessment methodologies do not adequately assess the hazard associated with potentially endocrine active substances. To test this hypothesis, we compared the effects of three doses of the fungicide vinclozolin including a low dose in a peri-postnatal regulatory developmental and reproductive toxicity study. Vinclozolin was chosen for this investigation because it is a specific androgen receptor antagonist. The study design drew from the methods laid down in OECD TG 414, 416 and 443, as well as the corresponding OPPTS/OCSPP guidance. The resulting study closely resembled the peri-postnatal study design recommended by ICH (ICH 2005) and was enhanced by a number of endocrine-sensitive pathological and molecular endpoints. Doses were chosen to a low endocrine effect level, a no endocrine effect level and one dose which were 1000-fold below the no endocrine effect level to test for a non-monotonic dose–response relationship in the range of the acceptable daily intake level. These three doses represent nearly 5 orders of magnitude; thus, this study is designed to adequately describe not only the expected endocrine toxicity at the high doses, but also examine the relationship between low doses of endocrine active substances and their effects in an apical study.

Lowest observed adverse effect level

As expected, vinclozolin treatment at doses above the NOAEL resulted in signs of an anti-androgenic mode of action in the developing offspring. Males exposed to 20 mg/kg bw/d vinclozolin had a reduced nipple regression on PND 12. Interestingly, by PND 20, those nipple retentions had completely receded. These data indicate that the disruptive effects of vinclozolin may in fact be transitory, at least at LOAEL. However, the nipple retention described by Schneider et al. (2011) did not receded completely at 20 mg/kg bw/d but was also substantially reduced from 64 to 6 %. As the development of sexually dimorphic external genitalia and mammary function are controlled by androgens, together these findings lend support to the previously characterized role of vinclozolin as an androgen receptor antagonist (Kavlock and Cummings 2005).

Delays in sexual maturation were only observed in male, not female offspring treated with 20 mg/kg bw/d. The delay in the age of preputial separation observed in the 20 mg/kg bw/d dose group was mild and non-statistically significant, but relevant. With 45.9 days (versus 43.8 days in control) to reach the criterion, a delay of about 1 day beyond the historical control range (PND 39.7–44.8, mean of means 42.1) was determined. These findings closely match those of Schneider et al. (2011), in which the oral dose of 20 mg/kg bw/d vinclozolin caused a significant delay with 44.8 days until preputial separation versus 42.3 days in control. Again, without statistically significance, a higher body weight of males at sexual maturity was observed with 191.2 g at 20 mg/kg bw/d versus 178.2 g in control. The absolute difference in mean values was even smaller in the study of Schneider et al. (2011) (182 g at 20 mg/kg bw/d versus 171 g in control) but reached a statistically significant level. No difference in sexual maturation was observed for females at 20 mg/kg bw/d. This was consistent with the study of Schneider et al. (2011). They could only describe an anti-androgenic effect in females, i.e. reaching sexual maturity at an early age combined with a lower body weight at 100 mg/kg bw/d. These data indicate that doses of 20 mg/kg bw/d vinclozolin substantially disrupt androgen signaling during pubescence. Within the clinical observations, a good reproducibility of anti-androgenic effects in type and degree of vinclozolin described before (van Ravenzwaay 1992; Gray et al. 1994; WHO-JMPR 1995; Ostby et al. 1997; Hellwig et al. 2000; Matsuura et al. 2005) confirmed the suitability of the chosen study design to detect adverse manifestations of endocrine-related toxicity.

Clinical findings confirm the anti-androgenic potential of vinclozolin at the highest tested dose of 20 mg/kg bw/d. Considering the mode of action as an androgen receptor antagonist (Wong et al. 1995), one could expect that increased testosterone and androstenedione levels were observed, indicating that the androgen receptor was at least partially antagonized at this dose (the negative-feedback loop was activated). However, in the performed investigations, the inter-individual variation of plasma concentration

was too high (resulting in large standard deviations within each group) to describe potential treatment-related alterations of plasma hormone levels. As the measured hormone concentrations are quite small, often at or near the lower limit of quantitation at some time points, this means that both the detection and the interpretation of small perturbations in sex steroid hormone biosynthesis can be quite difficult. One should be aware that this does not mean that there are no testosterone or androstenedione alterations at different time points or in local tissue levels. However, it is likely that any vinclozolin-induced alterations in serum sex steroid hormone levels at doses at or below the LOAEL are too minute to be significant and/or biologically relevant.

Across all time points, no biologically relevant differences were observed between the sex organ weights of treated female animals and their age-matched controls. In contrast, a number of male reproductive organ weights were significantly reduced in animals exposed to 20 mg/kg bw/d vinclozolin at different time points. In general, these weight data describe treatment effects most likely linked to the anti-androgenic properties of vinclozolin. More specifically, it was noted that the test groups which demonstrated pathological changes in a single male sex organ also displayed similar effects in other male sex organs. Furthermore, a comparison of the organ weights at weaning (PND 21) or in young adults (PND 83 $\pm$ 2) revealed few differences between the absolute and relative organ weights. In addition, the most sensitive weight parameters in males for an anti-androgenic effect in this study were the relative weight of seminal vesicles, prostate (total and ventral only) and muscles bulbocavernosus with musculus levator ani.

However, the pubescent offspring have to be assessed more carefully, because each subset 2 offspring was killed at a slightly different age (i.e. on the day of puberty of each individual). This is particularly important at the 20 mg/kg bw/d dose, because the slight delay in sexual maturation prevents exact comparison with age-matched controls. As the male animals of this group, and therefore their organ weights, are slightly larger at the time of puberty, the absolute body and organ weights of these offspring vary as widely as the ages of the animals. Thereby potential treatment-related decrease of organ weights might be covered by increasing body weights during the time delay to sexual maturation at 20 mg/kg bw/d. Thus, only the relative organ weights are considered toxicologically relevant at this time point. For the purposes of this analysis, we focused on the relative weights of those male sex organs we observed to be most sensitive to anti-androgen treatment: prostate (total and ventral only), seminal vesicles and muscles bulbocavernosus and levator ani.

The secretory activity of the accessory sex glands is extremely sensitive to androgen exposure; thus, weight change is often related to altered secretory activity in the prostate and seminal vesicle and can be used as a sensitive, and relatively rapid, integrated indicator of altered androgen signaling (OECD 2009). Direct comparison of the organ weights over time shows the general increase in relative sex organ weight as a result of sexual maturation. However, an increase in relative sex organ weight was observed in all test groups, but it was less pronounced at 20 mg/kg bw/d. The total prostate showed only in the high-dose test group (20 mg/kg bw/d) a significant decrease in absolute organ weights (Subset 3), but not in relative organ weights at any dose. In contrast, the ventral portion of the prostate in animals of dose group 20 mg/kg bw/d at puberty (subset 2) and at early adulthood (subset 3) showed a clear significant decrease of relative organ weights.

Similarly, as the secretions of the coagulating gland, prostate and seminal vesicle are also androgen-dependent, they can be used as a marker of androgen effects (OECD 2009). No alterations to secretion were noted in any of the young–adult animals treated with 0.005 and 4 mg/kg bw/d vinclozolin; however, a number of changes were observed at 20 mg/kg bw/d. The seminal vesicles of the young-adult offspring were reduced in size with decreased secretion in 1 of 10 males and the prostate was also smaller, with decreased secretion in 2 of 10 animals. These findings correlate with the described organ weight reduction in the respective organs and/or are similar to those found in previous reports at 100 mg/kg bw/d (Schneider et al. 2011).

Furthermore, muscle development is also androgen-dependent, particularly muscles bulbocavernosus and levator ani (BC/LA). In gonadectomized males, the weight of the muscles BC/LA was decreased over 50 % within 30 days which could be attenuated by testosterone supplementation (Rand and Breedlove 1992). After sexual maturity, the testosterone-dependent development of these muscles was significantly decreased at 20 mg/kg bw/d in young adults.

Overall, the treatment-related decreases of organ weights are relatively small and questionable if permanent. It is known that vinclozolin has the potential to cause more severe effects at higher doses, as described for 100 mg/kg bw/d and above which are likely to cause permanent alterations during development (Matsuura et al. 2005, Schneider et al. 2011) but those are linked to high-dose exposure and not seen at low-dose exposures.

Macroscopic lesions were only observed in one young-adult male animal at 20 mg/kg bw/d which exhibited size reduction of prostate and seminal vesicle. Neither hypospadias, a small size of testis nor tubular atrophy or Leydig cell hyperplasia during histopathological analysis, as well as aspermia/oligospermia in the corresponding epididymis, was observed as described before at 100 mg/kg bw/d (Schneider et al. 2011; van Ravenzwaay 1992).

Taken together, these results are typical for the effects previously reported for toxicological studies in rats

(rodents) dosed with vinclozolin, though these studies used higher doses and therefore the findings were more pronounced. However, the used 20 mg/kg bw/d chosen as the LOAEL was causing some adverse effects which are in frequency and degree comparable as described before (van Ravenzwaay 1992; Matsuura et al. 2005; Schneider et al. 2011) demonstrating the sensitivity of the protocol used to detect anti-androgenic compounds. However, no adverse finding could be observed for vinclozolin exposure representing the dose level of NOAEL and ADI.

The reproductive organs of PND 21 males (weaning), i.e., left testis, left epididymis, seminal vesicles, coagulating glands and prostate, all exhibited an immature stage which was normal for their age and comparable to controls. By puberty, the seminal vesicles, coagulating glands and prostates of all control and treated offspring had matured to a comparably juvenile stage with a moderate amount of secretion, with no observable differences between the vehicle and treatment groups, with the exception of a decreased relative organ weight of ventral prostate observed in young adults that was already detectable at puberty in the 20 mg/kg bw/d group. Furthermore, at weaning, the seminal vesicles were decreased in relative weight at 20 mg/kg bw/d. However, at later time points in development, this potential effect of vinclozolin did not reached statistically significant level anymore.

When all these clinical, clinical chemistry and pathological findings are taken together, they provide clear evidence of anti-androgenicity at 20 mg/kg bw/d. As expected for an androgen receptor antagonist, effects were observed in males; treated females generally exhibited no obvious sign of an endocrine-dependent effect. The effects observed were all classic downstream consequences of interferences in androgen hormone signaling. Findings were only evident for the most sensitive parameters (increased numbers of male offspring with nipples/areolas, age at puberty, and relative sex organ weights). Neither alterations to anogenital distance/anogenital index nor histopathological findings were observed. In addition, some of the anti-androgenic findings were transient, e.g., exhibited at weaning, but not by the time the animals were pubertal or young adult. Thus, these data were sometimes inconsistent and difficult to interpret (e.g., transient nipple retention and unaltered relative weight of seminal vesicles) and represented the gray area between doses, which were clearly anti-androgenic and a true NOAEL, with only the minor effects indicative of a true LOAEL.

Dose levels at or below the NOAEL

In contrast to what was observed at 20 mg/kg bw/d, vinclozolin treatment at doses at the NOAEL (4 mg/kg bw/d) or at the ADI (0.005 mg/kg bw/d) resulted in no clinical signs, no alterations of organ weights, no histopathological findings or alterations to steroid hormone levels representing anti-androgenicity. All findings were observed singly and in the absence of any other corroborating observations at the same test groups or time points. Therefore, any assumption of a treatment-related effect stretches credulity. Thus despite our extensive attention to a wide variety of anti-androgenic parameters, we were unable to find an anti-androgenic effect in the dose groups at or below the NOAEL despite the marked effects at doses at the LOAEL.

While these observations are not novel to such a well-characterized member of this compound class, they do provide clear positive indications of the validity of the study. Regardless of the lack of findings at doses at or below the NOAEL, the present investigation is important because it conforms to current guidance for the evaluation of chemicals for endocrine disruption (OECD 2002, 2012). Moreover, the study design conforms in parallel to the investigation of flutamide (Fussell et al. 2015) and prochloraz (Melching-Kollmuss et al. 2016) that it fulfills the criteria for the acceptance of studies: the experimental system, species, or animal strain chosen must be responsive to endocrine effects, a negative control group must confirm the experiment is "free of background contamination" and a positive-effect group must indicate that the experimental system is capable of responding to a low-dose acting on the same pathway. An outbred Wistar rat experimental model was chosen for its genetic diversity and its documented sensitivity to endocrine effects (Diel et al. 2004). No endocrine effects were observed in the negative control groups, and a variety of endocrine-sensitive parameters was altered at the top dose. Thus, any lack of findings at the other two doses can be interpreted as a lack of endocrine effects at these lower dose levels. This is important because the results of all three doses when considered together indicate the absence of evidence for a non-monotonic dose–response curve (NMDRC) at any endpoint measured.

Conclusion

The current regulatory paradigm of toxicology precludes any adverse effects at doses below the NOAEL, but this has never been conclusively verified. The current investigation was therefore designed to investigate whether a threshold exists for endocrine-related adverse effects using vinclozolin as a compound with an anti-androgenic mode of action. This study tested three vinclozolin dose levels, one effect level (20 mg/kg bw/d), the NOAEL dose (4 mg/kg bw/d), and one further dose below the NOAEL (0.005 mg/kg bw/d).

In general, we observed anti-androgenic changes at the 20 mg/kg bw/d (chosen as a LOAEL) doses, but not at

lower exposures. Nipple/areola counts appeared to be the most sensitive measure of effect, followed by male sex organ weights at sexual maturation, and finally gross and histopathological findings. The quantification of hormone levels was found to be not sensitive enough to detect anti-androgenic effects in steroid hormones at 20 mg/kg bw/d. When taken together these endpoints data reveal a very important observation: no findings below the LOAEL.

However, the endocrine system is specifically designed to respond to environmental fluctuations and still maintain stable hormonal signaling. Such regulations generally are considered normal, adaptive, and necessary as long as they are transient and within the normal homeostatic range (Goodman et al. 2010; Rhomberg and Goodman 2012). It seems logical that the same adaptive processes which allow humans to reassert hormonal homeostasis in a changing natural environment might also compensate for exposure to endocrine active substances at low doses. As long as these processes remain truly adaptive, then they do not necessarily pose an increased hazard to humans. Therefore, it is important to determine not only whether effects are observed at human relevant exposures, but also whether any effects observed are adverse.

Importantly, no anti-androgenic effects were noted in the present study at a dose of or below 4 mg/kg bw/d; however, anti-androgenic changes were observed at the 20 mg/kg bw/d dose, thereby demonstrating the capability of the test system to fulfill all Klimisch criteria (Klimisch et al. 1997). Despite testing three logarithmically-spaced doses, which stretched up to 4000-fold below the 20 mg/kg bw/d LOAEL, no evidence for non-monotonic endocrine disruption by vinclozolin was observed. Notwithstanding the fact that few studies have been performed measuring exposures to comparably low doses of anti-androgens, missing evidence for a non-monotonic dose–response closely matches observations of several other low-dose exposures to endocrine disruptors (Christiansen et al. 2010; Gray et al. 1999a, b; Holsapple and Wallace 2008; Howdeshell et al. 2008; Imperato-McGinley et al. 1992).

Supported by the data in the current investigation, the authors suggest that caution should be exercised when assumptions and conclusions are drawn about non-monotonic dose–response curves and low dose effects. Evaluation of this hypothesis should only be based on evidence from valid animal data with an understanding of the underlying mechanisms of toxicity.

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Compliance with ethical standards

Conflict of interest This study was co-sponsored by BASF SE, Ludwigshafen, Germany. BASF is producer of vinclozolin.

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REPRODUCTIVE TOXICOLOGY

Investigations on the dose–response relationship of combined exposure to low doses of three anti-androgens in Wistar rats

Steffen Schneider[1] · Karma C. Fussell[2] · Stephanie Melching-Kollmuss[3] · Roland Buesen[1] · Sibylle Gröters[1] · Volker Strauss[1] · Xiaoqi Jiang[1] · Bennard van Ravenzwaay[1]

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Abstract The current investigation examines whether combined exposure to three anti-androgens (flutamide, prochloraz, vinclozolin) result in interference with endocrine homeostasis when applied at very low dose levels, and whether the results of combined exposure are more pronounced than to the individual compounds. A pre–post-natal in vivo study design was chosen with more parameters than regulatory testing protocols require (additional endpoints addressing hormone levels, morphology and histopathological examinations). Dose levels were chosen to represent the lowest observed adverse effect level (LOAEL), the no observed adverse effect level (NOAEL), and the acceptable daily intake for each individual substance. Anti-androgenic changes were observable at the effect level (LOAEL) but not at lower exposures. Nipple/areola counts appeared to be a sensitive measure of effect, in addition to male sex organ weights at sexual maturation, and finally gross findings. The results indicate the absence of evidence for effects at low or very low dose levels. No (adverse) effects were seen at the NOAEL dose. A non-monotonic dose–response relationship was not evident. Combined exposure at LOAEL level resulted in enhanced responses for anogenital index, number of areolas/nipples, delayed preputial separation and reduced ventral prostate weight in comparison to the individual compounds.

