# Testing for Human Effects

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# Talk Outline

- Regulatory requirements for ED testing
- Tests for effects on human health
  - Current pesticide tests
  - Specific tests for ED
- Examples of results obtained using vinclozolin as a case study
- Prospects for testing in the future

# Regulatory requirements for testing for EDs

- REACH (1907/2006)- Regulation concerning the Registration, Evaluation and Restriction of Chemicals

- Regulation on Plant Protection Products (EC 11/07/2009 replacing Directive 91/414/EEC)

- Regulation on Biocides

- US EPA Tier 1 screening

# Tests for effects on human health

# Standard regulatory toxicity tests

- Standard pesticide registration packages already include many tests with endpoints relevant to the assessment of ED potential.
- These include repeat dosing studies, carcinogenicity tests, studies in pregnant animals.
- Metabolism studies are also very important and may lead to additional toxicity studies on metabolites.

# Standard regulatory toxicity tests useful for ED assessment

| Test | Endpoints useful for endocrine assessment | Assessment |
|---|---|---|
| **Repeat dose toxicity studies: 28d, 90d 1yr studies in rat, mouse, dog** | Organ weights and histopathology of:<br>Ovary, uterus, vagina<br>Testes, sex accessory organs<br>Thyroid gland<br>Adrenals<br>Pituitary gland<br>Mammary gland<br>Liver | Hazard and risk |
| **Carcinogenicity studies: rat, mouse** | As above but including neoplastic analysis | Hazard and risk |

# Standard regulatory toxicity tests useful for ED assessment

| Test | Endpoints useful for endocrine assessment | Assessment |
|---|---|---|
| **Reproduction studies: rat** | Target organs (ovary, uterus, vagina, testes, sex accessory organs, thyroid, adrenals, pituitary gland, mammary gland, liver)<br>Sexual maturation (vaginal opening, preputial separation)<br>Reproduction<br>Fertility<br>Post-natal development | Hazard and risk |
| **Developmental studies: rat, rabbit** | Foetal development<br>Ovary, uterus, vagina (maternal) | Hazard and risk |

