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A Testing Strategy for the Identification of mammalian Endocrine Disruptors with particular focus on steroids

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Our understanding of endocrine disruptors

Weybridge 1996:

"An endocrine disrupter is an exogenous substance that causes **adverse health effects** in an intact organism, or its progeny, **secondary (consequent) to changes in endocrine function**".

WHO 2002:

"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes **adverse** effects in an **intact organism**, or its progeny, or (sub)populations."

„Endocrine disruption" is potentially a cut-off criterion without any risk assessment

1989: The beginning
Study case: Vinclozolin

Anti-androgenic effects in:

– prenatal developmental toxicity study: reduced ano-genital distance

2-generation study:

– a syndrome of changes resulting in a feminization of male rat offspring

reduced prostate & seminal vesicle weight and activity
hypospadia
vaginal pouch
delayed / incomplete testicular decent

can be explained by several modes of action:

1) **Reduction of hormone synthesis**
2) **Increased breakdown of hormones**
3) **Receptor block**

Overview of the Tier-1 EDSP battery

In vitro

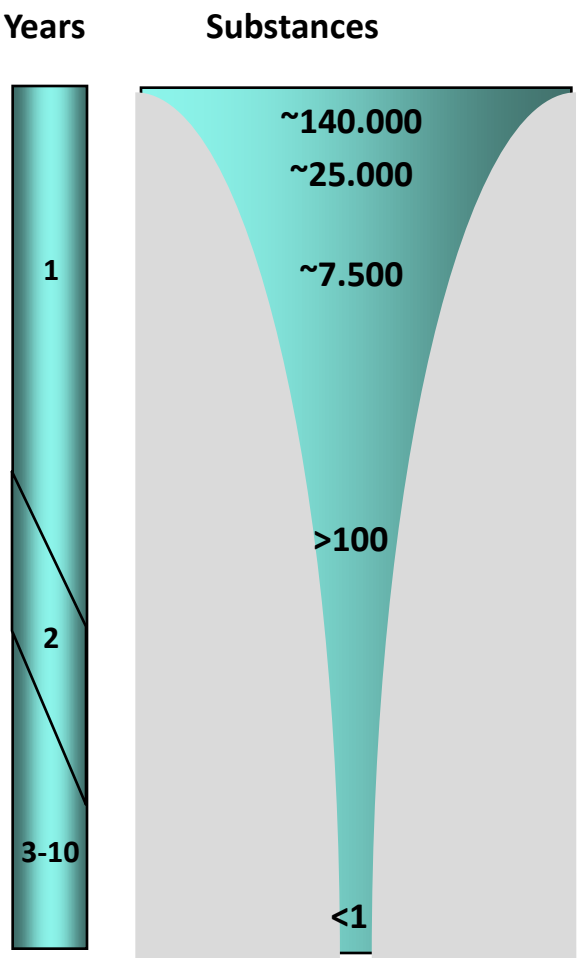
- Androgen receptor (AR) binding -rat prostate cytosol
- Estrogen receptor (ER) binding - rat uterine cytosol
- Aromatase - Human recombinant aromatase
- human estrogen receptor a transcriptional activation in HeLa-9903 line (OECD 455)
- Steroidogenesis in H295R line (OECD 456)

