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Investigation of Vinclozolin at Environmentally Relevant Concentrations

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LRI The Long-range Research initiative www.cefic-lri.org

BASF The Chemical Company

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 vinclozolin 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 flutamide 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 dose of vinclozolin (Table 3). Salient findings (and most sensitive endpoints to date) were:

- **Increased nipple retention**
- **Delayed male sexual maturation**
- **Reduced male sex organ weights**

Female offspring displayed no signs of developmental toxicity at any vinclozolin dose.

A NOAEL of 4 mg/kg bw/d was determined for pre-/post natal vinclozolin exposure in male offspring.

Table 3: Summary of anti-androgenic effects

Dose (mg/kg bw/day)		0.005	4	20
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			

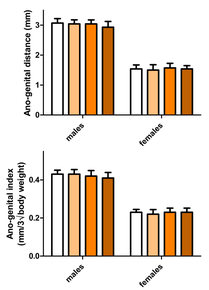


Figure 2: Prenatal vinclozolin treatment did not alter either the ano-genital distance or index of male or female pups.

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 0.005 (light orange), 4 (medium orange) or 20 mg/kg body weight/day vinclozolin (dark orange). 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. Neither parameter was statistically significantly reduced at any vinclozolin dose. Data are reported as mean ± standard deviation.

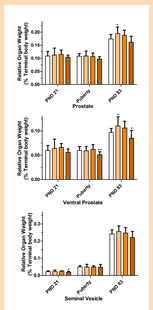


Figure 4: Increasing vinclozolin exposure reduced male, but not female, sex organ weights.

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), 0.005 (light orange), 4 (medium orange) or 20 mg/kg body weight/day (dark orange) vinclozolin doses at each timepoint. Although statistically significant reductions in the weights of the bulbo-urethral gland, cauda epididymis, epididymides, glans penis, bulbo-cavernous and levator-ani muscles, prostate, ventral prostate, and seminal vesicles were observed at the high-dose; 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.

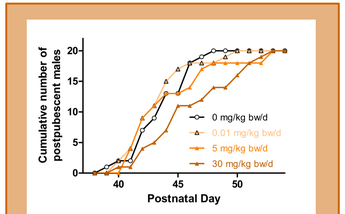


Figure 5: Vinclozolin treatment delays male, but not female sexual maturation.

Twenty male offspring which had been exposed to either vehicle only (black), or 0.005 (light orange), 4 (medium orange) or 20 mg/kg body weight/day (dark orange) vinclozolin 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 the low- and mid-dose groups; however, a statistically insignificant delay of about 1 day was observed at the high-dose. Female pubertal development (vaginal opening) remained unaffected by treatment. Nor were any developmental abnormalities observed at any dose in animals of either gender.

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).

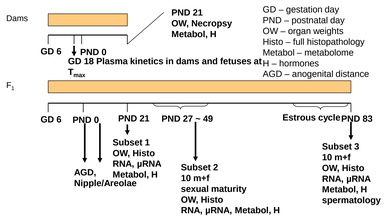


Figure 1: Overall experimental design. It should be noted that many of these parameters are evaluated in the same animals, for better comparison of the data.

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 (ADI)	Expected NOAEL*	Effect level
0.005 mg/kg bw/day	4 mg/kg bw/day	20 mg/kg bw/day

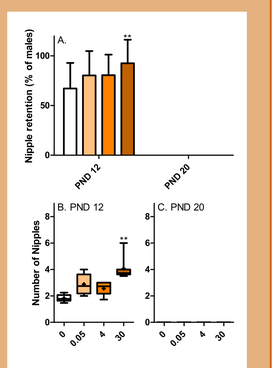
*anti-androgenicity

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.

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

Male pups exposed to either vehicle (white), 0.005 (light orange), 4 (medium orange) or 20 mg/kg body weight/day vinclozolin (dark orange) were examined for the presence of nipples or areolae on PND 12 and again at weaning on PND 21. While animals exposed to 20 mg/kg body weight/day vinclozolin, had a statistically significant increase in the incidence of retained nipples/areolae on PND 12, by PND 20 they had receded *(Panel A,* reported as mean ± SD). Furthermore, the number of nipples retained by each pup was also elevated on PND 12 *(Panel B)* but had disappeared by PND 20 *(Panel C)*. These plots contain information about the mean (+), median (bar), standard deviation (box), and 95% confidence interval (whiskers). 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 situations where this parameter is the only sign of endocrine disruption.



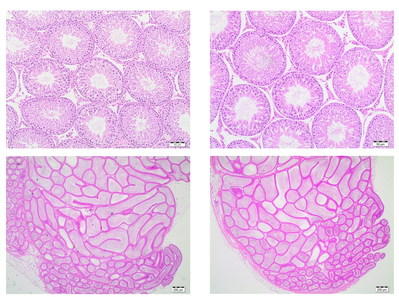


Figure 5: Increasing vinclozolin exposure altered neither sex organ size nor function in males.

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 at any developmental age. Similarly, the functionality of these organs remained unimpaired by vinclozolin treatment; no histopathological changes were detected in testis, which also corresponded to normal sperm analysis in these animals. These data suggest that treatment with up to 20 mg/kg body weight/day vinclozolin does not alter the size or function of the male sex organs.

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

Dose (mg/kg bw/day)		0.005	4	20
Apoptosis	Acin1			▲
	Maged1			▼
Meiosis	Dazl			▲
Transcriptional Regulation	BCL3			▲
	IRF3			▲
	HMG1L1			▲
	Mycn			▼
	Sin3a			▲

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.