Abstract ID: 105, Poster Board: 140

# Endocrine Exposure at Environmentally Relevant Concentrations

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## Introduction

Despite an increasing amount of attention, little is understood about how doses of endocrine disrupting chemicals (EDCs) at environmental concentrations affect homeostasis. The majority of studies (single or mixture) are conducted at effective doses at or close to the NOAEL of the single substances, but not at environmentally relevant doses or doses that are generally considered to be safe; these include legally binding reference values like ADIs, DNELs or RfC. To address these concerns, we launched a project to test the endocrine activity of three compounds at the LOAEL, NOAEL and ADI (or comparable). The first phase of the project has been to determine the effects of these doses and dose-response relationships of vinclozolin, flutamide, and prochloraz in an *in vivo* study design which was compliant with regulatory testing protocols. Endpoints like hormone level determination, as well as transcriptome (mRNA) and miRnome (miRNA) analyses, were added to investigate sensitive markers of endocrine activity. This work is the foundation for a subsequent study of comparable design, where compound mixtures will be administered. *This project is financed through BASF and a grant (EMSG56) from the CEFIC LRI program.*

## Conclusions

**Consistent anti-androgenic effects were detected in male offspring at the LOAEL doses of all 3 test substances and at the supposed NOAEL dose of flutamide (Table 3). Salient findings (and most sensitive endpoints to date) were:**

- **Decreased ano-genital distances**
- **Increased nipple retention**
- **Delayed male sexual maturation**
- **Developmental defects in males**
- **Reduced male sex organ weights**

**Female offspring displayed no signs of developmental toxicity at any dose of any test substance.**

**Another study using a dose of 0.025 mg/kg bw/d flutamide has been completed, as the assumed NOAEL of 0.25 mg/kg bw/d in this study still produced anti-androgenic effects in male offspring. The new NOAEL is 0.025 mg/kg bw/d.**

Table 3: Summary of anti-androgenic effects

| Dose Group | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | VIN | 0.005 | 4 | 20 | | | | | | |
| | FLT | | | | 0.0025 | 0.25 | 2.5 | | | |
| | PRO | | | | | | | 0.01 | 5 | 30 |
| Ano-genital Distance/Index | | | | + | | + | +++ | | | |
| Nipple Retention | PND 12 | | | +++ | | ++ | +++ | | +++ | +++ |
| | PND 21 | | | | | | +++ | | | |
| Developmental Abnormalities | | | | | | | +++ | | | |
| Male Pup Sexual Maturation | Age | | | + | | + | +++ | | | + |
| | Weight | | | | | | +++ | | | |
| Organ Weights | PND 21 | | | ++ | | | ++ | | | + |
| | Puberty | | | + | | ++ | +++ | | | + |
| | PND 83 | | | ++ | | | +++ | | | + |
| Histopathology | PND 21 | | | | | | | | | |
| | Puberty | | | | | | ++ | | | |
| | PND 83 | | | | | | +++ | | | |

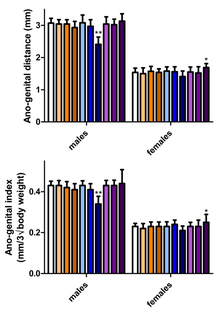
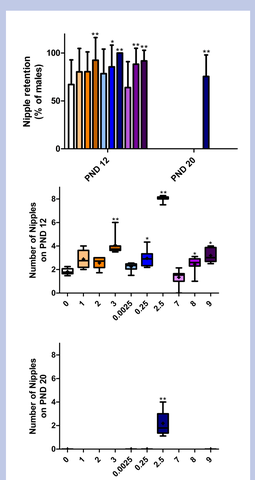



**Figure 2: Prenatal flutamide treatment decreased the ano-genital distance and index of male, but not female pups.**

Ano-genital distances *(top panel)* were measured in both male and female pups exposed *in utero* to either vehicle only (white), or low- (lightened), medium- (bright) and high-doses (darkened colors) of vinclozolin (orange), flutamide (blue), or prochloraz (purple), on PND 1. These data and the pup body weights were used to calculate the ano-genital indices *(bottom panel)*, a measure which corrects for pup size. Notably, female offspring of dams receiving 30 mg/kg bw/day prochloraz exhibited significantly larger ano-genital distances and indices than controls, similar to those previously described by Laier et al. in Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol. Appl. Pharmacol.* **213** 160-171 (2006) Data are reported as mean ± standard deviation.

