

Stand der Entwicklung von OECD-Tests für endokrine Effekte bei Säugern

Walter Lichtensteiger

GREEN Tox, Zurich

1. Das OECD-Framework
2. Nachweis endokriner Aktivität in vivo
3. Tests für Risk Assessment

NB: "*Work in Progress*": Änderungen im Guideline-Text vorbehalten!

1. OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

**Identification
and
Prioritization**

**Analysis of
existing data**

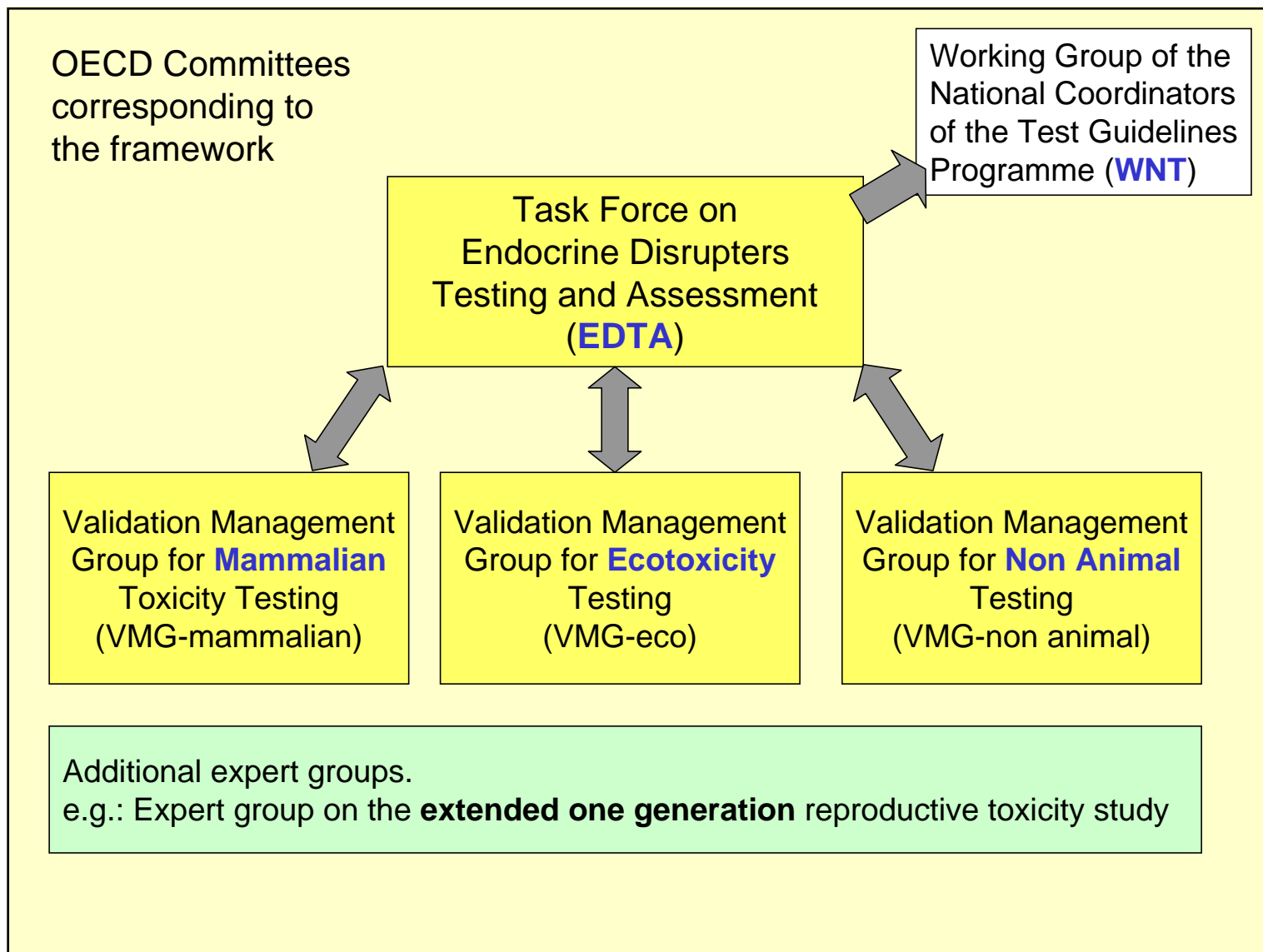
**Simple *in vitro* models
Short-term *in vivo* tests**