Keywords Mixture · Low dose · Non-monotonic dose–response · Anti-androgenic · Endocrine disruptor · Experimental test guidelines · Additive

Introduction

The regulation of substances is mostly based on single compound assessment. Moreover, human exposure is rarely to a single-substance, but rather to a variety of chemicals, cosmetic ingredients, drugs, biocides, pesticides and natural products. The need for mixture risk assessments has been discussed for several years and state-of-the-art reports on mixture toxicity—funded by DG Environment—were published (e.g., Kortenkamp et al. 2009). In the context of the regulation of pesticides in the EU (e.g., via the MRL Regulation (EC) No. 396/2005) due to the potential presence of pesticide residues in food "… account shall be taken of … known cumulative and synergistic effects, when methods to assess such effects are available." Similar text is included in Regulation (EC) No. 1107/2009, concerning the placing of PPPs on the market in the EU. Current work is ongoing to identify active ingredients with common target organs to be included in Cumulative Assessment groups (e.g., EFSA 2013a; External Scientific Report 2016).

It is generally acknowledged that the combined toxicological effects of two or more compounds can take one of three forms: independent action, dose addition or interaction (Wilkinson et al. 2000; Feron and Groten 2002). Independent action, also known as response addition, occurs when the toxicological effects of the individual compounds in a mixture are a consequence of separate mechanisms/modes

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✉ Bennard van Ravenzwaay
bennard.ravenzwaay@basf.com

[1] Experimental Toxicology and Ecology, BASF SE, 67056 Ludwigshafen, Germany

[2] Chemical Food Safety, Nestlé, Lausanne, Switzerland

[3] Product Safety, Regulations, Toxicology and Ecology, BASF SE, Ludwigshafen, Germany

of action. Dose addition, also referred to as simple similar action, occurs when the individual compounds in a mixture share the same mechanism/mode of action of their toxicological effects, and they differ only in their potencies. The term interaction includes all forms of joint action that depart from either dose- or response-addition. Hence, the combined effects of two or more interacting chemicals is either greater (synergistic) or lesser (antagonistic) than that predicted based on dose addition or response addition.

The basic assumption for conducting combined/cumulative risk assessment is dose addition for compounds with similar mode/mechanism of action, often simplified by "having effects on the same target organ" (EFSA 2013a, b). As the evidence for synergism is very weak (Boobis et al. 2008, ECETOC Monograph "low dose interaction"), the dose additivity assumption is considered protective for human health assessments; moreover, there is experimental evidence that dose additivity is a conservative assumption (Schmidt et al. 2016).

Anti-androgens are compounds with similar downstream effects on male sexual development. Specific modes of action may vary, but all involve disruption of androgen signaling within the endocrine system, generally via inhibition of androgen hormone biosynthesis or by blocking receptor-mediated signaling (Gray et al. 2006; Hellwig et al. 2000) and can lead to non-reversible effects in male offspring when exposure occurs during certain windows of development (Schneider et al. 2011). This signaling is important for the development and maintenance of male sexual health (Fridmans et al. 2005). Flutamide has been shown to cause reduced AGDs, increased nipple retention, pubertal delays, decreased sex organ weights, hypospadias and reduced penile length in male rat offspring (summarized in Fussell et al. 2015) and prochloraz was found to increase transient nipple retentions and caused delayed entries into puberty (Christiansen et al. 2009; Laier et al. 2006).

Mixture effects have been reported, when assumed anti-androgenic phthalates and pesticides have been simultaneously tested in vivo at individual effect or NOAEL levels (Boobis et al. 2008; Christiansen et al. 2008; Gray et al. 2007; Hass et al. 2007; Howdeshell et al. 2008a, b). Dose additivity has been found also for compounds with different modes/mechanisms of action, but displaying similar downstream in vivo effects (Blystone et al. 2009; Rider et al. 2010). Similarly, in none of the publications, adequate studies on individual compounds and on the mixtures have been conducted in terms of animal numbers, the guideline used, and the determined endpoints, which significantly hamper the overall assessment on the type of observed mixture toxicity (dose additivity or synergy). Thus, it is quite likely that some of the reported NOAELs in the literature, and consequently the literature-derived "NOAEL doses" used in published combination experiments, are actually effect levels, rather than true NOAELs (Jacobsen et al. 2012).

Human exposure to chemicals, pesticides, biocides are usually below safe threshold levels (e.g., ADIs, DNELs, RfC), which are in turn far below the NOAELs identified in the animal studies. However, mixture studies using realistic human mixture exposures are scarce (ECETOC TR 115 2012).

Here side-by-side extended pre/post-natal developmental toxicity studies on individual compounds and a mixture study were conducted, using vinclozolin and flutamide as androgen receptor antagonists and prochloraz as an inhibitor of steroid hormone biosynthesis (EFSA 2011) with weak androgen receptor antagonistic properties. Dose levels of the individual substances were selected representing a LOAEL and a NOAEL for anti-androgenic effects, as well as the acceptable daily intake (ADI, as determined by regulatory agencies and usually 100× below the lowest NOAEL), which were then combined together into a LOAEL-MIX, a NOAEL-MIX, and an ADI-MIX (dosing each compound at its specific LOAEL/NOAEL/ADI). Thus, the experiments were designed to test whether any combined effects occur at individual reference values, and to compare the extent or grades of the expected effects with the determined effects in the mixtures. The results of the single compound testing have been published previously, i.e. flutamide (Fussell et al. 2015), prochloraz (Melching-Kollmuss et al. 2017) and vinclozolin (Flick et al. 2016). Here we report on the effects of combined exposure to all three anti-androgens.

Materials and methods

The investigations reported herein exceeded the requirements of any specific regulatory guideline, but reference is made to OECD 414 and 416 (OECD 2001a, b) and 443 (Fegert et al. 2012; OECD 2011) as well as OPPTS 870.3700 (US Environmental Protection Agency 1998). The study was performed according to the OECD Principles of Good Laboratory Practice and the GLP Principles of the German "Chemikaliengesetz" (Chemicals Act) which (apart from few recognized differences) meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 160 (FIFRA) and Part 792 (TSCA)] (US Environmental Protection Agency 1972, 1976) . Hormone levels determination followed good scientific practice but was not according to formal GLP standards, but principally meeting EPA/FDA requirements. The study was performed in an AAALAC-approved laboratory. Permission for this study was obtained from the local regulatory agencies (permission number LRI-EMSG56-BASF/G11-3-013), and all study protocols were in compliance with the German Animal Welfare Act and the effective European Council Directive.

An overview of the experimental design is given in Fig. 1 as well as Table 1.

Table 1 Parameters investigated

Parameter	Dams (~PND 30)	Subset 1 (PND 21)	Subset 2 (Puberty)	Subset 3 (PND 83)
Full necropsy	+	+	+	+
Organ weights	+	+	+	+
Histopathology	+	+	+	+
Hormone levels	+	+	+	+
Estrous cycle				+
Spermatology				+

Test substances

Vinclozolin (VIN), (RS)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione (CAS number 50471-44-8), and prochloraz (PRO), *N*-propyl-*N*-[2-(2, 4, 6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide (CAS number 67747-09-5) were synthesized and fully characterized at BASF SE (Ludwigshafen, Germany) as a white powder of purity >99% and a brownish solid of purity 98 ± 1%, respectively. Flutamide, 2-methyl-*N*-(4-nitro-3-[trifluoromethyl] phenyl)propanamide (FLT), CAS number 13311-84-7, was purchased from Sigma-Aldrich (St. Louis, MO, USA) as a yellow powder of purity >99%. These solids were thoroughly dissolved in the corn oil vehicle to prepare each dosing solution. The correctness and the homogeneity of prepared gavage formulations were determined by HPLC–MS analyses of several aliquots sampled from the bottom, middle and top of the preparation vessels. Furthermore, the stability of vinclozolin, flutamide, and prochloraz in corn oil vehicle was proven by testing a sample stored at room temperature at intervals over a period of 7 days. Fresh gavage solutions were prepared weekly.

The mixtures data reported in this investigation were captured in the final of a series of three separate experiments of the same study design addressing the theme of single and mixed exposures to low dose levels of anti-androgens. Since we have previously published the effects of the single-substance exposures to the three test substances in prior reports (Fussell et al. 2015; Melching-Kollmuss et al. 2017; Flick et al. 2016), these outcomes will not be discussed here in detail. Instead, the included single-substance exposure data are presented only for the purposes of comparison to the mixed exposures tested in this specific investigation and will be discussed only in this context. Since these data arise from three separate experiments, all three concurrent control groups (called α, β, and γ) have also been included. However, all statistical tests are based on the comparison between a treatment group and its concurrent control group only. The three equivalent concurrent control groups were also separately compared with each other to establish a control range for each parameter (Table 2, Supplementary Figures 1–3 and all Supplementary Tables).

Parental female animals

Permission for animal studies was obtained from local regulatory agencies, and all study protocols were in compliance with German and EU animal welfare requirements. The study was performed in an AAALAC-approved laboratory. Time-mated outbred Wistar rats (WI:Han) were obtained from Charles River Laboratories (Sulzfeld, Germany) on gestational day zero (GD 0), defined by the presence of sperm or a vaginal plug in the vaginal canal. Throughout the study, all animals were maintained under standard

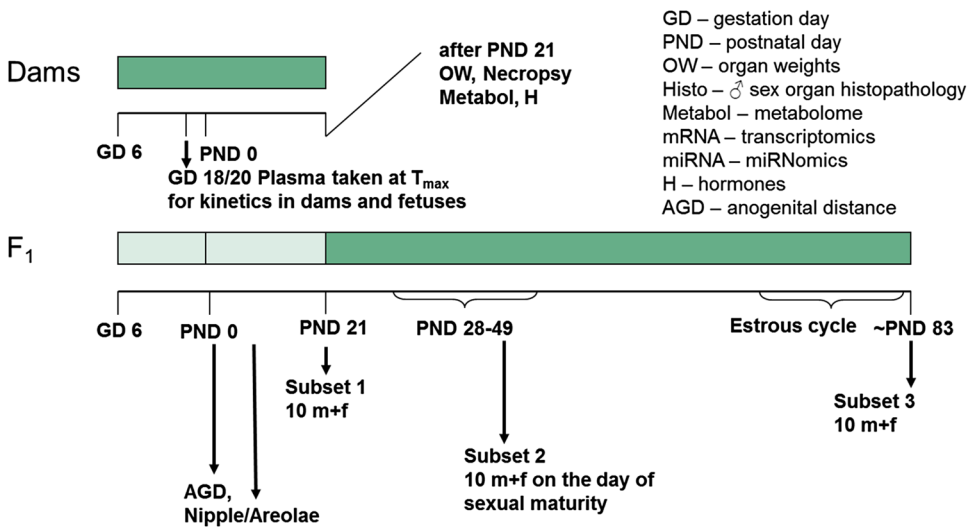


Fig. 1 Experimental design of the study. It should be noted that samples were taken for future molecular analyses (metabolome, miRNome, and transcriptome), the results of which are not described in this publication. Also, the plasma kinetics of dams and fetuses taken from an additional number of five animals killed on GD 18 are not described in this publication

Table 2 Summary of findings for VIN, FLT and PRO at (endocrine) LOAEL

Substance	LOAEL dose	Findings
VIN	20 mg/kg bw/d	Parental females No test substance-related adverse findings Pre-weaning Increased incidence of nipple/areolae in male pups on PND 12 (38% above control) Weaning Weight decrease of seminal vesicles Puberty Delay of preputial separation (about 1 day beyond the historical control range) Weight decrease of ventral prostate Juveno-adult transition in epididymides of two males Young adulthood Increased progesterone values in females around PND 83 in proestrus Weight decrease of cauda epididymis, epididymides, musc. bulb. lev. ani as well as total and ventral prostate Size reduction of seminal vesicles with decreased secretion in 1/10 male Size reduction of prostate with decreased secretion in 2/10 males
FLT	0.25 mg/kg bw/d	Parental females No test substance-related adverse findings Pre-weaning Increased incidence of nipple/areolae in male pups on PND 12 (28% above control) Weaning No test substance-related adverse findings Puberty Delay of preputial separation (about 1 day beyond the historical control range) Weight decrease of total/ventral prostate Young adulthood No test substance-related adverse findings
PRO	30 mg/kg bw/d	Parental females Decreased food consumption during GD 18–20 (about 11% below control) and during PND 0–21 (up to 23% below control) Decreased body weights during PND 0–21 (up to 8% below control) Increased duration of gestation (4% above control) Increased testosterone values in dams after weaning Decreased estradiol values in dams after weaning Pre-weaning Increased number of dams with stillborn pups (10 vs. 1 in control) Decreased number of live-born pups (30% below control) Increased number of stillborn pups (42 vs. 1 in control) Increased number of dead pups (8 vs. 0 in control) Increased number of cannibalized pups (7 vs. 0 in control) Increased incidence of nipple/areolae in male pups on PND 12 (37% above control) Decreased anogenital distance in female pups (10% below control) Decreased anogenital index in female pups (9% below control) Weaning No test substance-related adverse findings Puberty Juveno-adult transition in epididymides of three males Young adulthood Decreased food consumption in males during weeks 5–6 and weeks 7–8 (13% below control, respectively) Decreased mean body weights in males during weeks 5–8 (up to 12% below control, respectively) Decreased mean body weight change in males during several parts of the study phase (up to 31% below control, respectively) Increased testosterone values in females around PND 83 (in proestrus) Decreased estradiol values in females around PND 83 (in proestrus) Increased androstenedione and progesterone values in females around PND 83 (in proestrus) Decrease of terminal body weight in males Size reduction of seminal vesicles with decreased secretion in 1/10 male Size reduction of prostate with decreased secretion in 2/10 males

conditions: one animal (or litter) per Makrolon type MIII cage with LTE E001 bedding (ABEDD, Vienna, Austria) and maintained at 20–24 °C and 30–70% humidity with 15 air changes per hour and a 12-h light/dark cycle. All animals had free access to food (two batches of ground Kliba maintenance diet mouse/rat 'GLP' containing 102.5 and 122 ppm of total genistein equivalents [Provimi-Kliba, Kaiseraugst, Switzerland]), water and a wooden gnawing block as enrichment. The animals were allowed to acclimatize to the laboratory conditions until GD 6, when the dams were randomized into four dose groups of 25 animals per group.

Dams were treated with mixtures of vinclozolin, flutamide and prochloraz at three different dose levels representing the lowest observed adverse effect level (LOAEL), the no observed adverse effect level (NOAEL), and the acceptable daily intake (ADI) for each substance. The LOAEL-MIX contained 20, 0.25, and 30 mg/kg bw/d; the NOAEL-MIX 4, 0.025, and 5 mg/kg bw/d; and the ADI-MIX 0.005, 0.00025 and 0.01 mg/kg bw/d vinclozolin (see Flick et al. 2016), flutamide (see Fussell et al. 2015), and prochloraz (see Melching-Kollmuss et al. 2017), respectively. Each dam was administered its respective mixture in corn oil by gavage every morning from gestational day (GD) 6 until the day of killing [GD 20 or after weaning around post-natal day 30 (PND 30)], except during labor. In prior studies, these dose levels were also administered as single-substance exposures; a larger dose of flutamide (2.5 mg/kg bw/d) was also administered as a "clear effect level" positive control. These dose groups are also presented alongside the mixtures data for comparison purposes; however, as they have been previously reported, they are not discussed in detail as part of this investigation.

On GD 20, blood (1 mL with 10 µL of 10% EDTA as an anticoagulant) was collected from five dams in each dose group. Thereafter, these dams were killed by cervical dislocation under isoflurane anesthesia and necropsied. The pregnant uteri were dissected, opened, and the fetuses were removed. All implants and fetal weights were recorded before the fetuses were killed by snap freezing in liquid nitrogen and stored for future kinetic studies. The remaining dams were allowed to deliver and rear their pups until PND 21 (weaning).

During gestation and lactation, each dam was examined daily for clinical signs of morbidity and toxicity, as well as parturition and lactation behavior. The food consumption and body weight of each dam was evaluated on GD 0, 6, 13, 18, and 20. The food consumption was also determined weekly for each litter during lactation (PND 0–21) and later for every weaned F1 animal in subsets 2 and 3 (for explanation of subsets see 3.3). All females, which littered, were weighed on PND 0, 7, 14, and 21. Females, which did not litter, were killed and examined for gross abnormalities. The uteri were removed from these animals and stained with Salewski stain for implantation sites (Salewski 1964).

Offspring

The gender, status (live- or stillborn) and any gross morphological abnormalities of each delivered pup were recorded as soon as possible after birth. Pup viability, mortality and any clinical signs of toxicity or morbidity were determined at least daily. The pups were weighed on PND 1, 4, 7, 14, 21, as well as on the day of sexual maturation (vaginal opening or preputial separation). Anogenital measurements were obtained on all living pups on PND 1 using a measuring ocular in a blind, randomized fashion. All living, male pups were examined for the presence or absence of nipple/areola anlagen on PND 12, and re-examined on PND 20. Males of the control and LOAEL-MIX groups were also checked on PND 38, and again on the day of preputial separation.

Before weaning on PND 21, twenty pups of each gender per dose group were selected randomly to be allowed to mature (ten per gender in each of subsets 2 and 3). After blood sampling on PND 21 (at least 1 mL with 10 µL of 10% EDTA per mL as an anticoagulant and a further 300 µL without anticoagulant from the dams for estradiol measurement), the dams and a further ten male and ten female pups (subset 1) were killed under isoflurane/carbon dioxide anesthesia and dissected. The relevant tissues (Supplementary Table 1) were harvested from these animals for pathological and molecular analyses. Any surplus pups were also similarly killed on PND 21, but only macroscopically examined for gross abnormalities.