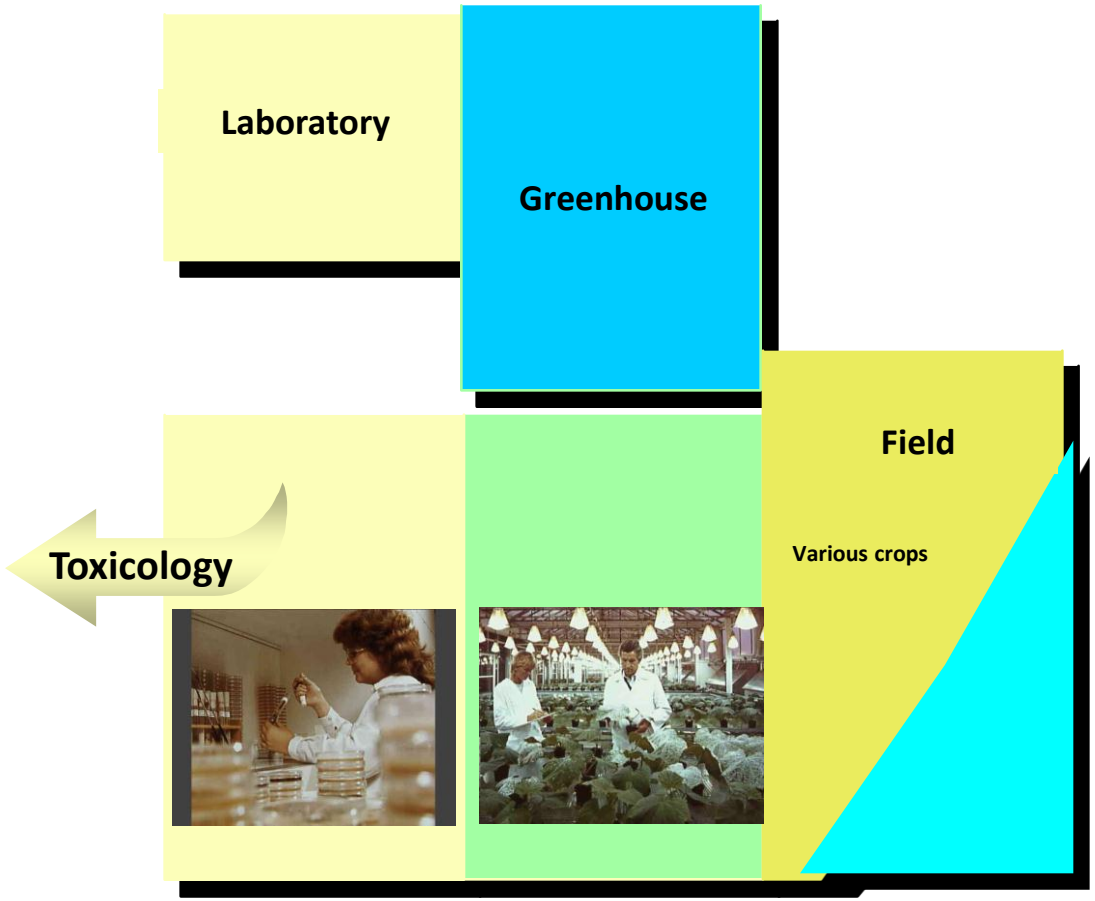
In vivo

- Uterotrophic (rat) (OECD 440)
- Hershberger (rat) (OECD 441)
- Pubertal female (rat)
- Pubertal male (rat)
- Amphibian metamorphosis (frog)
- Fish short-term reproduction

Summarized from EDSP, US-EPA homepage

Development and Registration of Agrochemicals: Screening and Selection





An integrated strategy for detection of Endocrine Modes of Action under 3Rs TIER 1

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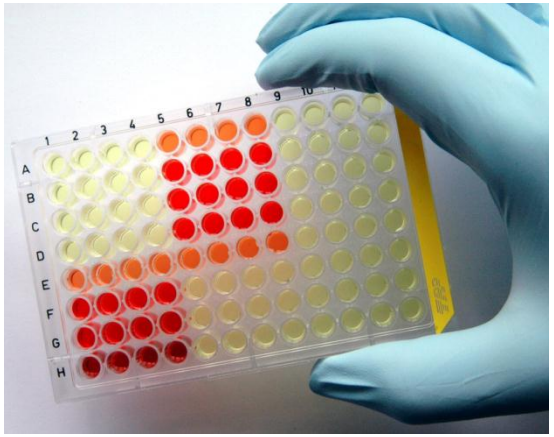
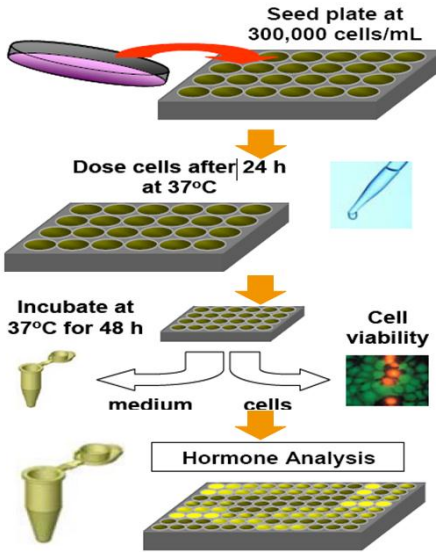
In vitro