**Risk Assessment
for selected
chemicals**

**Long-term studies:
Generation / Life cycle**

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

<p>Level 1 Sorting & prioritization based upon existing information</p>	<ul style="list-style-type: none"> - physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability, - human & environmental exposure, e.g., production volume, release, use patterns - hazard, e.g., available toxicological data 	
<p>Level 2 <i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> - ER, AR, TR receptor binding affinity - Transcriptional activation - Aromatase and steroidogenesis <i>in vitro</i> - Aryl hydrocarbon receptor recognition/binding - QSARs 	<ul style="list-style-type: none"> - High Through Put Prescreens - Thyroid function - Fish hepatocyte VTG assay - Others (as appropriate)
<p>Level 3 <i>In vivo</i> assays providing data about single endocrine mechanisms</p>	<ul style="list-style-type: none"> - Uterotrophic assay (estrogenic related) - Hershberger assay (androgenic related) - Non-receptor mediated hormone function - Others (e.g. thyroid) 	<ul style="list-style-type: none"> - Fish VTG (vitellogenin) assay (estrogenic related)
<p>Level 4 <i>In vivo</i> assays providing data about multiple endocrine mechanisms</p>	<ul style="list-style-type: none"> - enhanced OECD 407 (endpoints based on endocrine mechanisms) - male and female pubertal assays - adult intact male assay 	<ul style="list-style-type: none"> - Fish gonadal histopathology assay - Frog metamorphosis assay
<p>Level 5 <i>In vivo</i> assays providing adverse effects data from endocrine & other mechanisms for RA</p>	<ul style="list-style-type: none"> - 1-generation assay (TG415 enhanced)¹ - 2-generation assay (TG416 enhanced)¹ - reproductive screening test (TG421 enhanced)¹ - combined 28 day/reproduction screening test (TG 422 enhanced)¹ <p><small>¹ Potential enhancements will be considered by VMG mamm</small></p>	<ul style="list-style-type: none"> - Partial and full life cycle assays in fish, birds, amphibians & invertebrates (developmental and reproduction)



2. Identification of Endocrine Activity in vivo: Mammalian Test Systems

Concept: Validation of pre-existing protocols with the expectation of fast guideline development (except for metamorphosis assay).

Completed:

Estrogenicity:

Uterotrophic Bioassay in Rodents: new TG440 (2007)

Pending:

Androgenicity/Antiandrogenicity:

Hershberger Bioassay in Rats

Estrogenic, (Anti)androgenic and Thyroid Effects:

Revised TG407 in Rodents

Thyroid Effects:

Amphibian Metamorphosis Assay (Frog)

2.1. Uterotrophic Bioassay in Rodents (TG440)

- **Animal species:** normally Rat, Mouse possible (provide rationale)

- **Two versions:**

- A. **Immature intact** females prior to puberty

- B. **Ovariectomized** young adult females

- **Administration of test substance:**

- Oral gavage or subcutaneous injection.

- Minimum 2 dose groups + vehicle control on 3 consecutive days

- **Endpoint:**

- Uterine weight** (wet weight and blotted weight)

- **Critical Issues:**

1. **Developmental Time Window** in immature rat version:

- Last dose between postnatal day 21 and 24 (day of birth = postnatal day 0).

2. **Low control uterine weight.**

3. **Dietary phytoestrogen** levels not exceeding 350µg genistein equivalents/g diet.

4. Verification of **laboratory proficiency** with positive controls (periodic or concurrent)

- **Sensitivity:** Sensitive also for weak estrogens if properly performed.

- **Antiestrogenic activity:** Not included in validation procedure, but OECD Guidance Document available to use test for antiestrogenicity

Validation of the Uterotrophic Bioassay

Chemicals used in validation procedure:

Ethinylestradiol
Genistein
Bisphenol A
Methoxychlor
Nonylphenol
o,p'-DDT

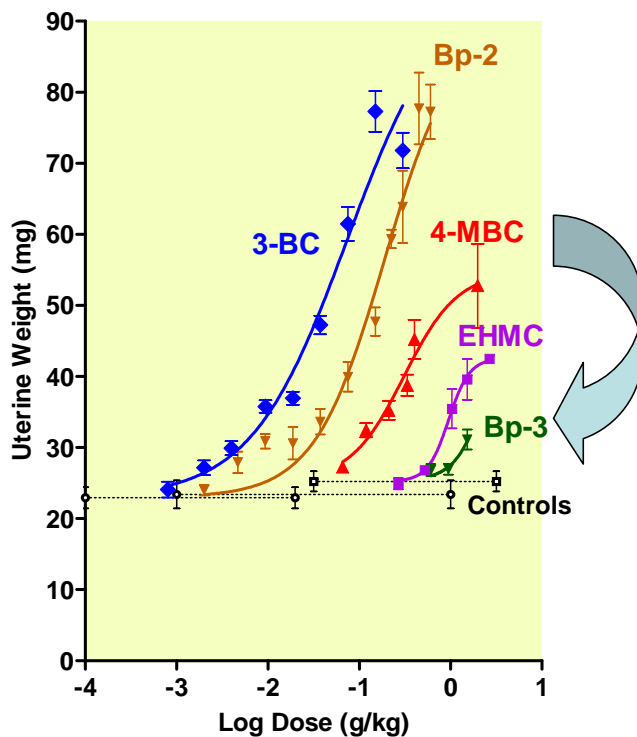
Study design:

1. **Dose-response** study with identified chemicals
(Kanno, J. et al., Environ. Health Perspect. 111: 1530-1549 (2003).
2. **Coded** single-dose study
(Kanno, J. et al., Environ. Health Perspect. 111: 1550-1558 (2003).

The Uterotrophic Assay in the Era of *in silico* and *in vitro* Analyses: Possible basis for **prioritization**

Example estrogenic UV filters: Similar activities *in vitro* but marked differences in acute activity *in vivo*.

Uterotrophic Assay in Immature Rats



In vitro versus in vivo Activity		
UV Filter	In vitro MCF-7 Cells EC50 (μ M)	In vivo Uterotrophic Assay ED50 (mg/kg)
3-BC	0.68	45
Bp-2	0.68	185
4-MBC	3.02	309
EHMC	2.37	934
Bp-3	3.73	> 1500
HMS	1.56	Inactive
OD-PABA	2.63	Inactive
BMDM	Inactive	Inactive

M. Schlumpf et al., 2004

2.2. Hershberger Bioassay in Rats

- **Animal species:** Rat

- **Principle:**

Castration of young adult (> postnatal day 42) male. 7 days later start of treatment with test chemical for 10 days. Age at necropsy < postnatal day 70.

- **Weanling rat version:** Not included: insufficient sensitivity for weakly active compounds

A. Test for Androgen-Agonists:

Test chemical (2 doses p.o. or s.c.) + negative control (vehicle) + positive control (testosterone propionate (TP)) on 10 consecutive days.

B. Test for Androgen-Antagonists and 5 α -Reductase Inhibitors:

Test chemical (3 doses) together with TP + negative control (TP alone) + positive control (TP together with flutamide) for 10 consecutive days.

- **Endpoints:**

Weight of **5 androgen-dependent accessory sex organs**: Seminal Vesicles, Ventral Prostate, Levator ani-bulbocavernosus Muscle, Cowper's Glands, Glans Penis.
Significant change in **one target organ = positive**.

- **Critical Issues:**

1. **Standard dissection:** OECD SOP manual for dissection. One person/tissue.
Performance criteria for organ weights given in guideline must be met.
2. Dietary phytoestrogen levels should correspond to uterotrophic guideline.

- **Sensitivity:** Very sensitive also for weak anti-androgens if properly performed.

Validation of the Hershberger Bioassay

Chemicals used in validation procedure:

Testosterone propionate (reference agonist)

17 α -Methyltestosterone

17 β -Trenbolone

Flutamide (reference antagonist)

p,p'-DDE

Linuron

Procymidone

Vinclozolin

Finasteride

Dinitrophenol (negative reference)

Nonylphenol

Study design:

1. **Dose-response** study with identified chemicals

(Owens, W. et al., Environ. Health Perspect. 115: 671-678 (2007))

2. **Coded** study

(Freyberger, A. et al., Toxicology 239: 77-88 (2007))

2.3. Revised TG 407 (Repeated Dose 28 Day Oral Toxicity Study in Rodents)

- **Animal species:** Rat preferred, other rodents possible

- **Principle:**

Enhancement of the **pre-existing** 28 day oral toxicity study guideline (**TG407**).

- **Oral administration** of test chemical to **young adult** animals of both sexes, daily for 28 days. At least 3 doses + control group.

- **Endocrine Endpoints** (draft 2008):

Gonads and Accessory Sex Organs: weight, histopathology

(testes, ovaries, epididymides, prostate+seminal vesicles+coagulating glands, uterus, vagina).

Male Mammary Gland

Vaginal smears on last 4 days before necropsy (Not = estrous cycle analysis!!)

Thyroid: histopathology (weight after fixation optional), thyroid hormones

- **Critical Issues:**

The validation **could not demonstrate sensitivity for weak endocrine disruptors:**

No sex hormone-specific effects with Nonylphenol and pp-DDE.

Some response to **Thyroid-active** compounds.

→ **Absence** of effect on reproductive tract **does not prove** lack of activity on sex hormone-dependent mechanisms.