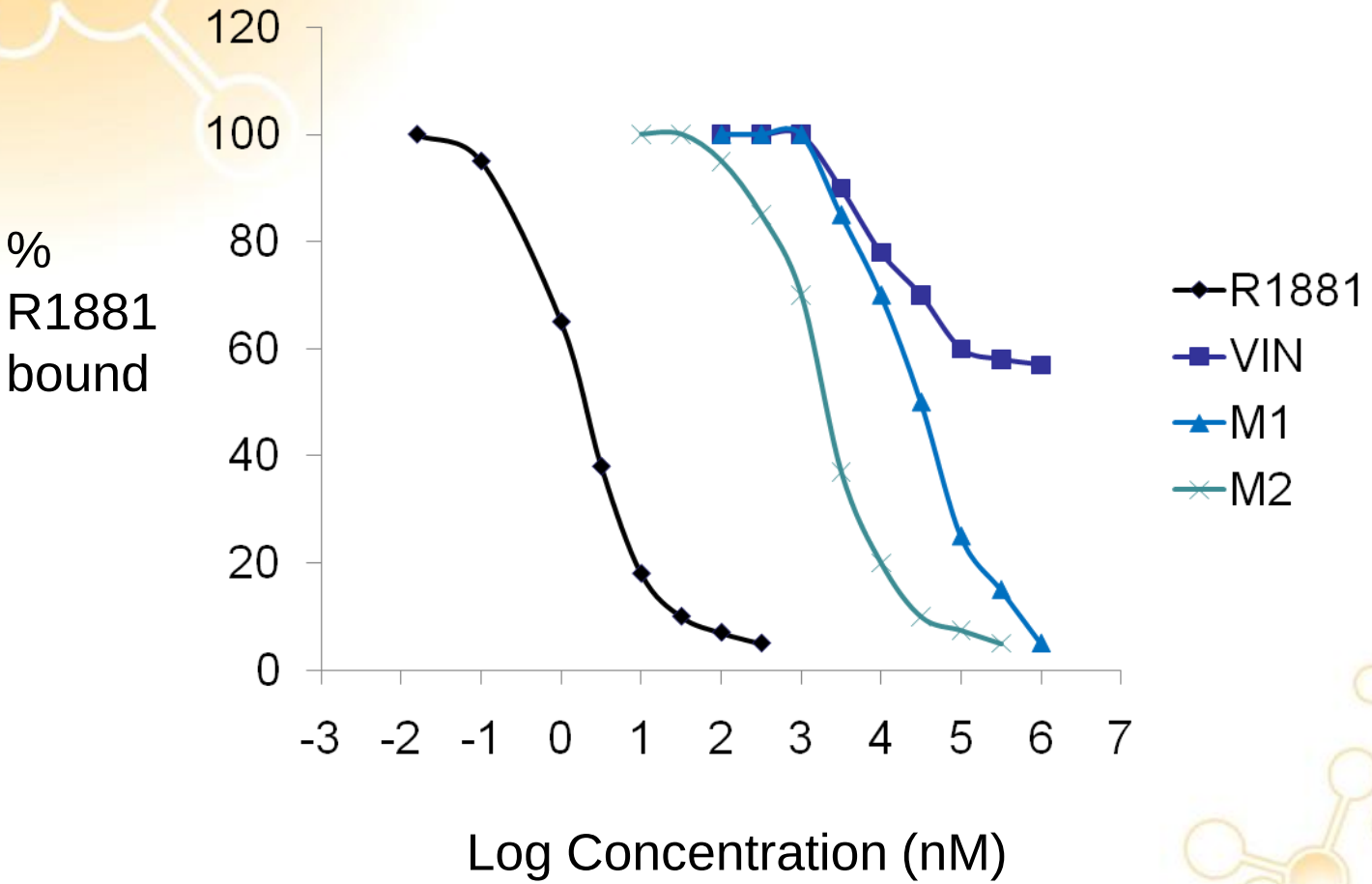
# Specific tests for ED

- OECD Conceptual framework
- US EPA EDSP tests
- Vinclozolin as a case study

# Specific tests for ED

| **Assay** | **Estrogen/Androgen Receptor Binding** |
|---|---|
| **Position** | OECD Level 2, US-EPA Tier 1 |
| **Purpose** | To identify chemicals that bind to ER/AR. |
| **Assessment** | Hazard assessment only |
| **Design** | ER from rat uterus or recombinant protein is incubated with estradiol and chemical.<br>AR from rat prostate or recombinant protein is incubated with R1881 (a strong ligand) and chemical. |
| **Endpoints** | Binding curves and IC50 (molar concentration of chemical which inhibits 50% of binding by estradiol/R1881). |

# AR binding: vinclozolin example




Kelce et al (Toxicol. Appl. Pharmacol. 1994)