Refined *in vivo*

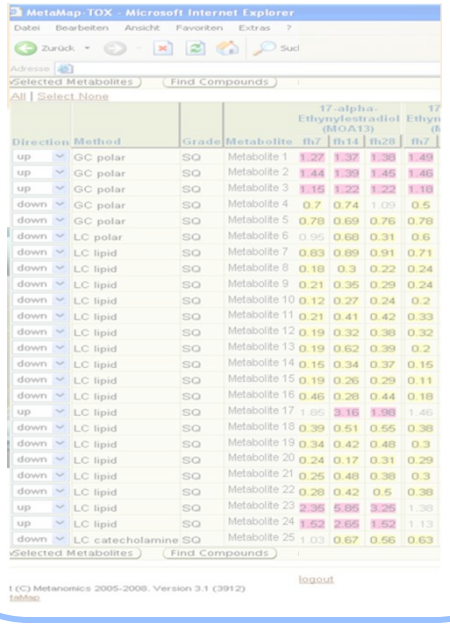
Receptor dependent



Hormone synthesis

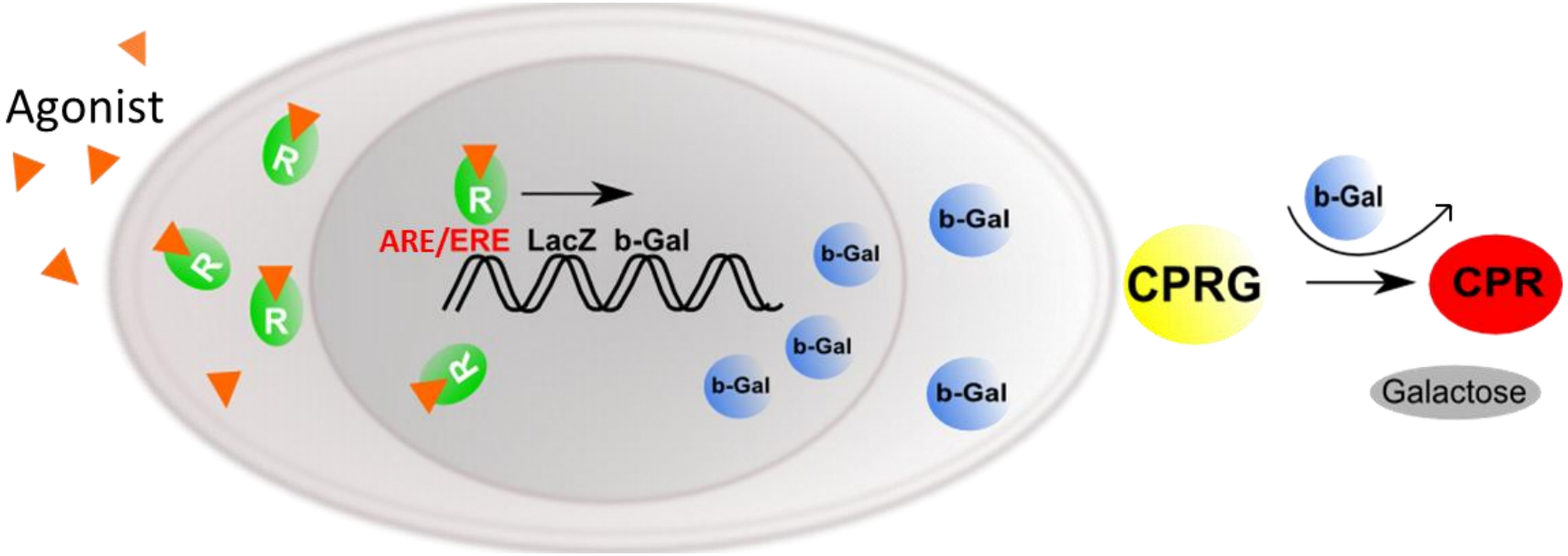


Metabolic profiling



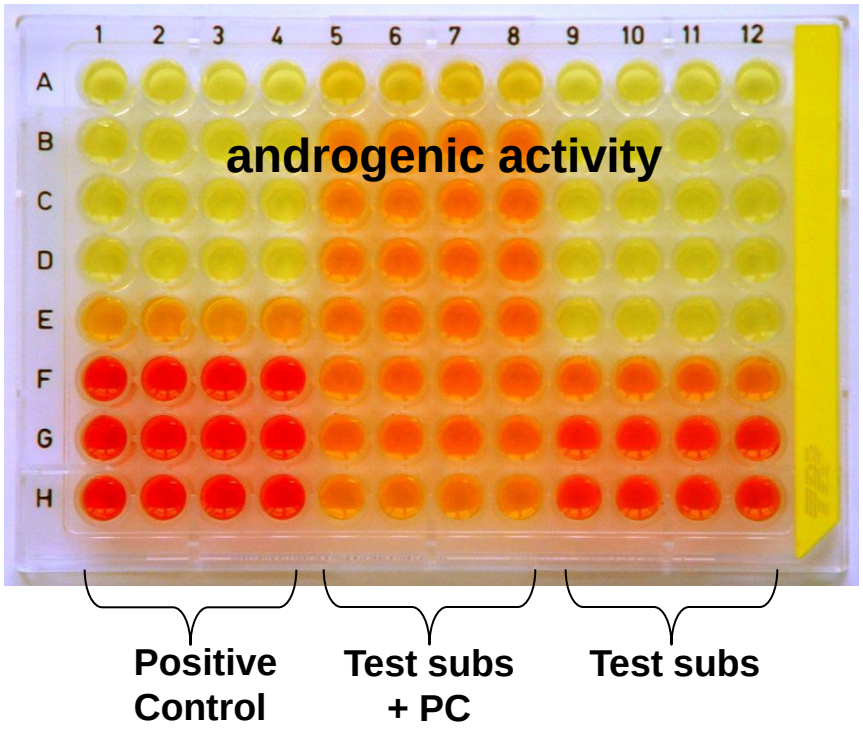
Assessment for Mammalian Endocrine Activity