From PND 21 until sexual maturity (subset 2) or young adulthood (subset 3), the maturing F1 pups were also orally treated with the mixtures by gavage. On the day of vaginal opening (females) or preputial separation (males), blood was sampled from each animal in subset 2 (about 500 µL with 5 µL of 10% EDTA as an anticoagulant) before it was killed and necropsied. The relevant tissues (Supplementary Table 1) were harvested from these animals for pathological and molecular (metabolome, miRNome and transcriptome) analyses. Similarly, blood was sampled from each F1 animal of subset 3 (again, approx. 1 mL with 10 µL of 10% EDTA as an anticoagulant and another 300 µL of female blood without anticoagulant) before killing as a young adult (PND 81–85). Again, the sexually dimorphic tissues were harvested.

Hormone analysis

Plasma/serum samples

The blood samples collected with EDTA after weaning (both parental and filial samples), sexual maturity, and during

young adulthood were centrifuged under refrigeration to separate out the plasma. This plasma was aliquoted (>200 µL per aliquot) and stored under nitrogen at −80°C for general hormone analysis. Serum was also similarly prepared and stored from the blood sampled without anticoagulant from all females, with the exception of the PND 21 pups, for the measurement of estradiol levels.

Steroid hormones (androstenedione, testosterone, progesterone and corticosterone) were measured in plasma by a proprietary online (solid phase extraction LC–MS/MS; Yamada et al. 2002; Zhang et al. 2011). Absolute quantification was performed by means of stable isotope-labeled standards.

Estradiol concentration in serum samples was determined using a commercially available ELISA kit from DRG Diagnostics (EIA-4399; Marburg, Germany) measured on a Sunrise MTP-reader (Tecan AG, Maennedorf, Switzerland) and evaluated by the Magellan Software of the instrument producer. Estradiol was not measured in subset 1 females or in males at any age, as estradiol concentrations in these animals are known to be below the technical lower limit of quantitation for this determination.

Testosterone in testes of male pups at gestation day 20 after ex vivo incubation

This investigation was solely conducted in the mixture experiment, because it is described as more sensitive method to measure endocrine effects (Borch et al. 2006). No equivalent data are available from the single-substance experiments, and can thus not be used for doing the combined toxicity assessment. Left and right fetal testes of all male fetuses of five dams per group were weighed and immediately incubated separately, each in 500 µL DMEM/F12 medium (without phenol red addition; with HEPES, 0.1 g/L gentamicin and 0.1% fetal bovine serum addition) for 5 h in a humidified incubator with 5%CO_2 enriched atmosphere at 37 °C on a horizontal rotator (150 rpm). The supernatant was stored at −80 °C until measurement.

Testosterone was measured after 1:4 dilution with DMEM/F12 medium with a Testosterone ELISA (DRG, cat no. EIA-1559) on a Sunrise MTP-reader (Tecan AG, Maennedorf, Switzerland) and evaluated with the Magellan Software of the instrument producer. The lowest quantifiable testosterone value is 1.2 nmol/L.

Tissue preparation and histopathological analysis

At all time points, the organs of dams and offspring were carefully trimmed of excess adhering fat and tissue and were weighed (fresh, unfixed). Adrenal glands, brain, cauda epididymis, epididymides, kidneys, liver, ovaries, spleen, testes and uterus were weighed without blotting to the nearest 0.001 g. Any other tissues (musc. levator ani together with musc. bulbocavernosus, Cowper's gland, glans penis, pituitary gland, prostate [total, ventral only], seminal vesicles with coagulating glands, thyroid glands) were weighed without blotting to the nearest 0.1 mg. The anesthetized animals were weighed to the nearest 0.1 g. Immediately after weighing, the ventral prostate was cut longitudinally in two halves (males of subsets 1 and 2). One of these halves, as well as the right testis and the right seminal vesicle, was snap frozen in liquid nitrogen and stored at −80°C for future analyses. In male animals of subset 3, both the ventral prostate and the right testis were halved after weighing. Half of each tissue plus the right seminal vesicle were snap frozen for possible future transcriptome analyses. In subset 3 males, sperm analysis was performed with the residual half of the right testis and the complete right epididymis: cauda epididymis sperm motility according to the method in Slott et al. (1991), sperm morphology, spermatid head count in the testes, and sperm head count in the cauda epididymis (Feuston et al. 1989, slightly modified).

All weighed tissues were preserved (except of glans penis, Cowper's gland, musc. levator ani together with musc. bulbocavernosus) and fixed in 10% neutral-buffered formalin or modified Davidson's solution (ovaries, left epididymis, and left testis). In addition to the organs mentioned above, oviducts and male/female mammary glands were sampled. Light-microscopical assessment was performed in all male offspring of all three subsets (all gross lesions, adrenal glands, left coagulating gland, left epididymis, pituitary gland, left prostate, left testis, left seminal vesicle). Therefore, the fixed tissues were trimmed, paraplast-embedded, cut with a thickness of 2–3 µm, mounted on glass slides and stained with routine hematoxylin–eosin stain. All work was done according to published literature ("Revised guides for organ sampling and trimming in rats and mice" (Ruehl-Fehlert et al. 2003; Kittel et al. 2004; Morawietz et al. 2004; Creasy et al. 2012) and assessment was performed by a board certified veterinary pathologist (DECVP) followed by an internal peer review.

Statistical analysis

Statistical analysis for the assessment of measured parameters

Means and standard deviations were calculated. In addition, the following statistical analyses were carried out.

Statistical analysis for the assessment of potential additivity of effects

For selected parameters (where applicable) the Loewe additivity model (Loewe 1953) was used for compound

combination evaluations to examine whether more than additive effects of the test substances at LOAEL concentrations occurred. For a combination of k ($k \geq 2$) compounds, the interaction index τ based on Loewe additivity model can be expressed as

$$\tau = \frac{d_1}{D_{y,1}} + \cdots + \frac{d_k}{D_{y,k}} \begin{cases} < 1, & \textit{synergy} \\ = 1, & \textit{additivity} \\ > 1, & \textit{antagonism} \end{cases},$$

where $d_1,\ldots d_k$ are doses of each compound in the mixture of k compounds resulting in effect y and $D_{y,1},\ldots D_{y,k}$, are the doses of compounds that result in the same effect y for each respective compound given alone. A confidence interval for the interaction index τ was constructed to account for variabilities in estimating dose-effect models. The combination dose is synergistic if the upper limit of the confidence interval is less than 1, antagonistic if the lower limit of the confidence interval is greater than 1, and additive if the confidence interval embraces the number 1. For the effect y observed in the mixture experiment, the corresponding doses of each single compound resulting in the same effect y can be estimated using inverse regression model. For AGI and day of preputial separation, a normalization procedure taking into account the concurrent study control data was performed. Specifically, the individual parameter value was divided by the mean for the corresponding control.

Dose–response modeling and calculation of interaction index based on Loewe additivity model The dose–response relationship is fitted using a non-linear regression model. The selection of regression model depends on the quality of a response. Modeling the probability of a binary response as a function of dose can be use a logistic regression. Gamma regression is suitable for modeling non-positive data and Poisson regression can be used to relate count responses to predictor.

Gamma regression was used to fit the relationship between dose and the weight of ventral prostate. The corresponding dose–response curve fits for the individual chemicals and mixture were displayed in Supplementary Figure 31. The horizontal lines indicate the mean weight of ventral prostate observed in the mixture experiment. The vertical lines indicate the estimated doses of each individual chemical resulting in the same mean weight of ventral prostate using the inverse regression model. The dose values (d) used in mixture experiment and the estimated dose values (D) of individual chemicals were substituted into the formula $\tau = d_1/D_1 + d_2/D_2 + d_3/D_3$ used for calculation of interaction index based on Loewe additivity model. The calculation results are shown in Supplementary Table 38.

Results

All concentration control analyses showed that the achieved substance concentrations were within acceptable limits, prepared dosing formulations were stable and homogeneous. Contaminants in the feed or water or changes in the environmental conditions, which might have influenced the outcome of the studies, were not observed (data not shown).

Gestation and littering

Despite a few idiopathic cases divided evenly among the dose groups, parental female fertility and mortality were unaffected by treatment; nearly all dams in this study were pregnant and survived until scheduled termination. A few clinical signs were observed in the dams after receiving either the LOAEL or NOAEL mixes or the LOAEL dose of prochloraz as a single-substance. These findings after treatment were minor and limited to piloerection, indicating stress, and salivation, likely a result of an unpleasant taste of the test substance or by local irritation of the upper digestive tract. Neither is considered to be a sign of systemic toxicity. Food consumption and body weight parameters were comparable to the concurrent control groups and within historical control ranges throughout the entire dosing period.

However, some alterations to reproductive performance were observed (Table 3). Statistically significantly, lengthened gestation was observed as a result of treatment with the LOAEL mixes. An increased duration of gestation was also seen for the NOAEL mixes but the value lies within the historical control range for the test facility (21.5–22.5 days) and is therefore not considered to be biologically relevant. Increased gestational length was also observed after treatment with the LOAEL of prochloraz, but not flutamide or vinclozolin, suggesting that this effect of the LOAEL mixture is specific to prochloraz.

Treatment with the LOAEL-MIX decreased the number of live-born and increased the number of stillborn pups across a number of the litters of these dose groups. A similar change was observed for the LOAEL dose of prochloraz (Melching-Kollmuss et al. 2017). However, in the current study, litter size and offspring weights remained unaffected. A statistically significant increase in pup death was observed in the first four post-natal days in animals exposed to the LOAEL of prochloraz, which, when combined with the statistically significant increase in cannibalization, contributed to reduced pup viability in this group. Therefore, it is not unlikely that prochloraz contributed to this observation. The sex ratio was unaffected in this study and in the single compound studies.

Pup mortality was increased in the NOAEL-MIX group, but as all dead pups and four of the six cannibalized pups were from the same litter, this effect is assessed as not related

Table 3 Summary of findings for FLT at positive control dose

Substance	Dose	Findings
FLT	2.5 mg/kg bw/d	Parental females No test substance-related adverse findings Pre-weaning Increased incidence of nipple/areolae in male pups on PND 12 (49% above control) and PND 20 (76% above control) Decreased anogenital distance in male pups (21% below control) and both sexes combined (20% below control) Decreased anogenital index in male pups (21% below control) and both sexes combined (18% below control) Increased body weight at criterion of preputial separation (33% above control) Weaning Weight decrease of cauda epididymis, epididymides, musc. bulb. lev. ani, total prostate and ventral prostate Puberty Delay of preputial separation (about 10 days beyond the historical control range) Increased testosterone and androstenedione values in males Weight decrease of total/ventral prostate Hypospadias in one male animal Juveno-adult transition in epididymides of all males Young adulthood Increased androstenedione levels in females around PND 83 in proestrus Weight decrease of bulbourethral gland, cauda epididymis, epididymides, glans penis, musc. bulb. lev. ani, total and ventral prostate as well as seminal vesicles Size reduction of testes, epididymides, prostate and seminal vesicles in one animal Size reduction of seminal vesicles with decreased secretion in 9/10 males Size reduction of prostate with decreased secretion in 5/10 males Unilateral minimal multifocal testicular tubular degeneration in 1/10 male Unilateral severe diffuse testicular tubular degeneration with Leydig cell hyperplasia in 1/10 male

to the test substance administration. No other maternal findings or effects on reproductive performance were observed.

Offspring development

Anogenital distance and index

As pup development was also monitored post-partum, a number of test-substance-related clinical findings were observed in the developing F1 offspring. On the day after birth, a small, but statistically significant increase in the anogenital distance (AGD) of female pups, as well as a statistically insignificant decrease in that of the male pups, were both observed in offspring exposed to the LOAEL-MIX (Fig. 2 and Supplementary Table 3). This increase in female AGD mirrored that observed because of the single-substance exposure to the LOAEL of prochloraz, but not the other substances; thus it is assumed that this effect of the mixture is specific to prochloraz. Non-statistically significantly, decreased male AGD were observed at the LOAEL doses of vinclozolin and flutamide, but not with prochloraz. No effects on the AGD were observed at the NOAEL or ADI dose levels.

These results are largely in concordance with those of the calculated anogenital index (AGI), a parameter where the weight of the animal is also taken into account, except that the statistical significance was reversed. In this case, the increase in the AGI of female pups was statistically insignificant after LOAEL-MIX exposure, while the decrease in the AGI of male pups with the LOAEL mixture was significant (Fig. 3 and Supplementary Table 3). The AGI in male pups was decreased in the LOAEL doses of flutamide and vinclozolin, but not in the prochloraz group and did not reach statistical significance. The AGI in female pups was increased at the prochloraz LOAEL dose (see Melching-Kollmuss et al. 2017).

No effects on the male or female AGI were observed at the NOAEL-MIX or ADI-MIX dose levels. When the Loewe additivity model was applied, the effects on male AGI of the LOAEL mixture group were more than expected assuming additivity (see boxplot in Supplementary Figure 28). It should be noted, however, that this was solely contributable to the absence of an effect on AGI in the prochloraz-treated male animals. Model calculations for vinclozolin and flutamide demonstrated approximate additivity of effects. Using the dose–response relationship for flutamide (including the positive control dose of 2.5 mg/kg bw/d) it can be calculated that the effect of the LOAEL mixture group on AGI is equivalent to a flutamide dose of 1.08 mg/kg bw/d.

Areolas/nipples

While male rats are born with mammary areolae like their female siblings, shortly after birth they undergo a postnatal increase in androgen levels, sometimes termed ‘mini puberty’, which has been shown to influence male external

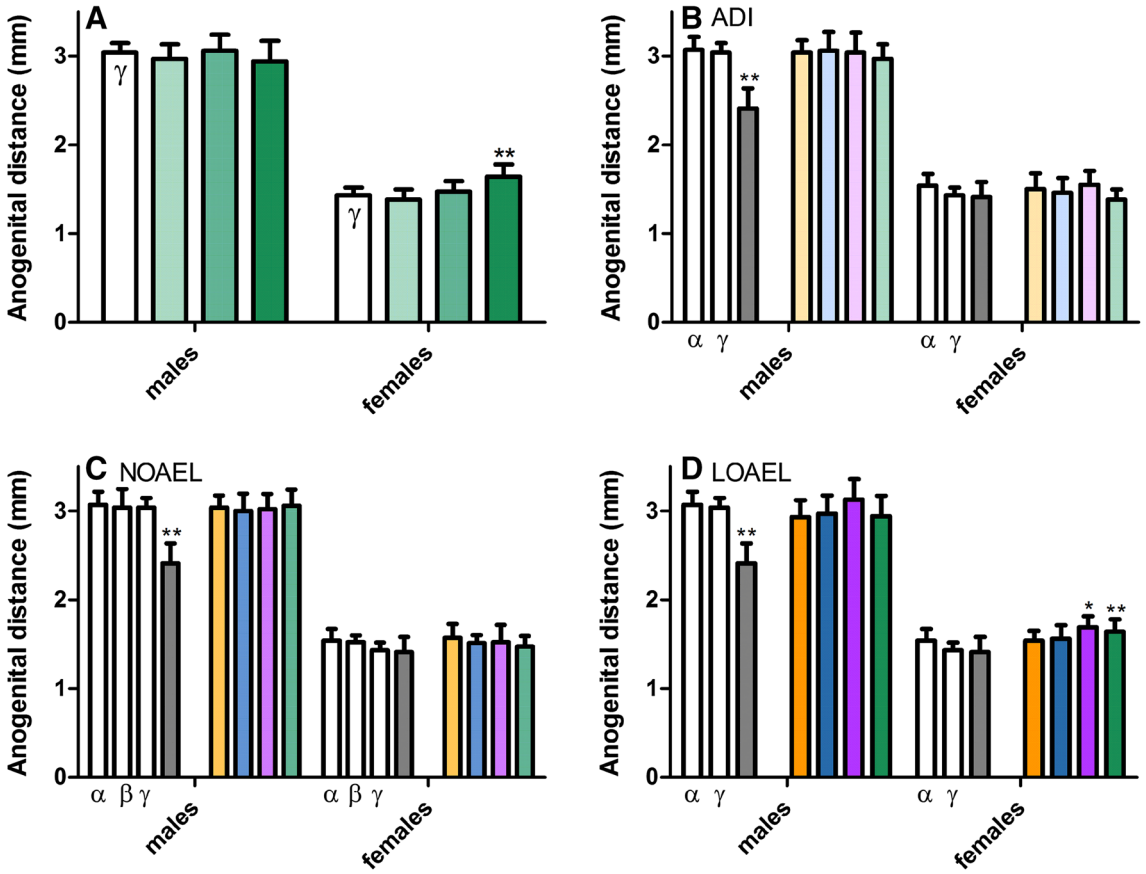


Fig. 2 Effect of increasing dose of mixtures of vinclozolin, flutamide, and prochloraz on anogenital distance. On PND 1, the anogenital distance (AGD) of all live-born pups exposed in utero to the vehicle control *open rectangle*, ADI-MIX *filled very light green rectangle*, NOAEL-MIX *filled light green rectangle* and LOAEL-MIX *filled thick green rectangle* was measured (**a**). These mixture results were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle*, and prochloraz *filled pink rectangle* at each dose level: ADI (**b**), NOAEL (**c**) and LOAEL (**d**), as well as vehicle *open rectangle* and positive controls *filled grey rectangle*. (Data are shown as mean ± SD.) Prenatal exposure to LOAEL dose levels of these anti-androgens, but not ADI or NOAEL levels, significantly altered AGDs. These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent controls relating to the exposures at each dose level are depicted in each graph. A comparison of the three control datasets can be found in **a** of Supplementary Figure 1 (color figure online)

development, including triggering areola regression (Kratochwil 1971; Kratochwil and Schwartz 1976; Foster and McIntyre 2002). Peak regression occurs around PND 12–14; therefore, significant retention of the areolae beyond this age may be an external indication of disturbances in androgen signaling. All male pups exposed to the LOAEL-MIX had retained at least one areola or hairless spot along the milk line until PND 12; this was consistent with the areola retention observed after single-substance LOAEL exposures (Fig. 4 and Supplementary Table 3). The numbers of areolae or hairless spots were also substantially increased on PND 12 in the male pups of the single compound LOAEL dose groups (Fig. 5 and Supplementary Table 3), compared to the controls. In the single-substance exposure groups pups retained 3–4 of the possible 12 areolae (compared to approximately 2 in the control animals), whereas in the LOAEL-MIX males had on average 6–8 areolae remaining on PND 12. These data reveal that areola regression occurred in all LOAEL groups, but was less in the LOAEL-MIX group indicating a contribution of multiple compounds, rather than a single dominating compound. Due to the nature of the data, the Loewe model could not be used to assess the quantitative nature of the interaction (i.e. additive, less than additive or more than additive).