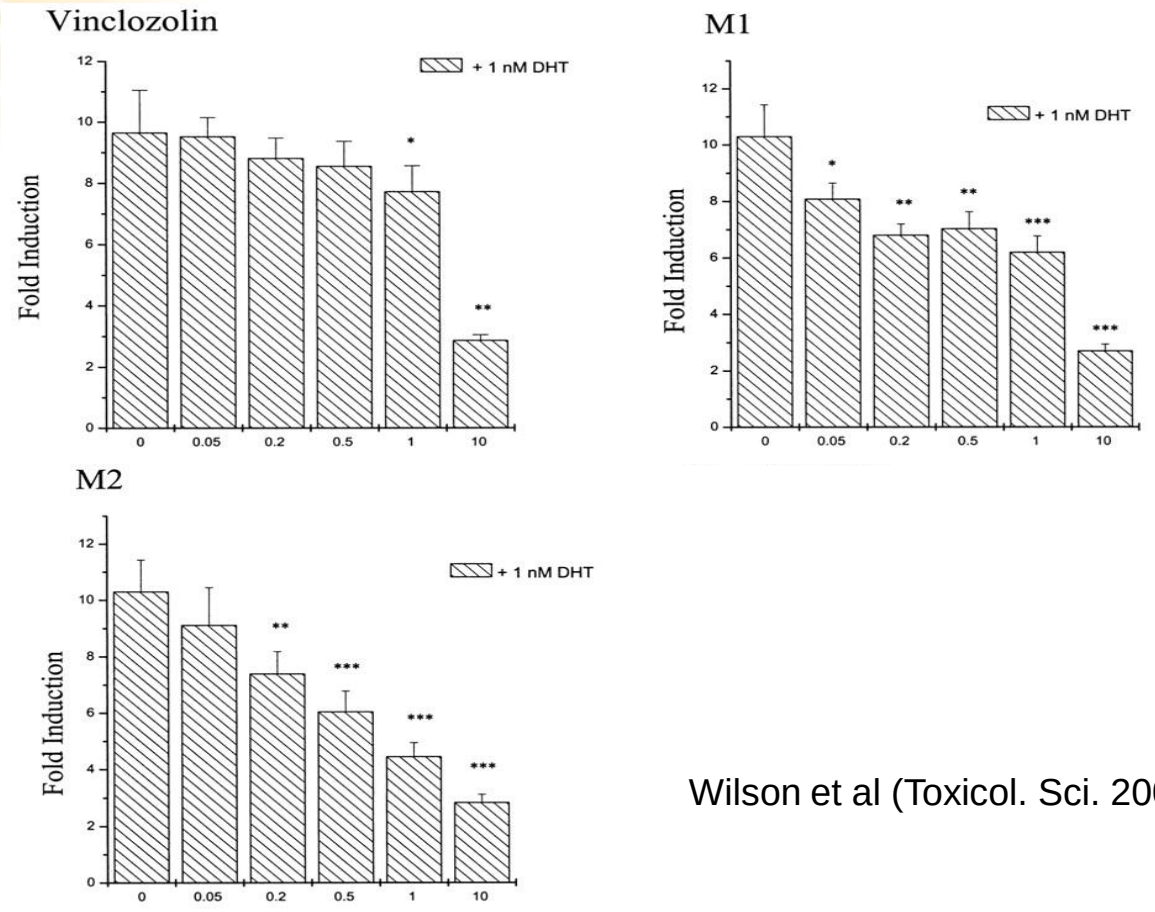
# Estrogen/Androgen Receptor binding

| What it tells you | What it doesn't |
| --- | --- |
| Chemical binds to receptor<br><br>Potency of binding | Whether it is an agonist or antagonist.<br><br>Whether this occurs in vivo.<br><br>What the phenotypic consequences may be in vivo.<br><br>Whether it has other activities. |

# Specific tests for ED

| **Assay** | **ER α and AR Transcriptional Activation** |
|---|---|
| **Position** | OECD Level 2, US-EPA Tier 1 |
| **Purpose** | To identify chemicals that bind to ER/AR and alter gene transcription. |
| **Design** | HeLa cells (stably transfected with hERα/AR expression construct) are incubated with chemical. |
| **Endpoints** | Measurement of bioluminescence reflecting changes in gene transcription. |

# AR transcriptional activation: vinclozolin example




Wilson et al (Toxicol. Sci. 2002)