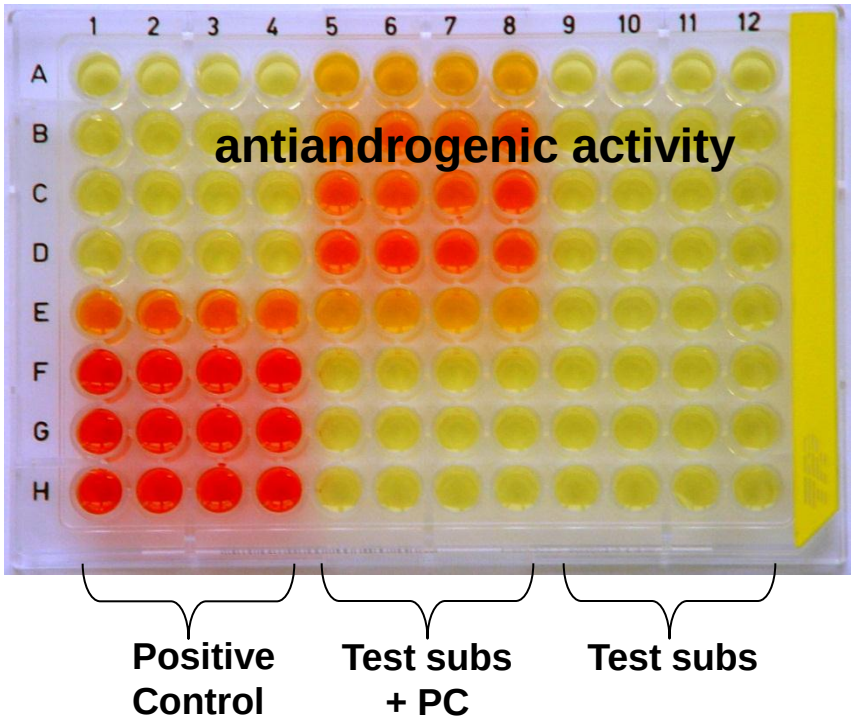
Tier 1:
Yeast Estrogen/ Androgen Screening Assays (YES/YAS)



- Yeast estrogen/ androgen screening assay
- Recombinant yeast strains expressing **human ER** or **AR** and reporter gene.