**Figure 3: Pre- and postnatal anti-androgen treatment slows male nipple regression.**

Male pups exposed to either vehicle only (white), or low- (lightened), medium- (bright) and high-doses (darkened colors) of vinclozolin (orange), flutamide (blue), or prochloraz (purple) were examined for the presence of nipples or areolae on PND 12 and again at weaning on PND 21. While animals from dose groups 3 (20 mg/kg body weight/day vinclozolin), 5, 6 (0.25 and 2.5 mg/kg body weight/day flutamide, respectively), 8 and 9 (5 and 30 mg/kg body weight/day prochloraz) had a statistically significant increase in the incidence of retained nipples/areolae on PND 12, by PND 21 they had receded, with the exception of those of the high-dose flutamide group *(top panel,* reported as mean ± SD*)*. Furthermore, the number of nipples retained by each pup was also similarly elevated on PND 12 *(middle panel)* and PND 20 *(bottom panel)*. These plots contain information about the mean (+), median (bar), standard deviation (box), and 95% confidence interval (whiskers). Clearly the nipples retained until PND 21 are an outward manifestation of adverse endocrine effects; however, in light of the high nipple retention in the control males, a question arises as to the toxic relevance of nipple retention on PND 12, particularly in the groups where nipple retention is the only sign of endocrine disruption.



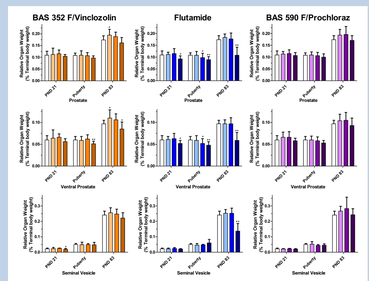




**Figure 4: Increasing anti-androgen exposure dose-dependently reduced male, but not female, sex organ weights.**

On PND 21 (Subset 1), the day of preputial separation (Puberty, Subset 2) and PND 83±2 (Subset 3), the sex organs of 10 male and 10 female rats were asservated, weighed and reported as relative organ weights at control (white), low- (lightened), medium- (bright) and high-doses (darkened colors) for each timepoint. Although similar effects were observed in the bulbo-urethral gland, cauda epididymis, epididymides, glans penis, bulbo-cavernous and levator-ani muscles, prostate, ventral prostate, and seminal vesicles; only the three most sensitive male sex organs are shown here. No change was observed in testes weight. Data are reported as mean ± standard deviation.

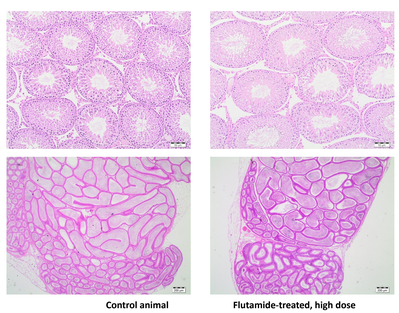



**Figure 5: Increasing flutamide exposure altered secondary male sex organ size, but not function.**

On PND 21 (Subset 1), the day of preputial separation (Puberty, Subset 2) and PND 83±2 (Subset 3), the sex organs of 10 male and 10 female rats were asservated, fixed, and evaluated histopathologically. Representative micrographs of left testis *(Puberty, upper panels)* and epididymis *(PND 83, lower panels)* tissues are shown here. No treatment-related histological differences were noted between the male sex organs, either on PND 21 or on the day of sexual maturation. But by PND 83, reduced prostate, ventral prostate and seminal vesicle sizes were apparent in animals exposed to 2.5 mg/kg bw/d flutamide, closely matching the sex organ weight data in Figure 4. Despite the changes, the functionality of these organs remained unimpaired. As in the organ weights, no histopathological changes were detected in testis, which also corresponded to normal sperm analysis in these animals. Furthermore, no change was detected in steroid hormone levels. These data suggest that 2.5 mg/kg bw/d flutamide treatment affects male secondary sex organ size without functional consequence.

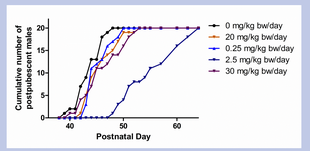



**Figure 6: Anti-androgen treatment delays male, but not female sexual maturation.**

Twenty male offspring which had been exposed to either vehicle only (black), or varying concentrations of vinclozolin (VIN, orange), flutamide (FLT, blue), or prochloraz (PRO, purple), were examined for preputial separation daily from PND 38 to 64. From these data, a Kaplan-Meier plot was generated for all test groups comparing the cumulative number of sexually mature males over time. For simplicity, only dose groups with delayed sexual maturation beyond historical control ranges are shown here. In most groups this statistically insignificant delay was about 1 day, however that resulting from treatment with 2.5 mg/kg body weight/day flutamide was both longer (almost two weeks) and statistically significant ($p \leq 0.01$). Furthermore, 4 incidences each of hypospadia and small penis developed in this dose group. On the other hand, female sexual maturation was unaffected by treatment.