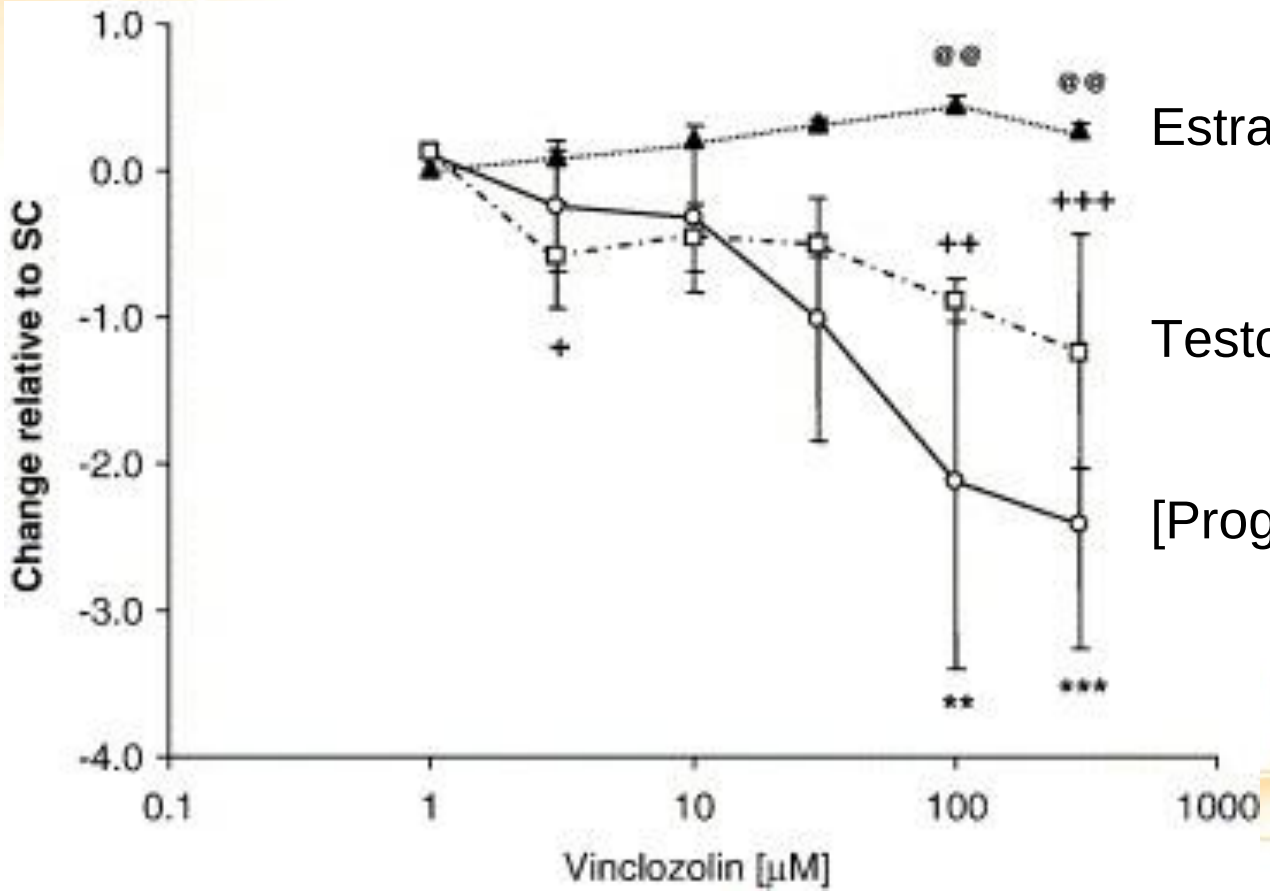
# ER and AR transcriptional activation

| What it tells you | What it doesn't |
|---|---|
| Chemical binds to receptor and triggers transcription.<br><br>Whether it is an agonist or antagonist<br><br>Potency of transcriptional activation | Whether this occurs in vivo.<br><br>What the phenotypic consequences may be in vivo.<br><br>Whether it has other activities. |

# Specific tests for ED

| | |
|---|---|
| **Assay** | **Steroidogenesis H295R Assay** |
| **Position** | OECD Level 2, US-EPA Tier 1 |
| **Purpose** | To detect chemicals that affect the synthesis of sex steroid hormones. |
| **Design** | H295R cells contain steroid hormone synthesis pathways. Cells are incubated with chemicals. |
| **Endpoints** | Estradiol and testosterone production from H295R cells. |

# Steroidogenesis assay: vinclozolin example




Hecker et al (Toxicol. Appl. Pharmacol. 2006)