- **Agonistic** and **antagonistic** effects measureable
- Determination of effect by **reporter assay**

Tier 1:
Yeast Estrogen/ Androgen Screening Assays (YES/YAS)





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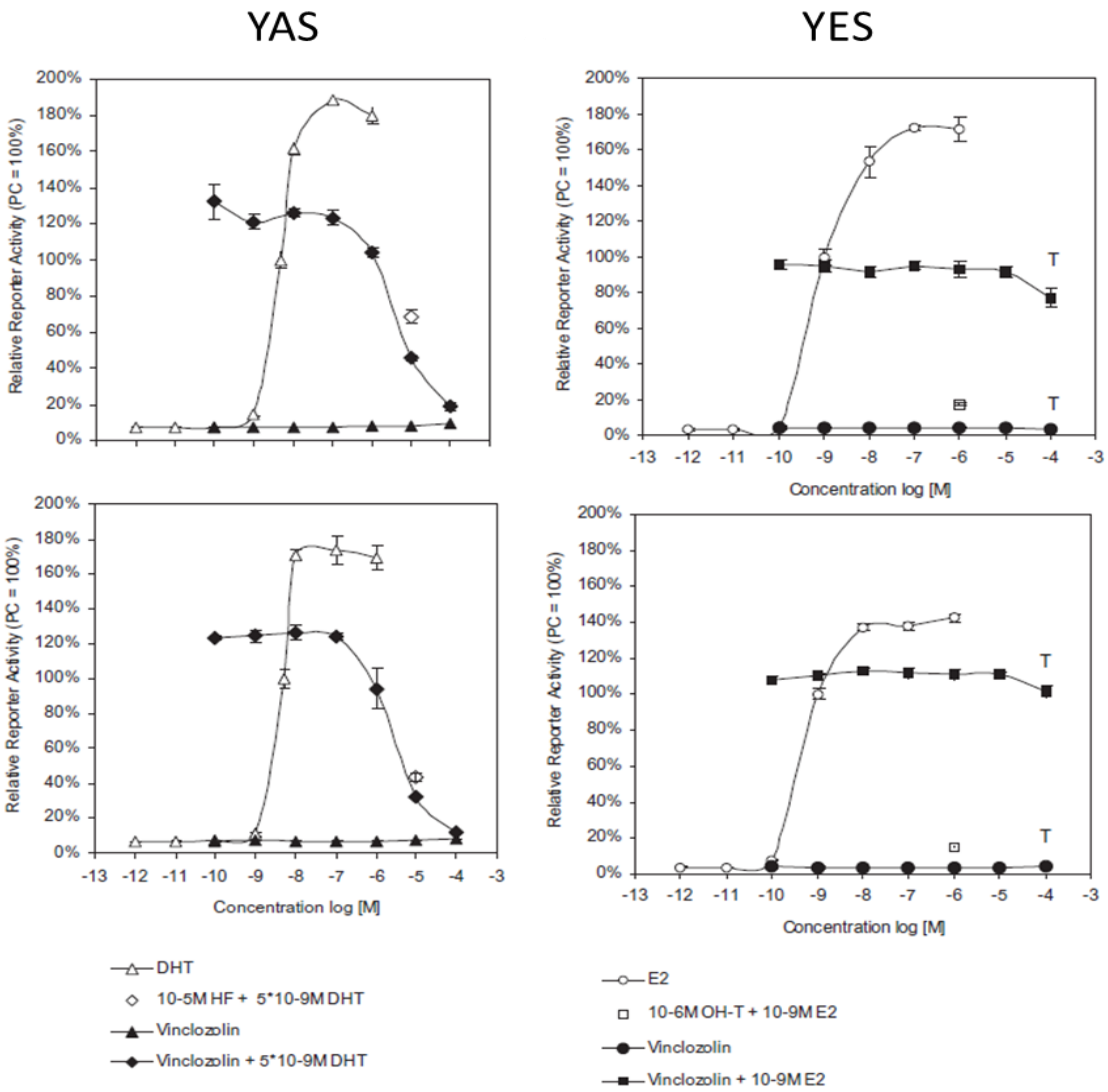