Increased nipple retention was not observed after exposure to the NOAEL or ADI mixes. The incidence of males with at least one nipple/areola remaining was slightly elevated in the NOAEL prochloraz group, but the number of nipple/areolae retained is similar to the other NOAEL exposures, as well as the range provided by the three concurrent control groups. Moreover, as no increase in nipple parameters was noted with the current NOAEL-MIX treatment this observation is not considered to be biologically relevant (could be a consequence of the lower variation in the

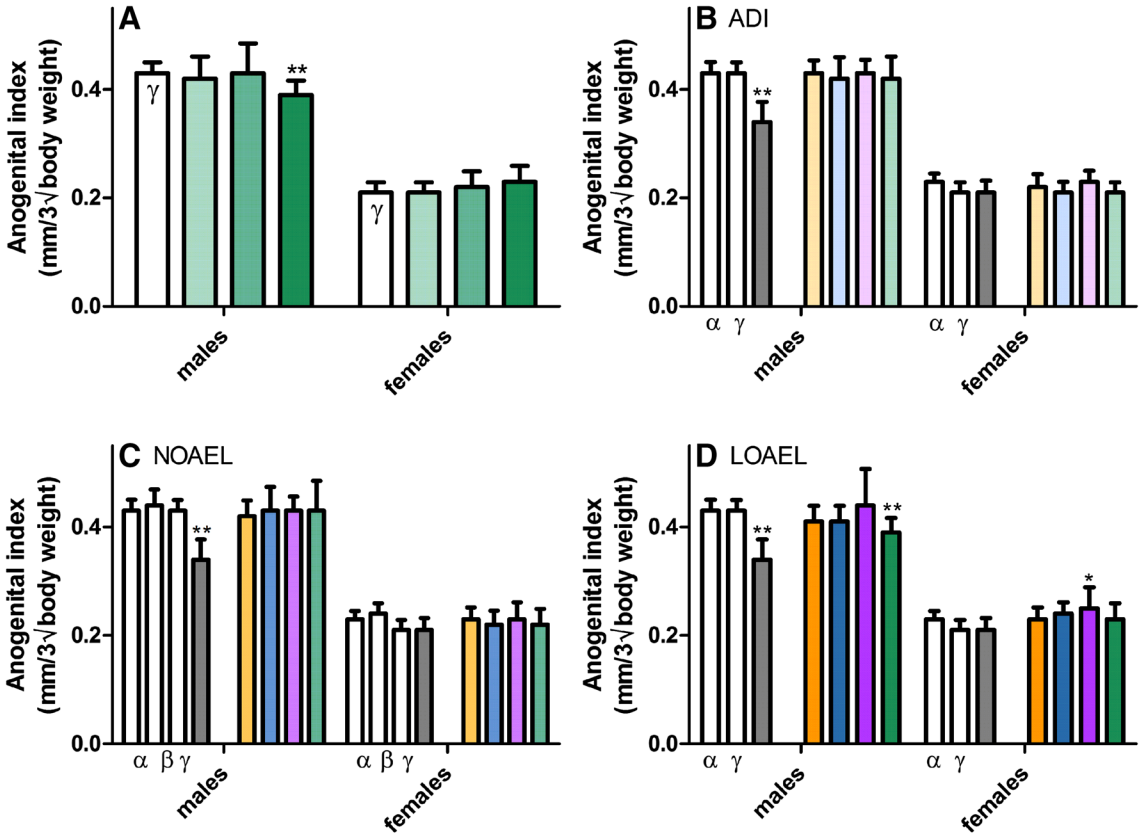


Fig. 3 Effect of increasing dose of mixtures of vinclozolin, flutamide, and prochloraz on the anogenital index. The anogenital index (AGI), a parameter which accounts for any differences in animal size, was calculated from the AGDs of all live-born pups exposed in utero to the vehicle control *open rectangle*, ADI-MIX *filled very light green rectangle*, NOAEL-MIX *filled light green rectangle* and LOAEL-MIX *filled thick green rectangle* (**a**). These mixture results were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle*, and prochloraz *filled pink rectangle* at each dose level: ADI (**b**), NOAEL (**c**) and LOAEL (**d**), as well as vehicle *open rectangle* and positive controls *filled grey rectangle*. (Data are shown as mean ± SD.) Prenatal exposure to LOAEL dose levels of these anti-androgens, but not ADI or NOAEL levels, significantly altered calculated AGIs. These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent controls relating to the exposures at each dose level are depicted in each graph. A comparison of the three control datasets can be found in **b** of Supplementary Figure 1 (color figure online)

concurrent control alpha, rather than a true anti-androgenic effect of prochloraz treatment at this dose).

Owing to the high background incidence of areola retention on PND 12, we habitually reexamine all male pups for residual areolae on PND 20, by which age the process of regression should be completed. Indeed, no males in the vehicle control, ADI-MIX, NOAEL-MIX, or any of the single-substance exposure groups still had areolae. However, around half the males of the LOAEL-MIX exposure group still had at least a single areola, but the number of areolae retained had reduced dramatically to an average of one per individual. Overall, these data indicate that the areolae did recede substantially by PND 20 and that much of the areola retention is a transient effect. For the individual compounds, areola/nipples were not observed at LOAEL LEVEL at PND 20.

To follow-up on these observations, the male offspring of this exposure group (as well as their corresponding concurrent controls) selected for Subsets 2 and 3 (all other offspring having been killed on PND 21) was re-examined for areolae on PND 38 and again on the day of sexual maturation. No change in the incidences of areola retention or the areola counts were observed at either time point in these animals. These data suggest that the areolae retained until PND 20 are permanent. Thus, the LOAEL mixture of vinclozolin, flutamide, and prochloraz was able to permanently alter male areola regression, while only transient effects observed after single-substance exposures. Otherwise, permanent nipples were only observed in the positive control group (i.e. flutamide dosed at 2.5 mg/kg bw/d). Therefore, the lack of disappearance of the areolae in the LOAEL-MIX treatment group indicates a combination effect of the substances when these are mixed. The nature of the data (no areolae in all

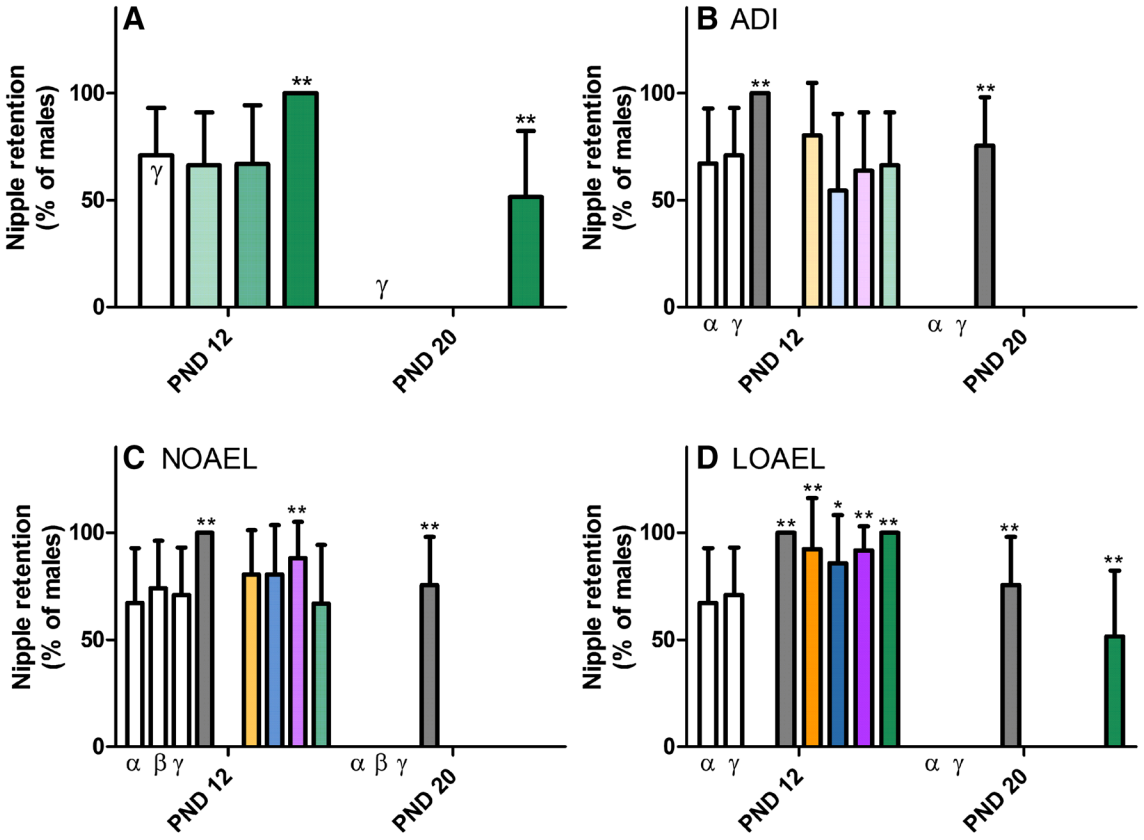


Fig. 4 Effect of increasing single-substance and mixed anti-androgen exposures on the incidence of male pups with nipples or areolae. On PND 12, the number of male pups exposed to the vehicle control *open rectangle*, ADI-MIX *filled very light green rectangle*, NOAEL-MIX *filled light green rectangle* and LOAEL-MIX *filled thick green rectangle*, which had retained nipples or areolae was counted. These animals were then recounted on PND 20 (**a**). These mixture results were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle* and prochloraz *filled pink rectangle* at each dose level: ADI (**b**), NOAEL (**c**) and LOAEL (**d**) as well as vehicle *open rectangle* and positive controls *filled grey rectangle*. (Data are shown as litter mean ± SD.) Despite the relatively high background rate of areola retention in control animals, prenatal and/or lactational exposure to the LOAEL-MIX, but not the ADI or NOAEL mixes, statistically significantly increased the number of male pups with nipple/areola retention on PND 12. This effect was somewhat transient; by PND 20, only the positive control and LOAEL-MIX dose groups had males with nipples/areolae. Exposure to the LOAEL-MIX of these anti-androgens, but not the single-substance exposures, significantly increased areola retention in male pups past PND 20. These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent controls relating to the exposures at each dose level are depicted in each graph. A comparison of the three control datasets can be found in **c** of Supplementary Figure 1 (color figure online)

single-substance exposure groups and two mixture groups), unfortunately, does not allow for the determination on the type of mixture toxicity.

Sexual maturation

Each pup, which was selected to become a part of subset 2 or 3 was also evaluated for commencement of sexual maturity, as indicated by vaginal opening in the females and balanopreputial separation in the males. No effect on the age at female vaginal opening was observed in any dose group, nor were any delays in preputial separation noted as a consequence of NOAEL- or ADI-level treatments. However, a statistically significant delay in the onset of preputial separation was observed because of LOAEL-MIX treatment (Fig. 6 and Supplementary Table 3); the mean age at male sexual maturation was nearly 10 days beyond the historical control range and about 9 days beyond the statistically insignificant delays resulting from the single-substance LOAEL exposures.

Using the Loewe additivity model to assess the quantitative nature of the interaction, with data being normalized to the concurrent study controls, the obtained value of 0.772 (95% confidence interval being 0.569–0.976) indicates a slightly more than additive effect. The scatter plot in Supplementary Figure 29 exhibits that the effect of LOAEL-MIX treatment approaches the effect of positive control dose of flutamide. Using the dose–response relationship for flutamide (including the positive control dose of 2.5 mg/kg bw/d it can be calculated that the effect of the mixture group on

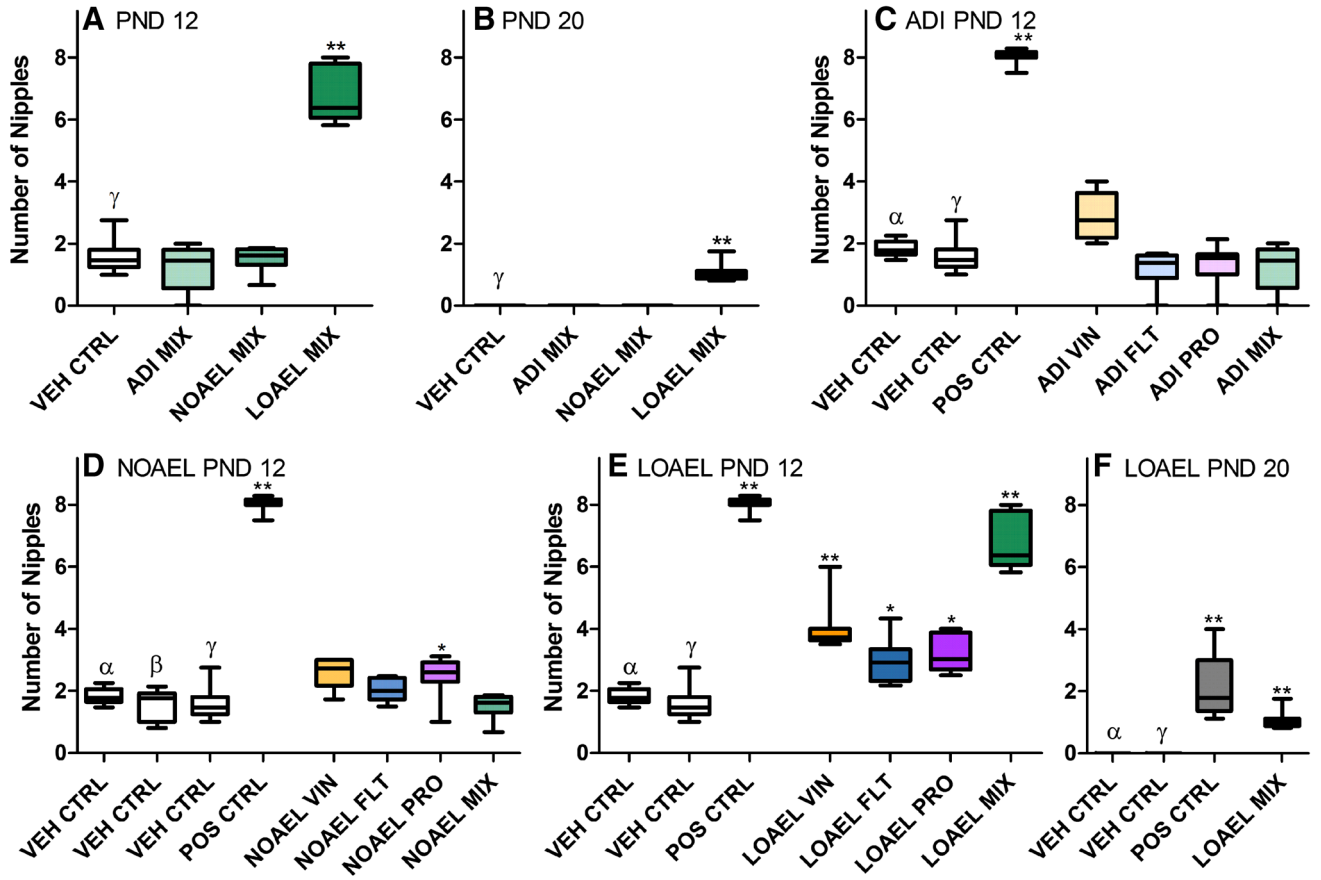


Fig. 5 Effect of increasing dose of mixtures of vinclozolin, flutamide, and prochloraz on the number of nipples/areolae on the male pups. On PND 12, the number of remaining nipples or areolae on each male pup exposed to the vehicle control *open rectangle*, ADI-MIX *filled very light green rectangle*, NOAEL-MIX *filled light green rectangle* and LOAEL-MIX *filled thick green rectangle* were counted (**a**). These animals were then recounted on PND 20 (**b**). The mixture results on PND 12 were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle* and prochloraz *filled pink rectangle* at each dose level: ADI (**c**), NOAEL (**d**) and LOAEL (**e**), as well as vehicle *open rectangle* and positive controls *filled grey rectangle*. (Data are presented as *boxplots* indicating the median [*bar*], interquartile range [*box*] and range [*whiskers*].) Prenatal and/or lactational exposure to the LOAEL-MIX, but not the ADI or NOAEL mixes, statistically significantly increased the number of nipples or areolae in male pups on PND 12. By PND 20 (**f**), most of these had completely receded. Only the positive control and LOAEL-MIX males still had nipples/areolae, but the number remaining was significantly reduced. Nonetheless, no retained areolae were observed in the single-substance exposure groups on PND 20, thus this observation represents an important difference between the LOAEL-MIX and its anti-androgen components. These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent controls relating to the exposures at each dose level are depicted in each graph. A comparison of the three control datasets can be found in **d** of Supplementary Figure 1 (color figure online)

the onset of preputial separation is equivalent to a flutamide dose of 1.72 mg/kg bw/d.