# Steroidogenesis H295R Assay

| What it tells you | What it doesn't |
|---|---|
| Chemical interferes with enzymes of steroidogenesis<br><br>Whether it induces or inhibits<br><br>Potency of interference | What the exact mechanism is<br><br>Whether this occurs in vivo.<br><br>What the phenotypic consequences may be in vivo.<br><br>Whether it has other activities. |

# Specific tests for ED

| **Assay** | **Uterotrophic and Hershberger Assays** |
|---|---|
| **Position** | OECD Level 3, US-EPA Tier 1 |
| **Purpose** | To detect (anti)-estrogenic/ (anti)-androgenic chemicals. |
| **Assessment** | Hazard assessment only |
| **Design** | Immature or castrated adult rats are administered chemical for 3/10 days using two test groups. |
| **Endpoints** | Uterine/SAT weight is measured and compared with controls. |

# Hershberger assay: vinclozolin example

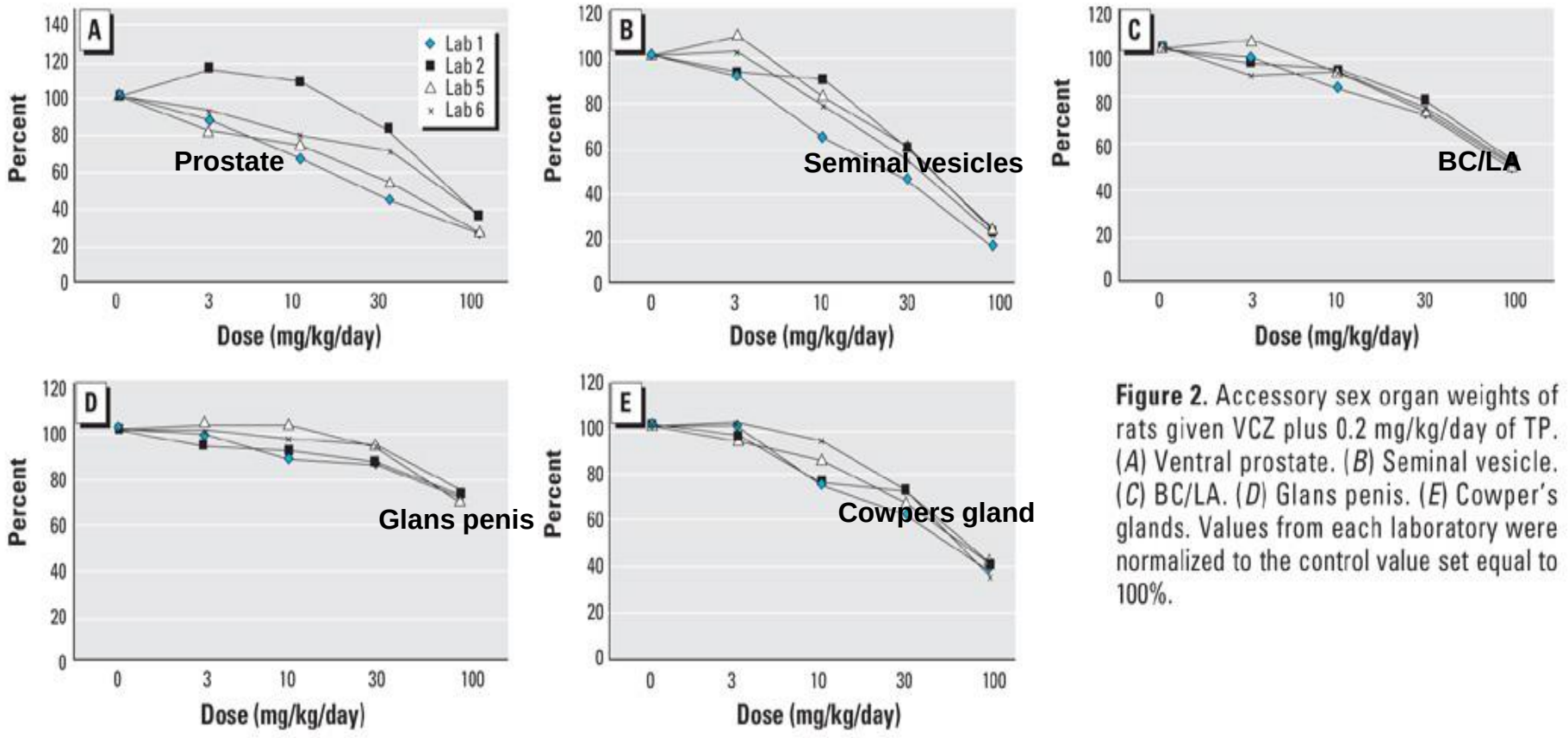



**Figure 2.** Accessory sex organ weights of rats given VCZ plus 0.2 mg/kg/day of TP. (*A*) Ventral prostate. (*B*) Seminal vesicle. (*C*) BC/LA. (*D*) Glans penis. (*E*) Cowper's glands. Values from each laboratory were normalized to the control value set equal to 100%.

Yamasaki et al (Env Hlth Persp 2003)

# Hershberger and uterotrophic assays

| What it tells you | What it doesn't |
|---|---|
| Chemical interacts with AR/ER to increase/decrease reproductive organ growth in vivo<br><br>Whether it is agonist or antagonist<br><br>Potency of effect<br><br>ADME effects may also be demonstrated | Whether the effects will be seen in "intact" animals<br><br>Whether the effects are adverse (ie affect reproduction, development etc)<br><br>Whether it has other activities. |

# Specific tests for ED

| **Assay** | **Pubertal Male Assay** |
|---|---|
| **Position** | OECD Level 4, US-EPA Tier 1 |
| **Purpose** | To detect chemicals with antithyroid, androgenic, or antiandrogenic activity or that alter pubertal development via changes in gonadotropins, prolactin, or hypothalamic function. |
| **Design** | Male rats are administered chemical from post-natal day (PND) 23 to PND 53 (31 days) using two test groups. |