(Kolle et al., 2012, RTP)

In house validation of the YES/YAS with 105 substances

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Androgens/antiandrogens: reported activity and test results.[a,b]

Name	CAS	Class	YES/YAS Result				Literature			
			E	AE	A	AA	E	AE	A	AA
1a,25-Dihydroxyvitamin D3 (Calcitriol)	32222-06-3	Other chemicals	–	+	–	+	o	+	o	+
2,4-Dihydroxybenzo-phenone	131-56-6	Antiandrogen	+	[d][–]	–	+	+	+	–	+
4,4-Biphenol	92-88-6	Antiandrogen	+	–	(+)	+	+	+	–	+
4,4′-DDD (Dichlorodiphenyl-dichlorethane)	72-54-8	Antiandrogen	–	–	–	+	+	o	o	+
4-Androstene-3,17-dione	63-05-8	Other chemicals	–	+	+	–	–	o	+	–
4-*n*-Octylphenol	1806-26-4	Other chemicals	–	–	–	(+)	–	–	–	+
4-*tert*-Octylphenol	140-66-9	Other chemicals	+	[d][–]	–	+	+	+	–	+
6α-Methyl-17αhydroxy-progesterone(Medroxyprogesterone)	520-85-4	Androgen	–	+	+	–	–	+	–	+
Apigenin	520-36-5	Estrogen	(+)	–	–	–	+	–	o	+
Benzophenone	119-61-9	Antiandrogen	(+)	(+)	–	+	(+)	o	o	+
Benzylbutylphthalate	85-68-7	Antiandrogen	(+)	–	–	+	+	–	–	+
Bicalutamide	90357-06-5	Antiandrogen	–	+	+	[d][–]	+	o	+	+
Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	Other chemicals	–	–	–	–	o	o	+	–
Bisphenol A	80-05-7	Estrogen	+	–	–	+	+	–	–	+
Bisphenol A-dimethacrylate	3253-39-2	Estrogen	(+)	–	–	–	+	–	o	+
Corticosterone	50-22-6	Other chemicals	–	(+)	–	+	–	+	–	+
Cyproteronacetate	427-51-0	Antiandrogen	–	+	+	(+)	–	o	+	+
Dibenz[a,h]anthracene	53-70-3	Other chemicals	–	–	–	–	–	+	+	–
[c]Dibutylphtalate	84-74-2	Other chemicals	+	–	–	+	–	–	–	+
Dihydrotestosterone	521-18-6	Androgen	+	–	+	–	+	o	+	–
Dihydroxymethoxychlor (HPTE)	2971-36-0	Estrogen	+	–	–	+	+	–	–	+
Diisononylphthalate (DINP)	28553-12-068515-48-0	Other chemicals	–	–	–	–	–	–	–	+
Di-*n*-amylphthalatedipentyl phthalate	131-18-0	Antiandrogen	–	+	–	+	–	–	–	+
Estrone	53-16-7	Estrogen	+	–	+	–	+	–	+	–
Ethinylestradiol	57-63-6	Estrogen	+	–	(+)	+	+	o	–	+
Fenarimol	60168-88-9	Estrogen	+	–	–	[d][–]	+	–	o	+