Role of hormone disruption in anti-androgen delayed sexual maturation

While it is true that treatment-related delays in preputial separation may be indicative of an endocrine mechanism of toxicity, impaired general growth has also been shown to alter the onset of puberty (Melching-Kollmuß et al. 2014). To separate any specific substance-dependent effects on preputial separation from the non-specific substance-dependent effects on general growth, we evaluated the body weights of individual animals on the day of sexual maturation. These individual results were then compared to the mean normal body weight development of the control animals from subset 3 (PND 83 ± 2), which were chosen because they were the only ones to be raised beyond puberty. To generate this growth curve, the ages and body weights from the PND 83 controls from the three similar studies were pooled (gray diamonds, shown as mean ± standard deviation) and analyzed by least mean squares regression. Thus, by this method we are able to assess whether changes observed in the day

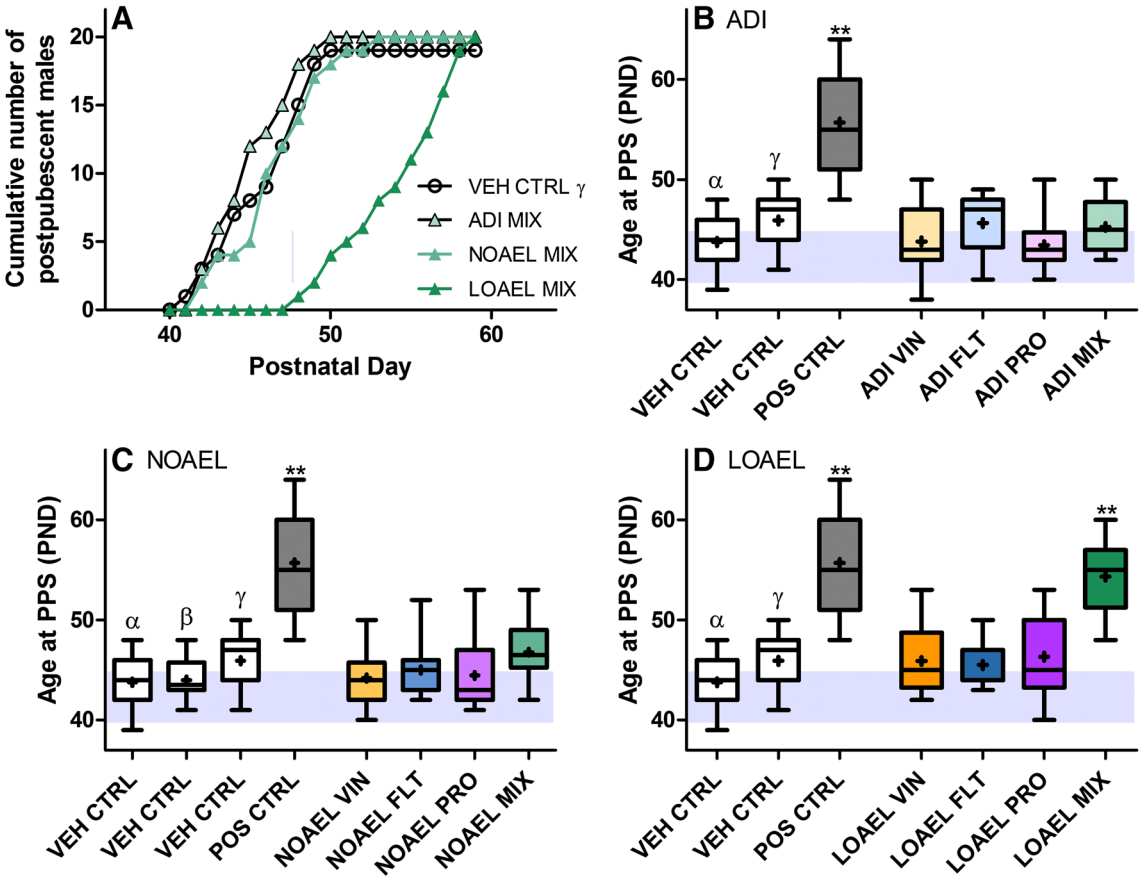


Fig. 6 Sexual maturation of male rats exposed to mixtures of vinclozolin, flutamide, and prochloraz. Twenty male offspring per exposure group were examined daily for the onset of external puberty from PND 38–64. The exact day of preputial separation was recorded for every male exposed to vehicle (*open circle*), ADI-MIX (*filled light green triangle with black border*), NOAEL-MIX (*filled light green triangle*), or LOAEL-MIX (*filled thick green triangle*); the cumulative results are presented here as a Kaplan–Meier plot (**a**). These mixture results were then compared to the effects of the single-substance exposures to vinclozolin, flutamide and prochloraz at each dose level: ADI (**b**), NOAEL (**c**) and LOAEL (**d**), as well as vehicle and positive controls. (Data are presented as *boxplots* indicating the median [*bar*], interquartile range [*box*] and range [*whiskers*].) Exposure to the LOAEL-MIX, but not the ADI or NOAEL mixes, statistically significantly delayed preputial separation in the developing offspring. Although statistically insignificant, the means (*plus sign*) of the males from the LOAEL single-substance exposures were also delayed beyond the historical control range (*shaded area*) for this rat strain. While biologically relevant, these delays were not nearly as dramatic as the delay observed when these substances were mixed. These data were collected in the course of three different experiments with similar study design. For clarity, only the concurrent controls relating to the single-substance exposures at each dose level are depicted in each graph. A comparison of the three control datasets can be found in **e** of Supplementary Figure 1 (color figure online)

of preputial separation are secondary to alterations in body weight.

Overall, the evaluation performed, as shown in Fig. 7, indicates that the males with delayed sexual maturation were heavier in proportion to their increased age, suggesting that these delays were not a secondary effect of systemic toxicity. However, a closer examination of the individuals exposed to the LOAEL dose of prochloraz reveals that these animals are slightly underweight for their ages. Thus, while much of the delay in preputial separation in this dose group is due to the anti-androgen effects of this substance, it is probable that there is also some contribution from a generally slowed development due to non-specific prochloraz toxicity, as has been discussed previously (Melching-Kollmuss et al. 2017).

Estrous cycle and sperm analyses

Estrous cycle data were generated for all young adult females during the 3 weeks prior to necropsy. These observations revealed regular cycles in the females of all test groups including the control. The mean estrous cycle duration was also similar across all test groups: 5.2 days after treatment with the vehicle, 6.3 days in the ADI-MIX, 6.0 days in the NOAEL-MIX and 6.8 days in the LOAEL-MIX groups. These data correlated very well with similar observations

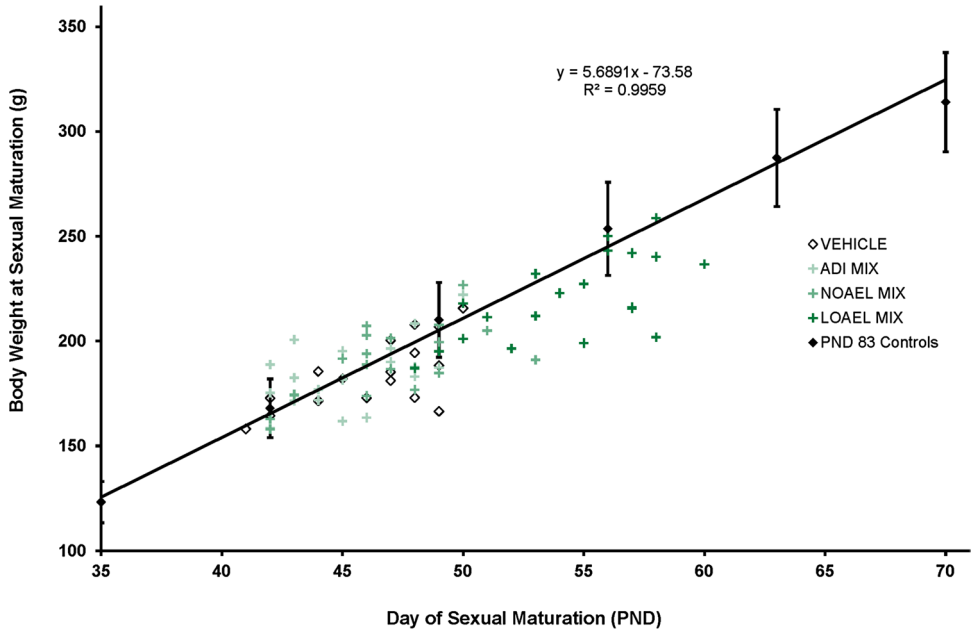


Fig. 7 Comparison of age and body weight of individual male offspring at sexual maturation (Subsets 2 and 3). The body weights (*y* axis) of individual male offspring exposed to the vehicle control (*open diamond*), positive control (*filled black diamond*), the LOAELs of vinclozolin (*filled yellow square*), flutamide (*filled blue triangle*), or prochloraz (*filled pink diamond*), or the LOAEL-MIX (*filled green plus sign*) were plotted against their corresponding ages on the day of preputial separation. These scatterplots were then compared to the normal body weight development of Subset 3 control animals (*filled grey diamond* ± SD). For clarity, only the controls or treatment groups with delayed preputial separation are shown. Overall, this plot indicates that the males with delayed sexual maturation were heavier in proportion to their increased age, suggesting that these delays were not a secondary effect of systemic toxicity. However, a closer examination of the individuals exposed to the LOAEL dose of prochloraz reveals that these animals are slightly underweight for their ages. Thus, while much of the delay in preputial separation in this dose group is due to the anti-androgen effects of this substance, it is probable that there is also some contribution from a generally slowed development due to non-specific prochloraz toxicity (color figure online)

from the single-substance exposures. Similarly, sperm and spermatids were collected from young adult (PND 83 ± 2) male offspring at killing, and were analyzed as a metric for male fertility (Tables 4, 5 and 6). The results from these analyses indicate no change in sperm parameters with mixtures treatment at any dose level. For prochloraz tested as

Table 4 Statistical analyses used in the assessment of measured parameters

Parameter	Statistical test
Food consumption, body weight and body weight change (parental animals and pups); estrous cycle length; duration of gestation; number of delivered pups per litter; developmental landmarks (days up to preputial separation or opening of the vagina); anogenital distance and index; implantation sites; post-implantation loss, weight of the fetuses, implantations, pre- and post-implantation losses, resorptions and live fetuses	DUNNETT's test (two-sided)
Number of live and dead pups and different indices (e.g., mating index, fertility index and gestation index) and number of litters with necropsy findings in pups; developmental landmarks (preputial separation or opening of the vagina), sperm morphology, incidence of males with a specific amount of abnormal sperm (cutoff value: 0.9-quantile [90%] of control groups)	FISHER's exact test
Proportion of pups with necropsy findings per litter, presence of areolas/nipples, sperm evaluation (with Bonferroni–Holms correction.	WILCOXON test (one-sided)
Weight of the anesthetized animals and absolute and relative organ weights (all organs excl. organs listed below); hormones	KRUSKAL–WALLIS and WILCOXON or Mann–Whitney *U* test (latter for hormones)
Weight parameters of ventral prostate (VP), seminal vesicles with coagulating gland (SVCG), Musc. levator ani together with Musc. bulbocavernosus (LABC), Cowpers gland (Bulbourethral gland) (COW), glans penis (GP)	DUNNETT's test (one-sided)

Table 5 Effects of single-substance and mixed exposures to anti-androgens on reproductive performance

	Remaining dams pregnant	Duration of Gest. (d)	Litters with livebirths/stillbirths/all stillbirths	Live-born/stillborn pups	Pups died/cannibalized	Males PND 0/PND 21 (%)
Vehicle control$^{\alpha}$	20	22.1	20/1/0	208/1	0/0	50.0/50.0
Vehicle control$^{\beta}$	19	21.7	19/0/0	183/0	0/0	53.0/53.0
Vehicle control$^{\gamma}$	20	22.0	20/1/0	192/1	0/0	46.9/46.9
Positive control$^{\alpha}$	20	22.0	20/0/0	202/0	3/2	44.1/43.9
VIN ADI$^{\alpha}$	19	22.0	19/0/0	191/0	0/1	52.9/52.6
VIN NOAEL$^{\alpha}$	18	22.1	18/0/0	177/0	0/2	52.0/52.0
VIN LOAEL$^{\alpha}$	19	22.1	18/1/0	182/1	0/1	45.6/45.9
FLT ADI$^{\gamma}$	19	22.1	19/2/0	172/5	1/1	48.3/48.8
FLT NOAEL$^{\beta}$	19	21.7	19/0/0	171/0	1/1	49.7/50.6
FLT LOAEL$^{\alpha}$	20	22.2	20/1/0	205/5	2/**7****	48.3/49.5
PRO ADI$^{\alpha}$	20	22.0	20/2/0	201/2	0/1	50.2/50.3
PRO NOAEL$^{\alpha}$	19	21.9	19/2/0	205/2	0/0	54.6/54.6
PRO LOAEL$^{\alpha}$	19	**22.9****	17/**10****/2	**146****/**42****	**8****/**7****	55.5/57.7
ADI-MIX$^{\gamma}$	20	21.8	20/1/0	198/1	0/0	42.9/42.9
NOAEL-MIX$^{\gamma}$	20	**22.3***	20/2/0	200/2	**7****/**6***	51.0/51.9
LOAEL-MIX$^{\gamma}$	20	**22.9****	17/**8****/2	**153****/**26****	0/3	53.6/54.0

Data are presented as mean ± SD (*N*)

* $p \leq 0.05$

** $p \leq 0.01$

$^{\alpha}$ indicates that statistical comparison was performed against concurrent control group α

$^{\beta}$ indicates that statistical comparison was performed against concurrent control group β

$^{\gamma}$ indicates that statistical comparison was performed against concurrent control group γ

a single substance, there was a borderline, but statistically significant decrease in sperm motility at the LOAEL dose. Taken these results of the mixture study into account, it is unlikely that this observation was test substance related, as previously assessed (Melching-Kollmuss et al. 2017).

Hormone analysis

The steroid hormones were graphed as boxplots for easy comparison, although due to the volume of data, only selected hormone levels in young adult offspring (PND 83 ± 2) are shown here (Figs. 8, 9). The remaining data can be found in Supplementary Figures 7–27 and Supplementary Tables 24–37.

A global assessment of the serum hormone measurements from all dose groups and developmental time points revealed a few key observations about this dataset. For the most part, the effect of continued development was clear in the sex hormone levels of both male and female offspring. Passage through puberty increased male androstenedione and testosterone levels, as well as female progesterone and estradiol levels. This trend was observed largely regardless of the dose group. In other words, the chance in hormone level over time was more profound than any treatment-related effects.

When treatment and control groups were compared at the same developmental stage, relatively few changes in the serum steroid hormone concentrations were observed. Moreover, little consistency was noted when comparing these treatment-related changes with those from same dose group at other developmental stages; and when the mixed- and single-substance exposure data were compared.

With all this in mind, we noted only a few changes in the juvenile animals, which seemed to be due to treatment. For instance, decreased progesterone levels were observed in male, but not female; NOAEL-MIX and LOAEL-MIX offspring killed on PND 21. No findings were observed in the ADI-MIX group in either gender, nor were any effects at all observed at puberty.