| **Endpoints** | Growth (body weight) Age and weight at preputial separation. Organ weights and histology including sex accessory tissues and thyroid. Serum hormones. |

# Pubertal male assay: vinclozolin example

Vinclozolin (orally administered from pnd 23-53, 30-100 mg/kg/day):

- Delayed pubertal maturation
- Reduced growth of sexual accessory organs and epididymides
- Increased levels of sex hormones (LH, testosterone, estradiol).

Monosson et al (Toxicol Ind Hlth, 1999)

# Pubertal male assay

| What it tells you | What it doesn't |
| --- | --- |
| Chemical causes phenotypic changes in reproductive/sex organs and thyroid growth *in vivo* in intact animal via various mechanisms<br><br>Effects on sexual maturation<br><br>Possibly whether it is agonist or antagonist<br><br>Potency of effects<br><br>ADME effects may also be demonstrated | Whether the effects are adverse (ie affect reproduction, development etc)<br><br>What the mechanism of action is. |

# Specific tests for ED

| **Assay** | **Updated OECD TG407 (28 day rodent oral toxicity study)** |
|---|---|
| **Position** | OECD Level 4 |
| **Purpose** | Provides information on possible health hazards likely to arise from repeated exposure 28d, including effects on the nervous, immune and endocrine systems. |
| **Assessment** | Hazard and risk |
| **Design** | Male and female rats/mice are administered chemical (orally) for 28d. Dosing starts at approx 7 weeks of age. Three treatment groups per sex. |
| **Endpoints** | As for TG407 but now including sex organ and accessory tissue weights and histology. Some optional endpoints including thyroid hormones. |

# Enhanced 28d oral toxicity test (TG 407), male and female rat: vinclozolin example

Vinclozolin (3-200 mg/kg/day):

- Reduced growth of sexual accessory organs and epididymides (no histopathological changes)
- Caused slight increase in estrus cycle length in females
- Altered levels of sex hormones in both sexes (in males LH & estradiol increased; in females LH increased & testosterone decreased).
- Thyroxine decreased, TSH increased but no thyroid histopath changes.

