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Investigation of Flutamide 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 flutamide at doses comparable to the LOAEL, NOAEL and ADI. The first phase of the project has been to determine the effects of these doses 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. Companion studies were also performed using vinclozolin and prochloraz. 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 top two doses 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 flutamide dose.

A NOAEL of 0.025 mg/kg body weight/day was determined for pre-/post natal flutamide exposure in male offspring.

Table 3: Summary of anti-androgenic effects

Dose (mg/kg bw/day)		0.0025	0.025	0.25	2.5
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				+++

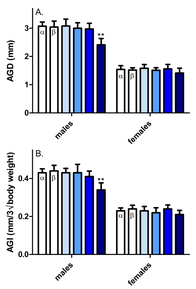

Figure 2: Prenatal flutamide treatment decreased the ano-genital distance and index of male, but not female pups.

Ano-genital distances *(top panel)* were measured on PND 1 in both male and female pups exposed *in utero* to either vehicle only (white), or 0.0025 (light blue), 0.025 (medium blue), 0.25 (bright blue) or 2.5 mg/kg body weight/d flutamide (dark blue). As these data were collected in two separate experiments, two sets of identical controls are shown. These data and the pup body weights were then used to calculate the ano-genital indices *(bottom panel)*, a measure which corrects for pup size. Data are reported as mean ± standard deviation; α and β represent two groups of concurrent controls.

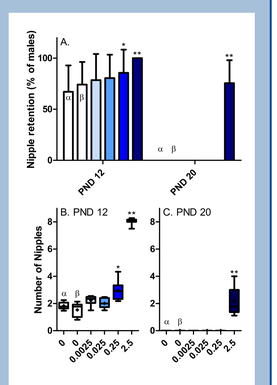

Figure 3: Pre- and postnatal flutamide treatment slows male nipple regression.

Male pups exposed to either vehicle (white), or 0.0025 (light blue), 0.025 (medium blue), 0.25 (bright blue) or 2.5 mg/kg body weight/d flutamide (dark blue) were examined for the presence of nipples or areolae on PND 12 and again at weaning on PND 21. As these data were collected in two separate experiments, two sets of identical controls are shown. While animals exposed to 0.25 and 2.5 mg/kg body weight/day flutamide, had a statistically significant increase in the incidence of retained nipples/areolae on PND 12, by PND 20 they had receded, except in the high-dose group *(top panel,* reported as mean ± standard deviation*)*. Furthermore, the number of nipples retained by each pup was also similarly elevated on PND 12 *(lower left panel)* and PND 20 *(lower right 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 if nipple retention is the only sign of endocrine disruption. In this figure, α and β represent two groups of concurrent controls.

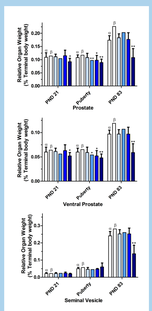

Figure 4: Increasing flutamide exposure dose-dependently reduced male, but not female, sex organ weights.

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 0 (white), 0.0025 (light blue), 0.025 (medium blue), 0.25 (bright blue) or 2.5 mg/kg body weight/d (dark blue) flutamide doses at 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; α and β represent two groups of concurrent controls.

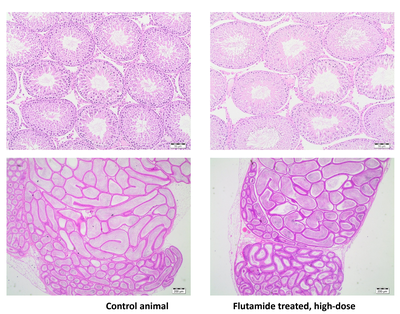

Figure 5: Increasing flutamide exposure altered secondary male sex organ size, but not function.

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. These data suggest that 2.5 mg/kg bw/d flutamide treatment affects male secondary sex organ size without functional consequence.

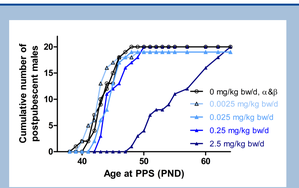

Figure 6: Flutamide treatment delays male, but not female sexual maturation.

Twenty male offspring which had been exposed to either vehicle only (black), or 0.0025 (light blue), 0.025 (medium blue), 0.25 (bright blue) or 2.5 mg/kg body weight/d (dark blue) flutamide 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. No delay in preputial separation was observed in animals of the 0.0025 or 0.025 mg/kg bw/d dose groups. A statistically insignificant delay of about 1 day was observed in the 0.25 mg/kg bw/d group; however the delay resulting from treatment with 2.5 mg/kg bw/d 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 vaginal opening remained unaffected by treatment.

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

Dose (mg/kg bw/day)		0.0025	0.025	0.25	2.5
Apoptosis	Acin1	▲	Data not yet measured	▲	▲
	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).

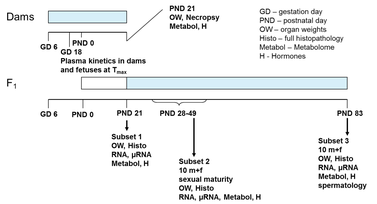

Figure 1: Overall experimental design. It should be noted that many of these parameters are evaluated in the same animals, for better comparison of the data.

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 a low-effect, the no observed adverse effect level (NOAEL) for endocrine effects, and the Acceptable Daily Intake (ADI).

Table 1: Experimental dosing of parental female animals

Reference value	Possible NOAEL	Expected NOAEL*	Effect level
NOAEL / 100 = 0.0025 mg/kg bw/day	0.025 mg/kg bw/day	0.25 mg/kg bw/day ††	2.5 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 was performed to ascertain a true 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 asservated and weighed. Blood was also collected for miRNA, metabolome, and hormone analyses. In addition, sperm analysis was also performed on male Subset 3 offspring. Blood and tissue samples were used to investigate sensitive markers of endocrine activity and to identify sub-pathological anti-androgenic effects on the metabolome.