The clearest picture of hormonal changes was observed in adult animals. Androstenedione levels were statistically insignificantly increased and estradiol levels mildly but statistically significantly decreased in the parental female animals treated with the LOAEL-MIX. Estradiol levels were also found to be decreased in the females of the NOAEL-MIX group. When compared to the prochloraz-only results, which is known to have aromatase inhibiting properties, statistically significantly increased testosterone levels and not significantly decreased estradiol levels were seen in the

Table 6 Analysis of sperm and spermatids of male offspring (PND 83 ± 2)

Treatment	Motile sperm	Abnormal sperm morphology	Spermatid count (×10^6/g testes)	Spermatozoa count (×10^6/g cauda epididymis)
Vehicle control$^{\alpha}$	88 ± 4% (10)	3 ± 3% (10)	190 ± 31.37 (10)	566 ± 114 (10)
Vehicle control$^{\beta}$	89 ± 4% (10)	6 ± 0% (10)	132 ± 29 (10)	515 ± 103 (10)
Vehicle control$^{\gamma}$	91 ± 6% (9)	2 ± 1% (9)	199 ± 23 (9)	643 ± 100 (9)
Positive control$^{\alpha}$	**82*** ± 6% (9)	6 ± 6% (9)	186 ± 76 (10)	493 ± 185 (10)
VIN ADI$^{\alpha}$	89 ± 5% (10)	3 ± 1% (10)	192 ± 17 (10)	622 ± 101 (10)
VIN NOAEL$^{\alpha}$	87 ± 6% (10)	4 ± 2% (10)	215 ± 31 ((10)	638 ± 144 (10)
VIN LOAEL$^{\alpha}$	84 ± 6% (10)	3 ± 2% (10)	193 ± 23 (10)	544 ± 178 (10)
FLT ADI$^{\gamma}$	92 ± 6% (10)	2 ± 1% (10)	233 ± 49 (10)	677 ± 125 (10)
FLT NOAEL$^{\beta}$	90 ± 5% (10)	6 ± 0% (10)	118 ± 14 (10)	488 ± 84 (10)
FLT LOAEL$^{\alpha}$	86 ± 5% (10)	3 ± 2% (10)	188 ± 28 (10)	616 ± 138 (10)
PRO ADI$^{\alpha}$	85 ± 5% (10)	2 ± 1% (10)	191 ± 19 (10)	641 ± 156 (10)
PRO NOAEL$^{\alpha}$	86 ± 4% (10)	3 ± 2% (10)	191 ± 32 (10)	535 ± 127 (10)
PRO LOAEL$^{\alpha}$	**83**** ± 3% (10)	4 ± 3% (10)	198 ± 20 (10)	590 ± 129 (10)
ADI-MIX$^{\gamma}$	88 ± 8% (10)	2 ± 2% (10)	209 ± 32 (10)	614 ± 110 (10)
NOAEL-MIX$^{\gamma}$	92 ± 4% (10)	2 ± 1% (10)	213 ± 27 (10)	725 ± 188 (10)
LOAEL-MIX$^{\gamma}$	92 ± 6% (10)	2 ± 1% (10)	198 ± 22 (10)	731 ± 262 (10)

Data are presented as mean ± SD (*N*)

* $p \leq 0.05$

** $p \leq 0.01$

$^{\alpha}$ Indicates that statistical comparison was performed against concurrent control group α

$^{\beta}$ Indicates that statistical comparison was performed against concurrent control group β

$^{\gamma}$ Indicates that statistical comparison was performed against concurrent control group γ

prochloraz LOAEL group, while significantly increased testosterone was also seen in the prochloraz NOAEL group (Melching-Kollmuss et al. 2017). However, without any concurrent effect on reproductive parameters or other apical end points, these hormone level changes are regarded as suggestive of a start of an effect of the compound on the organisms, but not as adverse in nature, because of no functional or anatomical alteration. The hormone changes were mirrored in the subset 3 offspring which lived to early adulthood (PND 83 ± 2), where serum androstenedione concentrations were statistically insignificantly elevated in both male and female offspring treated with the LOAEL-MIX, but not the NOAEL- or ADI-mixes (Table 7). All other findings were considered spurious, as explained in the Supplementary Information.

A clear picture was also obtained by the measurement of testosterone in testes of male pups at GD 20 after ex vivo incubation. The mean testosterone values were statistically significantly decreased in the animals treated with the LOAEL-MIX, but not the NOAEL- or ADI-mixes (Fig. 8), and the variability of the data was lower than seen with serum hormone concentrations.

Pathology

Sex organ weights

After killing, a number of organs, including the reproductive organs, were removed from both the male and female animals of all dose groups and weighed. These absolute organ weights were then used to calculate organ weights relative to animal body weight (Supplementary Figures 5–6 and Supplementary Tables 4–23). A number of organ weight alterations were noted; generally though only the ones observed in the male sexual reproduction organs were considered to be related to treatment. Most were significantly altered as a result of LOAEL-MIX exposure. A variety of these organ weights were consistently reduced either on PND 21 (relative ventral prostate) or in early adulthood (PND 83 ± 2), including bulbourethral gland, cauda epididymis (absolute only), glans penis (absolute only), bulb. and lev. ani muscles, total and ventral prostates and finally the seminal vesicles. The most sensitive of these organ weights are shown as relative weights in Figs. 10, 11 and (dose response) and 12 (comparison to the single-substance exposures); all remaining organ weights are located in Supplementary Figures 5–6 and Supplementary Tables 4–23.

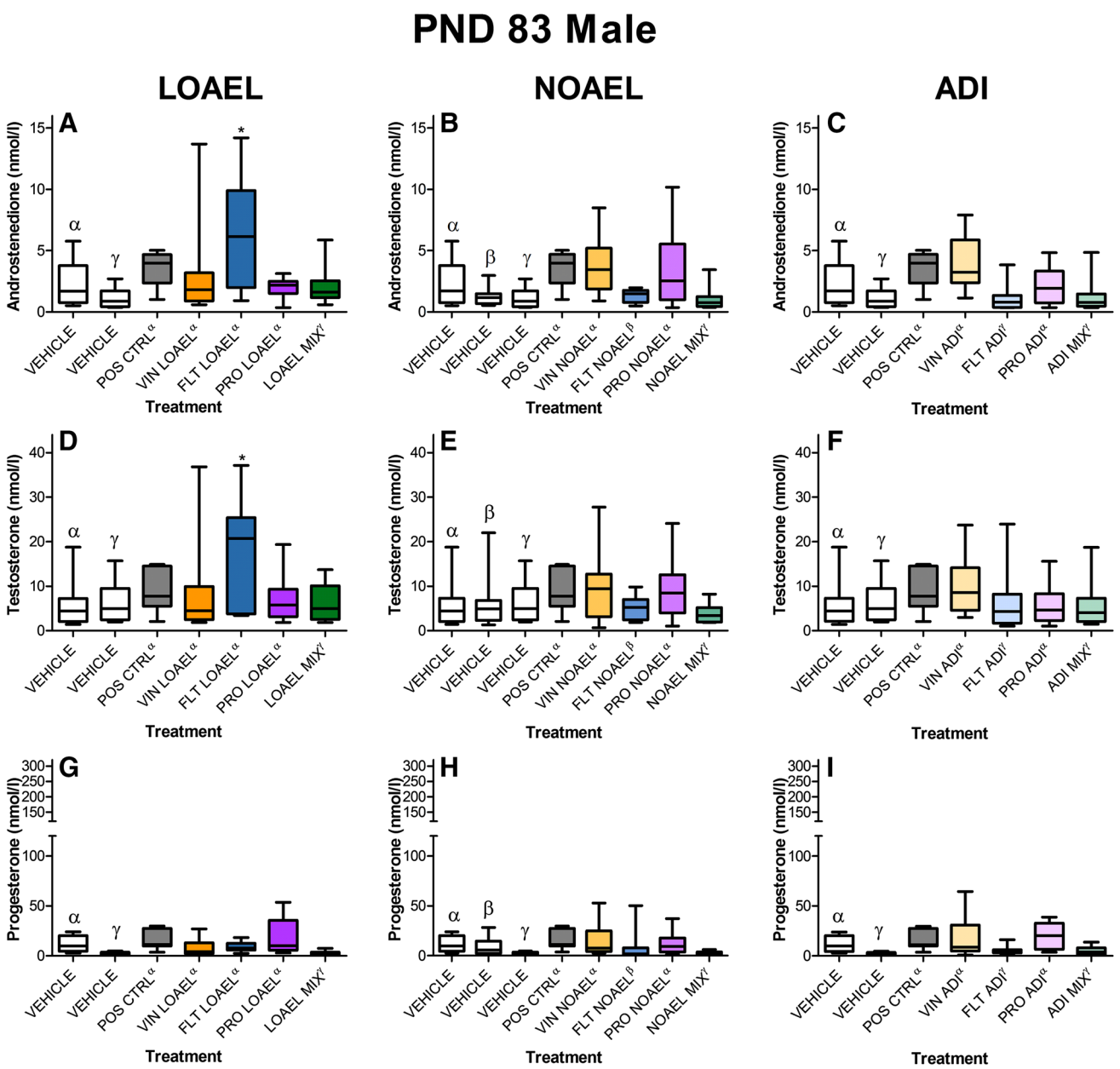
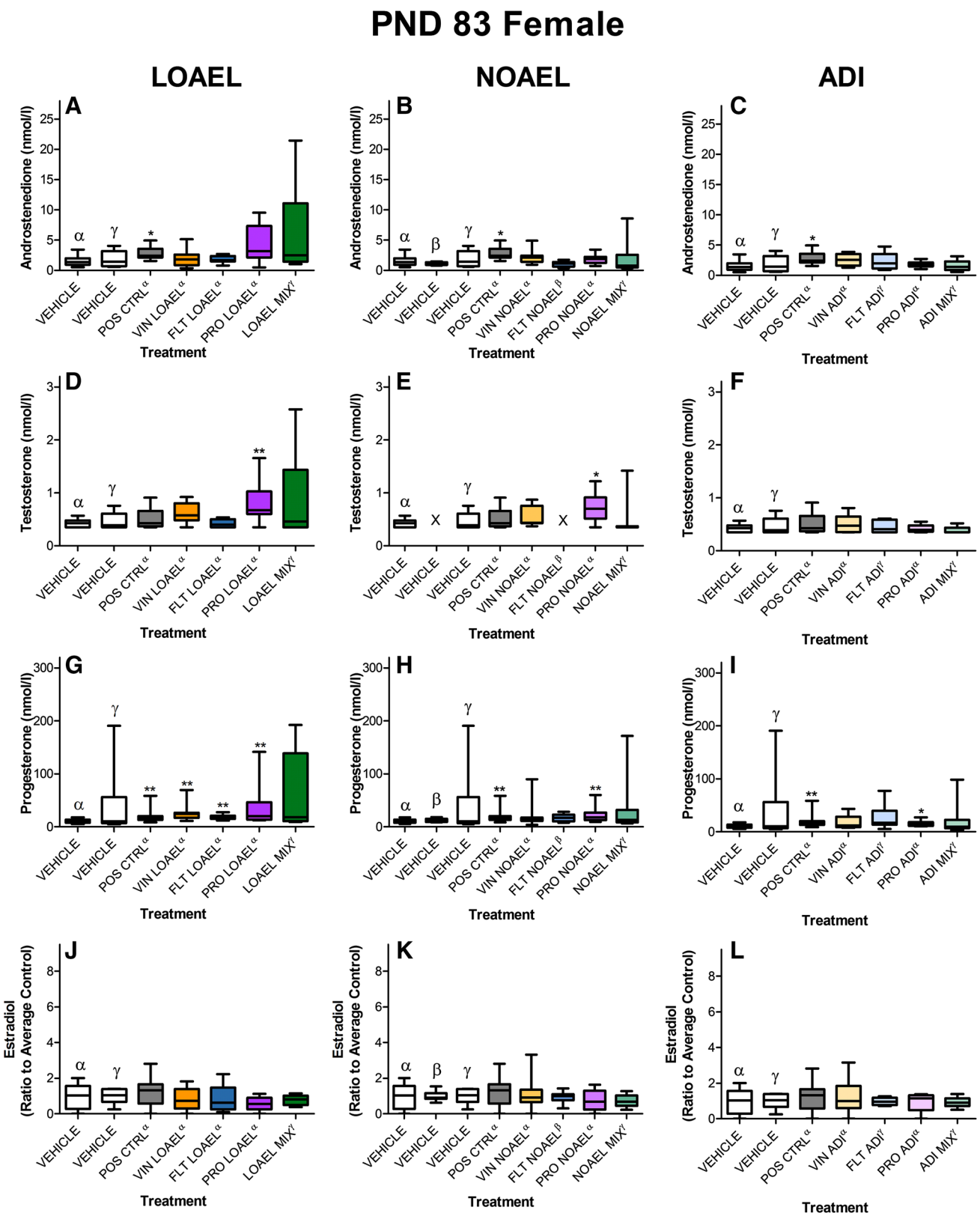


Fig. 8 Concentrations of selected steroid hormones in young adult males exposed to anti-androgens. Circulating steroid hormone concentrations were measured in the serum taken from ten male offspring on PND 83 ± 2 using LC–MS/MS. The serum concentrations of the mixture-treated animals *filled thick green rectangle* were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle* and prochloraz *filled pink rectangle* at each dose level: LOAEL (*left panels*), NOAEL (*center panels*) and ADI (*right panels*) as well as vehicle *open rectangle* and positive controls *filled grey rectangle*. Data are graphed as *boxplots* representing the median (*bar*), interquartile range (*box*) and range (*whiskers*) for each treatment group. Each graph represents a different steroid hormone and dose level. $^{\alpha,\beta,\gamma}$These data were collected in the course of three different experiments with similar study design; for the purposes of statistical testing, each treatment group was only compared to its corresponding concurrent control as indicated by the superscript. As not all dose levels were evaluated in all studies, only the concurrent controls relating to the exposures are depicted in each panel. Due to the data volume, only the most relevant sex steroids are shown here; a complete set may be found in the Supplementary Figures 7–27. A comparison of the hormone levels in control males (Supplementary Figure 2) is also available (color figure online)

The same is true of the male organ weights at puberty, although these must be appraised somewhat cautiously, as they were measured at the same developmental stage (preputial separation) rather than at the same post-partum age, which complicates the issue of biological significance. Given the pubertal delay in the males treated with the LOAEL-MIX, it is understandable that these animals would also be heavier (in proportion to their increased age), resulting in



the observed increased terminal body weight at killing when compared to the control group. Furthermore, the increased adrenal gland-, kidney-, liver- and thyroid gland weights in the LOAEL-MIX group are probably also effects of the increased animal size at killing, and therefore, also secondary to the delay in preputial separation. This is confirmed

◂**Fig. 9** Concentrations of selected steroid hormones in young adult females exposed to anti-androgens. Circulating steroid hormone concentrations were measured in the serum taken from ten female offspring on PND 83 ± 2 using LC–MS/MS. The serum concentrations of the mixture-treated animals *filled thick green rectangle* were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle* and prochloraz *filled pink rectangle* at each dose level: LOAEL (*left panels*), NOAEL (*center panels*) and ADI (*right panels*) as well as vehicle *open rectangle* and positive controls *filled grey rectangle*. Data are graphed as *boxplots* representing the median (*bar*), interquartile range (*box*) and range (*whiskers*) for each treatment group. Each graph represents a different steroid hormone and dose level. $^{\alpha,\beta,\gamma}$These data were collected in the course of three different experiments with similar study design; for the purposes of statistical testing, each treatment group was only compared to its corresponding concurrent control as indicated by the superscript. As not all dose levels were evaluated in all studies, only the concurrent controls relating to the exposures are depicted in each panel. Due to the data volume, only the most relevant sex steroids are shown here; a complete set may be found in Supplementary Figures 7–27. A comparison of the hormone levels in control females (Supplementary Figure 3) is also available (color figure online)

by the fact that the relative adrenal gland-, kidney-, liver- and thyroid gland weights remain unaltered. Thus, while the increased terminal body and absolute organ weights in this dose group are undoubtedly treatment-related effects of the LOAEL-MIX, they should be considered to be indirect effects and not indicative of organ toxicity.

In contrast, no alterations to male sex organ weights were observed in the ADI-MIX dose group. Moreover, it is unlikely that any change observed at the NOAEL-level is related to treatment. A small, but statistically significant decrease was observed in both the absolute weights of the cauda epididymis and the relative weights of bulbourethral glands in young adult male offspring (Subset 3) treated with the NOAEL-MIX. These very slight weight deviations are most likely unrelated to treatment because the absolute and relative weights of these organs are inconsistent with each other, because these organs are not thought to be the organs most sensitive to anti-androgens, and because no other organs are affected in this test group. Moreover, these parameters fell within the range formed by the means of the control animals of the other two preliminary single-substance exposure studies (0.020–0.024). Finally, the histology of epididymides was comparable between control animals and NOAEL-MIX animals, in contrast to differing morphology observed in the epididymides of those offspring treated with the LOAEL-MIX. Taken together, a treatment-related etiology for the decrease in male sex organ weights after NOAEL-MIX exposure seems unlikely.

Using the Loewe additivity model, the effects on ventral prostate weight of the LOAEL mixture group in three subsets were additive in nature, based on their 95% confidence intervals for the interaction index including 1 (see also boxplot in Supplementary Figure 30).

Gross and histopathology

All gross pathological findings occurred individually and were of the usual type for Wistar rats. The histopathological investigations of these lesions revealed no ontological cause; therefore, they were considered to be idiopathic without any relationship to treatment. The reproductive organs were also excised from the male offspring at each developmental stage, fixed, stained, and evaluated histopathologically. This assessment also included developmental staging of each tissue.