- **Sensitivity:** Low for weakly active chemicals interacting with sex hormones

Validation of the Enhanced TG 407

Chemicals used in validation procedure:

Ethinylestradiol

Genistein

Nonylphenol

Tamoxifen

CGS 18320B (non-steroidal aromatase inhibitor)

17 α -Methyltestosterone

Flutamide

p,p'-DDE

I-Thyroxine

Propylthiouracil

3. Risk Assessment in Mammals: Generation Tests

Purpose:

Evaluation of exposure **across all life stages** into subsequent generations.

Present Situation:

Framework Level 5

In vivo assays providing *adverse effects data* from endocrine & other mechanisms

TG 416 Two Generation Test (P, F1, F2)
= "Gold Standard"

TG 415 One-Generation Test (P, F1)
Existing protocol **outdated**.

TG 421 (**enhanced**): *Reprod./Developmental Toxicity Screening test: **Insufficient information on F1***
TG 422 (**enhanced**): *Combined Repeated Dose with Reproduction/Developmental Toxicity **Insufficient information on F1***

Initiative for a new Generation Test

Two unrelated problems:

1. Scientific:

*The existing tests (including TG416) have been **designed for reproductive toxicity testing**, not for a general analysis of interactions with endocrine systems.*

2. Animal Welfare and Logistic/Economic:

*Considering the fact that many Two Generation Tests may have to be conducted under **REACH**, the Two Generation test presents a problem because it involves a **heavy work load/costs** and **big number of experimental animals**.*

Project for an Extended One Generation Reproductive Toxicity Study (EOGRTS)

Proposal (SPSF) submitted to OECD (WNT) by Germany, Netherlands and United States in 2008 → **OECD Expert Group** on EOGRTS (1st Meeting in October 2008).
Also ECETOC-Meeting on Triggering and Waving Criteria for EOGRTS in April 2008.

Objectives:

(partly based on work by Ralph Cooper et al. for ACSA (Agricultural Chemical Safety Assessment))

- 1. Address classical endpoints of reproductive and developmental toxicity, but:
produce an **F2 generation only if triggered** (saves about 1000 animals/test)
(Retrospective studies point to **limited relevance of F2**, in discussion)*
- 2. Address endpoints associated with **endocrine, neuro- and immuno-toxicity**
→ 3 cohorts: Set 1 Reproductive toxicity cohort
Set 2 Neurotoxicity cohort
Set 3 Immunotoxicity cohort*

*The new guideline will include the possibility of breeding F1 offspring to **produce an F2. Triggers** for production of an F2 can be effects found in the F1 generation as well as other available pertinent information. This aspect is under discussion.*

Pros and Cons of EOGRTS – a personal view of “work in progress”

- The **new modules Developmental Immunotoxicity (DIT) and Developmental Neurotoxicity (DNT)** represent a considerable progress. For the first time, an analysis of developmental immunotoxicity will be included in a generation protocol. One weakness of the DNT module is that **endocrine impacts on CNS** receive little attention.
- The **suppression of the F2** could considerably reduce animal numbers and work load. **Replacement of the analysis of F1 fertility by other endpoints** remains a critical issue, however, particularly in females. Potential triggers for F2 are in discussion, and additional **retrospective analyses on the value of F2** are under way.
- From a **scientific point of view in the endocrine disruption context**, the EOGRTS is not really a modern protocol, in spite of its merits and innovative value (DNT, DIT modules). **New methods and data** from recent **basic research** on endocrine disruption are little reflected. The new guideline is built on classical test procedures/endpoints validated in other guidelines. In order to fill this gap in the risk assessment context and to introduce new endpoints, **more research** would be needed that **systematically addresses the link between mechanistic data and hazard** (morphological or functional damage), and this across a broad range of different types of chemicals.

Literature

Design:

Cooper RL et al. (2006). A tiered approach to life stages testing for agricultural chemical safety assessment. *Crit. Rev. Toxicol.* 36: 69-98.

Moore N et al. (2009). A modular approach to the extended One-generation reproduction toxicity study. *ATLA* 37: 219-225.

Role of F2 Generation:

Janer G et al. (2007). A retrospective analysis of the two-generation study: What is the added value of the second generation? *Reprod. Toxicol.* 24: 97-102.

Rudén C, Hansson SO (2008). Comment on “*A retrospective analysis of the two-generation study: What is the added value of the second generation? By G. Janer, B.C. Hakkert, W. Slob, T. Vermeire, A.H. Piersma*” [*Reprod. Toxicol.* 24 (2007): 97-102]. *Reprod. Toxicol.* 25: 397-405.

Myers DP et al. (2008). An analysis of the results from Two-Generation reproduction toxicity studies to assess the value of mating animals of the second (F1) generation for the detection of adverse treatment-related effects on reproductive performance. *Reprod. Toxicol.* 26: 47-50
Additional retrospective analyses are in preparation..