Shin et (Reg Tox, 2006)

# TG407 assay

| What it tells you | What it doesn't |
|---|---|
| Chemical causes phenotypic changes in reproductive/sex organs and thyroid growth *in vivo* in intact animal via various mechanisms<br><br>Possibly whether it is agonist or antagonist<br><br>Potency of effects<br><br>ADME effects may also be demonstrated | Whether there effects on sexual maturation<br><br>Whether the effects are adverse (ie affect reproduction, development etc)<br><br>What the mechanism of action is.<br><br>May be insensitive to weak chemicals<br><br>Some endpoints are optional |

# Standard reg test and ED test

| | |
|---|---|
| **Assay** | **Mammalian 2 generation (TG416 enh)** |
| **Position** | OECD Level 5, US-EPA Tier 2 |
| **Purpose** | To provide general information on the effects of a test substance on the integrity and performance of the male and female reproductive systems, and on the growth and development of the offspring. |
| **Assessment** | Hazard and risk |
| **Design** | Continuous administration of compound (orally), prior to and during mating, gestation, lactation, to termination after weaning of the F2 generation. Three treatment groups. |
| **Endpoints** | Reproduction, parturition, AGD, lactation, postnatal development. Puberty onset, sexual development, sperm parameters, oestrus cycle parameters, organ weights, histopathology. |

# Mammalian 2 generation (TG416 enh): vinclozolin example

2 generation study with dietary administration
40ppm=2-6mg/kg (L), 200ppm=11-30mg/kg (M), 1000ppm=57-150mg/kg (H)

| GEN | MALES | FEMALES |
|---|---|---|
| F0 | SAT wt↓ & histopath (H), LH&FSH↑, T3&T4↓(H) | Ovaries histopath (H), T3&T4↓(H) |
| F1 | SAT wt↓ & histopath (M&H), abnormal ext genitalia LH&FSH↑, T3&T4↓(H), bwt pnd21↓(H), perinatal AGD↓(H), nipples↑(M&H), age at PPS↑ Fertility index↓ (H) for 1st and 2nd F1 mating | Ovaries wt↑ & histopath (H), T4↓(H), bwt pnd21↓(H), |
| F2 | SAT wt↓(H), abnormal ext genitalia, perinatal AGD↓(M&H), nipples↑(M&H), | No changes observed |

Matsuura et al. J Tox Sci 2005 30 pp 163-188

# 2-Generation test

| What it tells you | What it doesn't |
| --- | --- |
| Chemical causes phenotypic changes in reproductive/sex organs and thyroid growth *in vivo* in intact animal via various mechanisms<br><br>Chemical has an adverse effect on reproduction.<br><br>Chemical has an adverse effect on development (post-natal, sexual).<br><br>Chemical has an adverse effect on sexual behaviour.<br><br>Potency of effects<br><br>ADME effects are demonstrated | What the mechanism of action is.<br><br>Older studies may not contain ED sensitive endpoints. |

# Summary

- ED has a high profile in regulations for human and environmental health
- Within EU, acceptable criteria for defining ED are needed in order to protect health but allow the use of valuable chemicals.
- Many tests for ED have now been validated.
- In the US, mandatory testing has started but in the EU there is no formal regulatory framework.
- Use in regulatory frameworks will lead to some tests being discarded in favour of more robust tests.
- All the tests are being used by research groups.

# What the future holds

- New endpoints for existing tests eg
  - Effects on mammary glands
  - Late onset changes eg premature reproductive senescence
- Addition of new tests for additional hormonal systems eg
  - Glucocorticoid system
  - Receptor cross talk (eg AhR, PPARs)
- Involvement of EDs in obesity, diabetes etc
- Developmental neuroendocrine effects

Thank-you for listening!

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