The reproductive organs of all male offspring on PND 21 (subset 1) were at an immature stage, which was common to all control and treatment groups. However, by the onset of puberty (subset 2), development of these tissues had advanced towards a juvenile stage. The seminal vesicles, coagulating glands as well as prostate (ventral and dorsolateral parts) of all control and treated animals were judged to be at a comparable juvenile stage with a moderate amount of secretion. The left testis was also at a juvenile stage with fully developed spermatogenic cycle. This corresponded to a juvenile stage, either without any sperms or only some sperms in the region of the head, in the epididymal tissues of control males and those exposed to the NOAEL and ADI mixes. In contrast, all animals treated with LOAEL-MIX were at the more advanced so-called juveno-adult transition stage characterized by many sperms in all compartments of the epididymis and a heightened epithelium.

This more advanced development was only a transitory consequence of treatment, as all male reproductive organs examined (left testis, left epididymis, seminal vesicles, coagulating glands, prostate) had developed to full maturity by early adulthood (subset 3, PND 83 ± 2). This mature stage was comparable between the animals of all treatment groups. Further examination of the prostate and seminal vesicles for signs of reduced secretion, revealed no morphological correlate in LOAEL-MIX treated males that might explain the reduced absolute and relative weights of these organs in this dose group. No other histopathological findings were observed in the male reproductive tissues. Furthermore, histopathologic examination of the adrenal and pituitary glands also revealed no findings at any time point.

Discussion

There are only very few studies available to assess whether mixtures of endocrine active substances alter sexual development at dose levels well below single-substance activity levels, or as mixtures thereof. To address this concern, we performed a series of side-by side pre-/post-natal reproductive toxicity studies to measure the developmental toxicity of low single and mixture doses of three substances with

Table 7 Blood hormone changes in dams after weaning in proestrus and in F1 male and female pups at PND21, sexual maturity and around PND83 (in females in proestrus) in dosed rats compared to controls

	Males			Females			
	Progesterone	Androsten-edione	Testosterone	Progesterone	Androsten-edione	Testosterone	Estradiol
ADI							
Prochloraz							
F0 dams				=	=	=	=
F1 PND22 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	=	=
Flutamide							
F0 dams				=	=	=	=
F1 PND22 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	=	=
Vinclozoline							
F0 dams				=	=	=	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	=	=
Mixture							
F0 dams				=	=	=	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	=	=
NOAEL							
Prochloraz							
F0 dams				=	=	↑	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	↑	=
Flutamide							
F0 dams				=	=	=	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	↑	=
Vinclozoline							
F0 dams				=	=	=	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	=	=
Mixture							
F0 dams				=	=	=	↓↓
F1 PND21 pups	↓↓	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	=	=	=	=
LOAEL							
Prochloraz							
F0 dams				=	=	(↑)	(↓)
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=

Table 7 (continued)

	Males			Females			
	Progesterone	Androsten-edione	Testosterone	Progesterone	Androsten-edione	Testosterone	Estradiol
F1 PND83	=	=	=	=	(↑)	↑↑	(↓)
Flutamide							
F0 dams				=	=	=	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	↑	↑	=	=	=	=
F1 PND83	=	=	=	=	↑	↑↑	=
Vinclozoline							
F0 dams				=	=	=	=
F1 PND21 pups	=	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	=	=	↑↑	=	=	=
Mixture							
F0 dams				=	(↑)	=	↓↓
F1 PND21 pups	↓↓	=	=	=	=	nm	nm
F1 sexual maturity	=	=	=	=	=	=	=
F1 PND83	=	(↑)	=	=	(↑)	=	=

Dose groups were established with one compound only, or with a mixture of the mentioned three compounds at levels of the ADI (prochloraz 0.01, flutamide 0.25, vinclozolin 0.005 mg/kg bw/d), NOAEL (prochloraz 5, flutamide 0.25, vinclozolin 4 mg/kg bw/d and LOAEL (prochloraz 30, flutamide 2.5, vinclozolin 20 mg/kg bw/d). Hormone levels were measured in about 20 dams per groups and in about 10 F1 pups per group and sex. Only dose-dependent hormone changes beyond the historical control ranges were regarded. = no hormone change, (↑) or (↓) relevant but not statistical significant change, ↑ or ↓ weak significant change ($p < 0.05$), ↑↑ or ↓↓ significant change ($p < 0.01$), nm: not measured. Kruskal–Wallis test followed by two-sided Mann–Whitney *U* or Wilcoxon test were applied. Details are mentioned in the supplementary tables

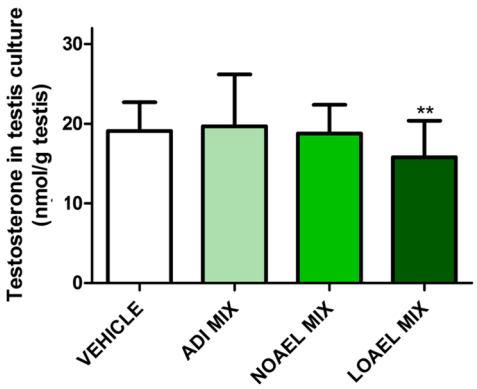


Fig. 10 Concentrations of testosterone in both testes of male fetuses at GD 20 exposed to anti-androgens (color figure online)

an anti-androgenic mode of action: vinclozolin, flutamide and prochloraz. Dose levels were selected to mimic the LOAEL as well as the NOAEL for anti-androgenic effects, and the acceptable daily intake (ADI) for each compound, which were then combined together into three mixtures of the LOAELs, NOAELs, and ADIs. Due to the complexity of the study design and the number of treatment groups in this investigation, this project has been sub-divided into a number of publications for analysis; the focus of the present publication will be the mixtures data. Therefore, as the single-substance exposures have been previously discussed in detail, they will only be discussed here insofar as they assist in the interpretation of the mixtures data (Fussell et al. 2015; Melching-Kollmuss et al. 2017; Flick et al. 2016).

Maternal and developmental toxicity

While no maternal toxicity was observed at any dose level, a number of endocrine-mediated adverse effects were observed at delivery and during development of the F1 generation after LOAEL-MIX treatment. Gestation was lengthened by almost a day, and increased numbers of stillborn pups (and litters with stillborn pups) were noted after parturition, resulting in a reduced live-birth index. A small, but statistically significantly lengthened gestation was observed as a result of NOAEL-MIX treatment, but this value lies within the historical control range of the test facility. Furthermore, no other effect on reproduction or delivery was observed at this NOAEL-MIX, nor was any similar effect on gestation observed after single-substance exposure. Thus, this effect is assessed as incidental and not a result of treatment. Absolutely no changes on reproduction and delivery were observed in the ADI-MIX dose group. Mortality was increased in the pups treated with the NOAEL-MIX, but

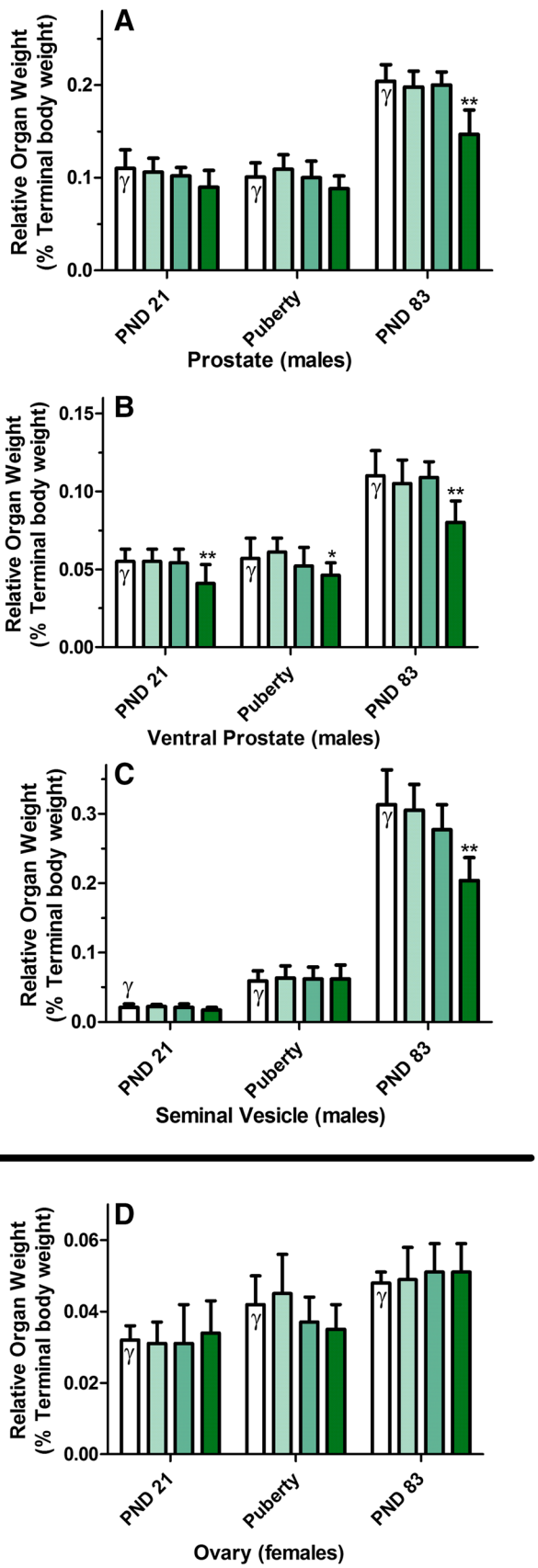


Fig. 11 Weights of selected sexual organs of rats exposed to mixtures of vinclozolin, flutamide, and prochloraz. On PND 21 (Subset 1), the day of preputial separation or vaginal opening (Puberty, Subset 2) and PND 83 ± 2 (Subset 3), the sex organs from each of ten male and ten female rats were preserved, weighed and reported as relative organ weights. These data are graphed as vehicle control *open rectangle*, ADI-MIX *filled very light green rectangle*, NOAEL-MIX *filled light green rectangle*, and LOAEL-MIX *filled thick green rectangle* at each time-point. In general, increasing anti-androgen exposures reduced male, but not female, sex organ weights dose-dependently. Three of the most sensitive male sex organs are shown (**a**–**c**) in comparison to a female sex organ (**d**) (color figure online)

all dead pups and 4 of the 6 cannibalized pups were from the same litter. This is a relatively common incidental finding in this rat strain and may occur for a number of reasons unrelated to treatment including difficult parturition and poor maternal post-partum care. No dose dependency was observed; moreover, the lower viability index in the NOAEL-MIX group was within the historical control range of the test facility. When this litter was excluded from analysis, pup mortality returned to control levels; further evidence that this is probably a spurious finding. These effects are all consistent with the previously observed single-substance effects of prochloraz at this dose level, but not of vinclozolin or flutamide, and therefore, are considered associated with aromatase inhibiting effects by prochloraz (Andersen et al. 2002).

No other effects on reproductive performance or mortality were observed as a result of exposure to either the NOAEL or ADI mixtures

Effects in female offspring

In addition, a small, statistically significant increase in the anogenital distance of female pups and a similar, but statistically insignificant increase in the anogenital index of female pups were observed in this dose group on PND 1. These findings are in line with findings in the offspring, exposed to prochloraz-only at its LOAEL dose, and therefore, are assessed to have arisen from this component of the mixture (Melching-Kollmuss et al. 2017; Laier et al. 2006). Regardless, these were the only clinical effects observed in the female offspring; no changes in either onset of puberty or the female sex organ weights were noted. All other findings occurred in the males only.

Effects in male offspring

Anogenital distance/index (AGD/AGI)

Clinical findings, typical for anti-androgenicity, were observed in the developing F1 male offspring after treatment with the LOAEL mixture. A small, but statistically significant decrease in the anogenital index as well as a statistically

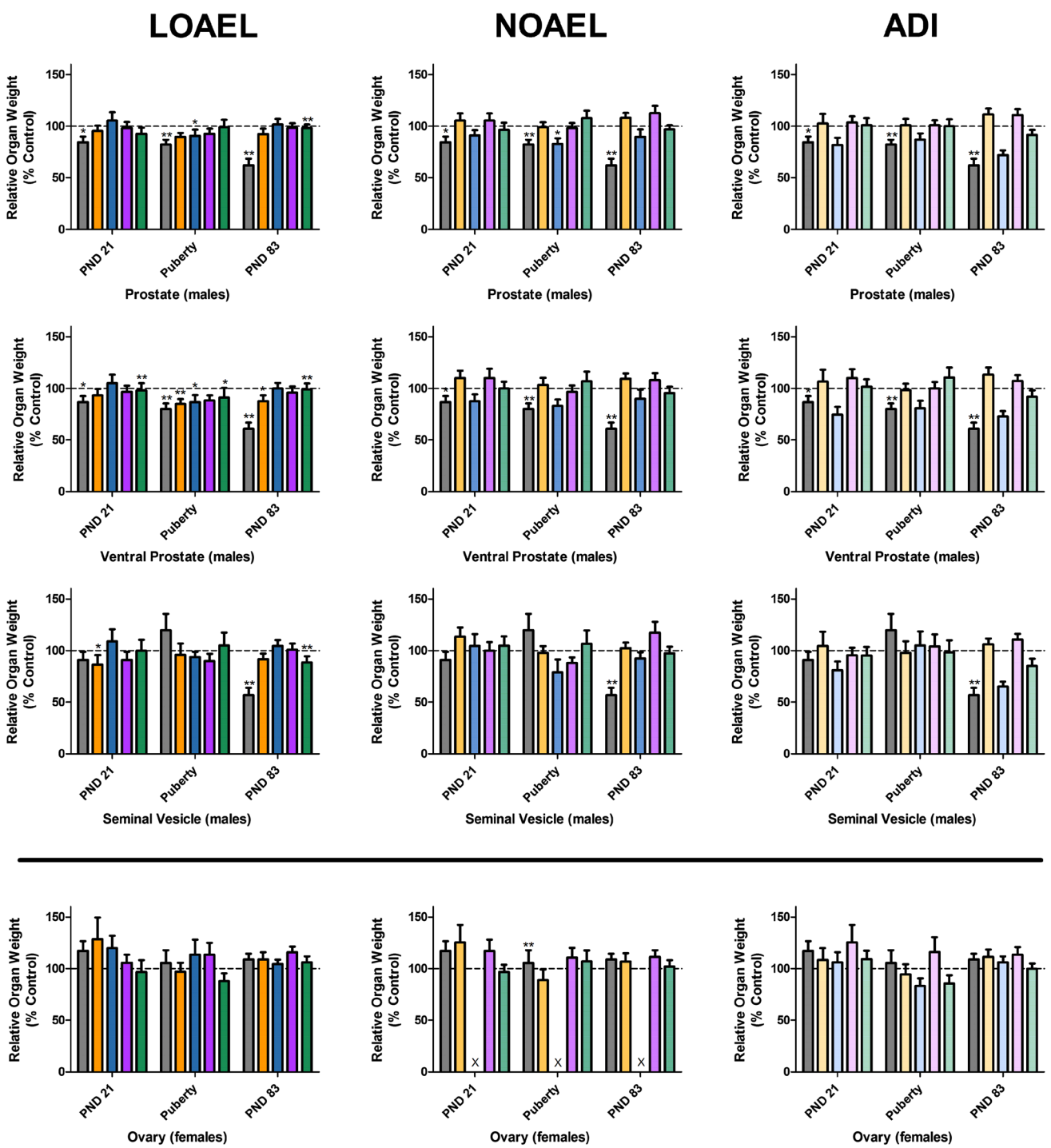


Fig. 12 Comparison of the weights of selected sexual organs of male rats exposed to anti-androgens. On PND 21 (Subset 1), the day of preputial separation or vaginal opening (Puberty, Subset 2) and PND 83 ± 2 (Subset 3), the sex organs from each of ten male and ten female rats were preserved, weighed and reported as relative organ weights. The relative organ weights of the mixture-treated animals *filled thick green rectangle* were then compared to the effects of the single-substance exposures to vinclozolin *filled yellow rectangle*, flutamide *filled blue rectangle* and prochloraz *filled pink rectangle* at each dose level: LOAEL (*left panels*), NOAEL (*center panels*) and ADI (*right panels*) as well as vehicle *open rectangle* and positive controls *filled grey rectangle* (Data are shown as mean ± SD). Generally, male sex organ weights were reduced in animals exposed to the anti-androgens at the LOAEL level, but not the NOAEL or ADI. For brevity, only selected organ weights are shown here, the remaining male sex organs can be found as part of Supplementary Figures 5 and 6. These data were collected in the course of three different experiments with similar study design. For clarity, all data have been normalized to the concurrent controls relating to the exposure. A comparison of the three control datasets for all male sex organs, as well as the female ovary weights used for comparison, can be found in Supplementary Figure 4. *X* no control data was available for normalization (color figure online)

insignificant reduction in the anogenital distance of male pups, were both observed in the LOAEL-MIX treatment group on the day after birth. Reduced anogenital distances and indices were also observed after vinclozolin and flutamide single-substance exposures (Fussell et al. 2015; Flick et al. 2016); however, these results were less pronounced and not statistically significant, when tested in single compound studies. According to the statistical evaluations using a Loewe additivity model, the degree of the effect on AGI in the LOAEL mixture was higher than what would have been expected; however, it has to be mentioned that the combination effect on the body weight development of the three compounds in the LOAEL mixture has confounded this observation. No respective effects on the male AGD, which does not take into account the body weights of the pups, in the LOAEL mixture has been seen accordingly.