Table 2: Selected gene expression modulations in the testes of PND 83 offspring

| Dose Group | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | VIN | 0.005 | 4 | 20 | | | | | | |
| | FLT | | | | 0.0025 | 0.25 | 2.5 | | | |
| | PRO | | | | | | | 0.01 | 5 | 30 |
| Apoptosis | Acin1 | | | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| | Maged1 | | | ▼ | | ▼ | ▼ | ▼ | ▼ | ▼ |
| | HMGB1 | | | | | ▲ | ▲ | ▲ | ▲ | ▲ |
| | Birc2 | | | | ▲ | ▲ | ▲ | ▲ | ▲ | |
| | Htra2 | | | | ▲ | ▲ | ▲ | | ▲ | ▲ |
| Meiosis | Sycp2 | | | | ▲ | ▲ | ▲ | | | ▲ |
| | Dazl | | | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| Transcriptional Regulation | BCL3 | | | ▲ | ▲ | ▲ | ▲ | | ▲ | ▲ |
| | IRF3 | | | ▲ | ▲ | ▲ | ▲ | | ▲ | ▲ |
| | PAX2 | | | | ▼ | ▼ | ▼ | | ▼ | ▼ |
| | JUN | | | | ▲ | ▲ | ▲ | | ▲ | ▲ |
| | TCEA1 | | | | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| | HMG1L1 | | | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| | Mycn | | | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ |
| | Sin3a | | | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| | XAB2 | | | | ▼ | ▼ | ▼ | | ▼ | ▼ |

In initial experiments, total RNA was extracted from 4 snap-frozen tissue samples per dose group, purified and reverse-transcribed to cDNA before being hybridized to Agilent Sureprint G3 8x60K microarrays. The resulting data were normalized by the print tip LOWESS method (intra-array) and by quantile normalization (inter-array) before evaluation using MeV (cluster analysis) and Ingenuity (pathways analysis). The preliminary results reported here show gene expression changes as attributed to the three most common gene ontologies. We are currently in the process of confirming these results by qPCR in all 10 testis samples per dose group.

## Experimental Design

**The aim of this study was to determine effects which go beyond adaptive processes to maintain homeostasis in the endocrine system. A pre- postnatal, *in vivo* study design was chosen which is compliant with standard regulatory testing protocols. The test design was improved by the addition of endpoints measuring hormone levels, morphology and histopathological examinations (Figure 1).**

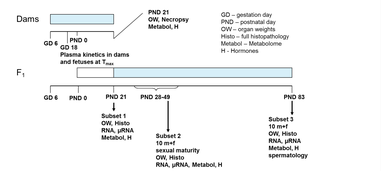



**Figure 1: Overall experimental design. It should be noted that many of these parameters are evaluated in the same animals, for better comparison of the data.**

**Briefly 10 groups of 25 presumed-pregnant female Wistar rats were administered test-substance daily by gavage from gestation day 6 (GD 6) until sacrifice. The tested doses (Table 1) were selected to mimic low-effect levels, the no observed adverse effect levels (NOAEL) for endocrine effects, and the Acceptable Daily Intake (ADI).**

**Table 1: Experimental dosing of parental female animals**

| | Reference value | Expected NOAEL* | Effect level |
|---|---|---|---|
| Vinclozolin | 0.005 mg/kg bw/day | 4 mg/kg bw/day | 20 mg/kg bw/day |
| Flutamide | NOAEL / 100 = 0.0025 mg/kg bw/day | 0.25 mg/kg bw/day †† | 2.5 mg/kg bw/day |
| Prochloraz | 0.01 mg/kg bw/day | 5 mg/kg bw/day | 30 mg/kg bw/day |

*anti-androgenicity

††As anti-androgenic effects were documented at this dose, a second identical study at 0.025 mg/kg bw/day is underway to ascertain the NOAEL for flutamide.

**One hour after dosing, 5 dams from each group were sacrificed on GD 18 and a caesarian section was performed. Dams and fetuses were collected for plasma/tissue kinetics. The remaining dams were sacrificed after weaning on postnatal day 21 (PND 21). A full necropsy was performed, including weighing the organs, and blood was collected for both metabolome and hormone analyses**

**20 male and 20 female offspring were selected to be raised until the day of sexual maturation (Subset 2, puberty) or early adulthood (Subset 3, PND 83±2). After weaning they were gavaged with the same test-substance as their mothers. A further 10 male and 10 female offspring were sacrificed at weaning (Subset 1, PND 21). All subset offspring were necropsied, organs were weighed and asservated. Blood was also collected for miRNA, metabolome, and hormone analyses. In addition, sperm analysis was also performed on male Subset 3 offspring, only. The blood and tissue samples were used to investigate sensitive markers of endocrine activity and to identify sub-pathological anti-androgenic effects on the metabolome.**