Retained nipples/areola

A full 100% of male pups treated with the LOAEL-MIX had retained at least one nipple, areola or hairless spot along the milk line at PND 12. The numbers of nipples, areolae or hairless spots were also substantially increased in this dose group. While the most of the areolae had substantially receded by PND 20, they did not disappear completely in all animals and persisted until puberty, suggesting that while much of the nipple retention is transient, some of the remaining areolae were permanent. This permanent retention is unique feature of the LOAEL mixture group as single-substance administration of the LOAEL dose levels of vinclozolin, flutamide, and prochloraz were unable to produce more than a transient delay in nipple regression. Only the 2.5 mg/kg bw/d flutamide positive control, chosen because it represents a clear anti-androgen effect level, was able to achieve similar permanence (Fussell et al. 2015). When taken together with the anogenital distances/indices recorded just after birth, these data suggest that the clinical effects of LOAEL-MIX exposure were consistent with those previously observed during the single-substance exposures to the individual LOAEL dose levels of vinclozolin, flutamide, and prochloraz, but no assessment can be made on whether this represents a dose-additive or a super-additive response. Importantly, no new, qualitatively different findings were observed in any dose group of the combined exposure study.

Preputial separation (entry into male puberty)

The mean age at male sexual maturation was nearly 10 days beyond the historical control range, much longer than the delay arising from the LOAEL single-substance exposures (flutamide: 1 day, vinclozolin: 1 day, prochloraz: 1 day) and close to the positive control group flutamide (nearly 2 weeks). In addition, the comparison of the expected with the observed effects on preputial separation using the Loewe additivity model revealed a slightly more than additive effect. Moreover, this delay in the age at preputial separation corresponded to an increase in body weight commensurate with the more advanced age of the animals. Thus, while the delays in preputial separation can be indicative of both specific (endocrine disruption) and non-specific (impaired general growth) forms of developmental toxicity (Melching-Kollmuß et al. 2014), the principle cause of the delay in sexual development of the LOAEL mixture was not slower body weight development but instead a specific mode of action, one which is consistent with the expected effects of an anti-androgen. The grade of the observed statistically significantly increased delay in preputial separation in the offspring exposed to the LOAEL-MIX compared to the expected effects gives some indication for a more than additive response in the mixtures. No effects were seen for entry into male puberty in the NOAEL mixture group. No effects on female puberty were observed.

Hormone measurements

Scattered and mild, but statistically significant, serum hormone levels were noted in male and female animals in the LOAEL mixture group. Increased androstenedione concentrations at killing were noted in both male and female PND 83 ± 2 (Subset 3) offspring as well as the parental female animals. Decreased estradiol levels were observed in the serum of the dams but not their young–adult offspring. Similar hormone changes were not observed in the developing offspring on PND 21 (subset 1) or at puberty (subset 2). These findings were analogous to, although less potent than, those of the single-substance exposure to the LOAEL of prochloraz, resulting in an increase in serum testosterone and reduction in estradiol levels in both the parental females and the PND 83 ± 2 female offspring (subset 3). As prochloraz is known to act via different modes of action which include overall obstruction of steroidogenesis (possibly via Cyp17), inhibition of androgen receptor and specific inhibition of aromatase activity; the increased androgen and decreased estrogen levels in females would be consistent with a prochloraz-specific activity of the mixture due to aromatase inhibition (Melching-Kollmuss et al. 2017).

The majority of the findings are generally compatible with antagonism of the androgen receptor and/or disruption of androgen signaling and subsequent activation of the hypothalamic–pituitary–gonad (HPG) negative-feedback loop (Stocco and McPhaul 2006). This explanation is also consistent with the decreased testosterone levels in fetal testis as a result of in utero LOAEL-MIX exposure, which has also been described by Blystone et al. (2007). Although this HPG feedback loop is only established post-natally, antagonism of the androgen receptors at this developmental

stage would be expected to result in decreased, rather than increased testosterone production by Leydig cells.

Although no hormone effects were observed after single-substance treatment with the receptor antagonists at LOAEL dose levels, a similar increase in androgen concentrations was observed in male and female rat serum after administration of a higher flutamide dose (2.5 mg/kg bw/d, used as a positive control). These serum hormone findings have also been described in the literature at a variety of vinclozolin and flutamide dose levels above the LOAEL (Chandolia et al. 1991).

Reproduction organs

No treatment-related findings were observed in the organ weights of the female parental animals of any test group; however, both absolute and relative weight changes were observed in the sex organs of the male offspring exposed to the LOAEL-MIX. Decreased reproductive organ weights were observed across a number of organs and are interpreted as an indication of altered androgen signaling (see supplement). More specifically, it is noted that a dose level capable of inducing an anti-androgenic effect in one organ, generally also induces similar effects in the other male sex organs. Furthermore, as exposure continued in the offspring, the number of affected organs increased; by adulthood these included most of the accessory sex glands. Although the most logical explanation for these findings in adults would be a reduced secretion by the glands, histopathological evaluations revealed no apparent morphological correlate and no reduced secretion in the accessory sex glands.

In this study, some differences in anti-androgen sensitivity between the organs were observed. For instance, on PND 21 (subset 1) and at puberty (subset 2), the responses of the relative ventral and total prostate weights to LOAEL-MIX treatment differed, suggesting that at different time points the ventral prostate weight is more sensitive than the total prostate weight followed by the weight of seminal vesicles. Using the statistical Loewe model, dose additivity was most accurately reflecting the changes seen in ventral prostate weights of the LOAEL-MIX compared to the single compound LOAEL dose groups.

The histopathologically observed differences in the left epididymis of LOAEL-MIX treated subset 2 (at puberty) animals correlated very well with the higher age of these animals and the delayed day of preputial separation (day of necropsy) leading to a more advanced stage of developed testis and epididymis. This may indicate that the local androgen level in the testes is still sufficient for testicular maturation and spermatogenesis.

When all findings in the male offspring (reduced anogenital distance/index, areola retention, delayed puberty, increased serum androgen/decreased serum estrogen levels and reduced reproductive organ weights) are considered in a quantitative way, there is evidence for a combined toxicity effect in the LOAEL mixture group, relative to the individual compounds. The overall quantitative nature of the interaction, as analyzed using the Loewe model can be best described as additive (reproductive organ weights, anogenital distance) or slightly more than additive (preputial separation and less convincing anogenital index) (see Supplementary Figures 28–30). Additivity of parameters sensitive to anti-androgenicity would be in concordance with previously described expectations of the additive effects of a mixture of endocrine modulators, even in case of different modes of actions (Birkhoj et al. 2004; Kjaerstad et al. 2010; Rider et al. 2008a, b, 2009; Blystone et al. 2009).

The three AR antagonists (vinclozolin, flutamide and procymidone) used by Hass et al. (2007) and Metzdorff et al. (2007) also showed dose addition for anogenital distance and nipple retention. However, a combination of two phthalates, i.e. DEHP and DEHA, did not show a mixture effect (Jarfelt et al. 2005). When administered from GD 14–18 a binary phthalate and 5-component phthalate mixture showed dose addition for several anti-androgenic parameters (Howdeshell et al. 2007, 2008a, b). Essentially the same picture was drawn after administration of mixtures of phthalates (BBP, DBP, DEHP) and pesticides (vinclozolin, procymidone, linuron, and prochloraz). According conclusions drawn from these studies on mixtures of anti-androgenic compounds with differing mechanisms of action, the concept of independent action would lead to underestimation of effects. An overall reasonable agreement is seen with concept of dose addition (Rider et al. 2008a, b). An anti-androgenic mixture with different mechanism of action (DEHP, vinclozolin, prochloraz, finasteride) in a perinatal study design (dosage from GD 7-PND 16) showed for the majority of endpoints the same result, however, for a single endpoint a synergistic mode of action was postulated (Christiansen et al. 2009).

Mixture effects were not observed at the NOAEL-MIX. This means that dose levels were too low to cause effects as single-substances, and in the present study, were also not able to jointly cause a substantial effect as a mixture. In particular, no effects were noted after examination of the same developmental endpoints used to clinically assess the anti-androgenic activity of the LOAEL-MIX. No decrease in anogenital distance or index was noted in male pups exposed in utero to either the ADI-MIX or NOAEL-MIX when compared to those of concurrent controls. Nipple retention was not increased by exposure to either mixture, not even transiently. Nor were any delays in male (or female) sexual maturation noted as a result of treatment with these mixtures. Similar results were obtained for non-endocrine effects with no adverse effects seen in NOAEL mixtures of four compounds (Schmidt et al. 2016).

Relevance of historical control data and adversity of effects

Typically, these standardized experiments are designed to model the effects of a test substance in humans, necessitating the choice of an outbred animal model to more accurately imitate the amount of genetic variation in a human population. This is not without experimental trade-offs; increased genetic variation in the animal model results in a larger range of biological responses to the test substance (higher biological variation) and even recessive genetic phenotypes that can manifest as clinical signs similar to substance toxicity but having nothing to do with substance exposure.

The historical control ranges should accurately reflect the amount of phenotypic variation around the time of the study and describe the inter-study variation for each endpoint, which is often larger than the intra-study variation described by the concurrent controls. When used in concert with the concurrent controls from the study, historical control data can provide some justification for why a statistically significant observation may or may not be biologically relevant.

Thus, a lack of historical control data to reference can seriously hinder the interpretation of inconclusive data. This is often the case when novel endpoints, non-standardized methodologies and alternative time points are employed in a study design. In the course of the current investigation, we found this to be true for a few observations: a statistically significantly reduced serum estradiol concentration in the parental females and decreased absolute cauda epididymis and relative bulbourethral gland weights in PND 83 ± 2 (Subset 3) males exposed to the NOAEL-MIX. In all cases, these observations were borderline effects with uncertain interpretations.

Chiefly, the problem with the interpretation of such data is the inconsistencies inherent in such observations, particularly if outbred animal strains are being used, as it is general custom for toxicological investigations. For instance, a reduced serum estradiol concentration in dams is consistent with aromatase inhibition by prochloraz; however, no decrease in serum estradiol level, not even a statistically insignificant one, was observed after treatment with the single-substance. While this effect was observed in NOAEL-MIX dams, it was not observed in the adult female offspring of the NOAEL-MIX group; this pattern is totally dissimilar to the pattern of estrogen and androgen hormonal changes that were observed at the LOAEL level in both parental and filial adult females.

These inconsistencies are also relevant to the interpretation of the decreased absolute cauda epididymis and relative bulbourethral gland weights in PND 83 ± 2 (subset 3) males. Decreased male reproductive organ weights make sense in the context of anti-androgenic treatment; however, usually any effects in adults would be noted in both absolute and relative organ weights. Moreover, this anti-androgenic response generally is noted in a variety of sex organs and secondary glands, rather than just two. Due to the comparative scarcity of other findings in this treatment group, we assess that these borderline findings have no biological relevance.

A more likely explanation lies in the random nature of observed events. If we compare these results to the control groups from the other studies in a sort of faux historical control comparison, we find that these hormone and body weight findings lie well within the "historical control range" described by just these two control groups. Thus, these findings are probably not considered relevant to NOAEL mixture treatment. Importantly, these observations also highlight the magnitude of the inherent variation between studies, even under controlled laboratory situations. This level of variation seems to be quite high at some measured endpoints, especially the serum hormone concentrations, suggesting that some biological processes have a wider tolerance for 'normal' responses while others are tightly controlled. This variation is not necessarily reflected in the standard deviations observed within a single study, as all the animals enrolled in the study are descendants of the same outbred colony in the same animal room at the same breeder. Thus, each parameter needs to be evaluated individually, both in the context of the concurrent control and the historical dataset.

One logical reason for this variation is the diversity of developmental triggers. Some parameters are controlled by time (e.g., somite development, neural tube closure), some by general developmental factors (e.g., body weight), some by hormonal influences (e.g., male external genital development, nipple regression), but most are controlled by a combination of these factors (e.g., onset of puberty, sexual behavior). The more apical and terminal the phenotypic event, the more developmental factors are likely to be involved. Thus, each individual represents a unique, but perfectly normal, solution to the problem of gene–environment interaction, but one solution which reflects both the gene pool from which it originates and the environment.

It is reasonable then to assume that this spectrum of biological variation will result in a spectrum of responses to a test chemical for each endpoint. Furthermore, this variation of biological response will be higher at some endpoints than others. This is partly due to the number and type of factors that might be involved at that stage of development, but also because of the performance involved in making the measurement or observation. This kind of variation may be more or less relevant at certain endpoints, but becomes extremely pertinent for others. For instance, anogenital distance measurements vary substantially depending on the exact technique used. Therefore, it is important to standardize the observations as much as possible to minimize the effect of the technical variation on the outcome of the study and understand the limitations of the technique chosen, to inform the ultimate data assessment and prevent over-interpretation

of the results. The combination of method standardization and the use of historical control ranges ultimately define the limits of what is 'normal', be it derived from the technical limitations of the study design or biological limitations of the animal model. This range must be taken into account when assessing the toxicological relevance of data.

The question of adversity also needs to be addressed. It is often unclear to what extent the observed findings adversely affect animal health (Lewis et al. 2002). As Foster and McIntyre (2002) reported, rare, permanent structural defects which compromise the quality of life (e.g., reproductive tract malformations) should be considered adverse findings. But they go on to note that the relationship between statistically significant changes in endpoints considered to be indicators of impaired androgen status and such malformations is uncertain. Moreover, these indicators of impaired endocrine status are functional, rather than toxicologically induced morphologic changes. Put another way, every individual organism interacts differently with its environment to solve the endocrine homeostasis problem; even when the environmental stimuli are exactly the same, one might produce more hormonal signal, while a second might upregulate the receptor to reassert homeostasis. Such regulations generally are considered normal, adaptive, and necessary as long as they are transient and within the normal homeostatic range (Goodman et al. 2010; Rhomberg and Goodman 2012). It seems logical that the same adaptive processes, which allow humans to reassert hormonal homeostasis in a changing natural environment, might also compensate for exposure to endocrine active substances at low dose levels individually or as mixtures. As long as these processes remain truly adaptive, then they do not necessarily pose an increased hazard to humans. Therefore, it is important to determine not only whether effects are observed at human-relevant exposures, but also whether any effects observed are adverse.

Conclusion

The present experiments were designed to test for combined effects at NOAEL and human relevant low doses of substances at the same site of action, as well as the possibility of synergism between different modes of action at effect levels. Actual side-by-side testing of single compounds and the mixed combinations were performed in experiments with an appropriate experimental design including a suitable number of test subjects, appropriate dosing, apposite time-windows and duration of exposure, and all relevant endpoints (OECD 2002).

Three compounds having anti-androgenic properties were chosen to represent two different modes of action, vinclozolin and flutamide, are both androgen receptor antagonists, thereby disrupting androgen signaling while prochloraz primarily disrupts steroid hormone biosynthesis, but also inhibits the aromatase and is an androgen receptor antagonist. Dose levels were selected to mimic a LOAEL as well as a NOAEL for anti-androgenic effects, and the acceptable daily intake (ADI) for each individual compound, which were then combined together into three mixtures of the LOAELs, NOAELs, and ADIs. Mixture effects would be established through direct comparison of the single-substance and mixture exposure groups.

In general, anti-androgenic changes were observed at the LOAEL-MIX (20, 0.25, and 30 mg/kg bw/d vinclozolin, flutamide, and prochloraz, respectively), but not at lower exposures (NOAEL-MIX 4, 0.025, and 5 mg/kg bw/d; ADI-MIX 0.005, 0.00025 and 0.01 mg/kg bw/d vinclozolin, flutamide, and prochloraz, respectively). Neither the small changes in serum androgen hormone concentrations nor the borderline reduction in single male sex organ weights observed with NOAEL-MIX treatment were noteworthy enough to be considered adverse. Thus, the NOAEL-MIX is truly a NOAEL. Furthermore, since there were absolutely no findings in the ADI-MIX, this dose level is definitely below the NOEL.

Nipple/areola counts appeared to be the most sensitive measure of effect, closely followed by age at sexual maturation, then anogenital distance/anogenital index and male sex organ weights, esp. ventral prostate weight, and finally gross and histopathological findings. This order generally coincides with the order of sensitivities seen in the literature (Borgert et al. 2014). The quantification of circulating hormone levels showed little consistency when comparing possibly treatment-related changes with those from same dose group at other developmental stages or when mixed- and single-substance exposure data were compared. However, adult serum hormone levels were mildly affected by LOAEL mixture treatment while similar hormone changes were not observed in the developing offspring on PND 21 (subset 1) or at puberty (subset 2). In contrast, testosterone changes in testes of male fetuses at GD 20 seems to represent an appropriate biomarker for potential anti-androgenic effects in a sensitive tissue during a critical developmental window. Combined exposure at LOAEL level resulted in more than additive responses for decreased male anogenital index (but not for anogenital distance), and delayed preputial separation in comparison to the expected effects of the individual compounds.

While these endpoints for anti-androgenicity had varying sensitivities, when taken together these data reveal two important observations: The dose–response curve clearly indicates a monotonic process and no evidence for an interaction of the compounds at the individual NOAEL or lower doses. In summary, endocrine toxicity is sometimes said to represent a special case with regard to dose–response at low dose levels, but in our experiments, we found no evidence for non-monotonicity.

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Compliance with ethical standards

Conflict of interest This study was co-sponsored by BASF SE, Ludwigshafen, Germany. BASF is producer of Vinclozolin and Prochloraz.

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