Detection of estrogenic compounds with accuracy of 87%, antiestrogenic with 90%, androgenic with 95% and antiandrogenic with 85%.

(Kolle, et al., Toxicol. In Vitro 24(7), 2030-2040).

Tier 1:
Steroidogenesis assay
OECD TG 456 and OPPTS 890.1550

- The objective of the steroidogenic screen assay is to detect any substance that would disrupt estrogen and/or androgen gonadal steroid hormone production.

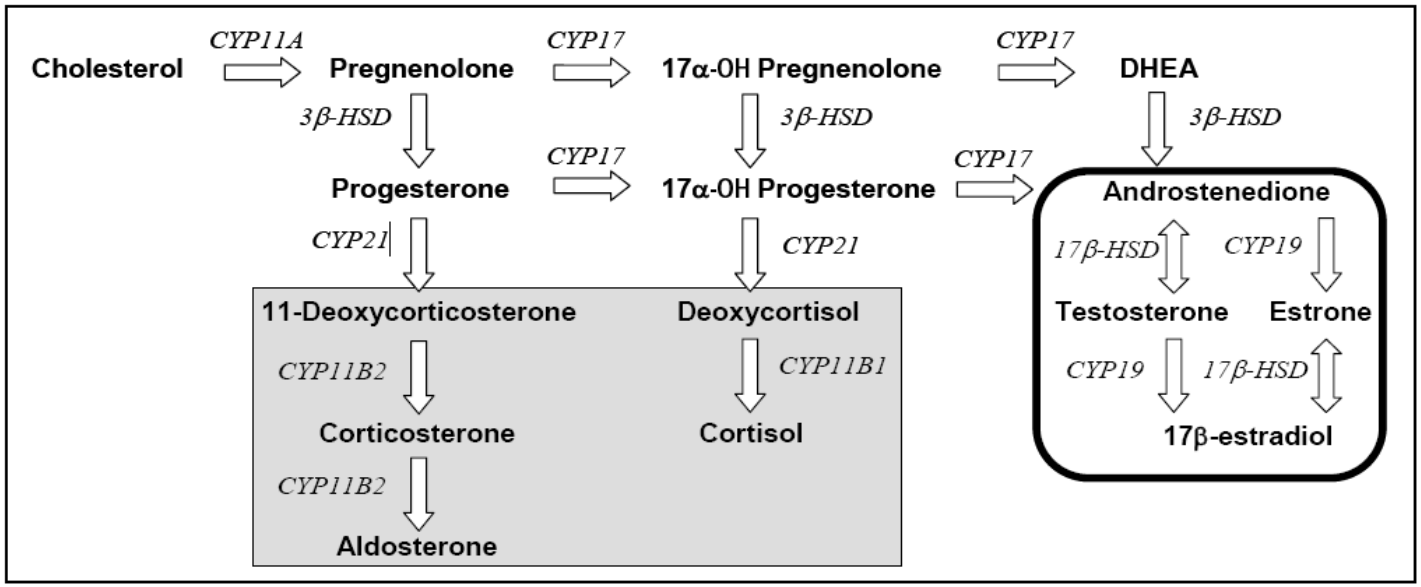
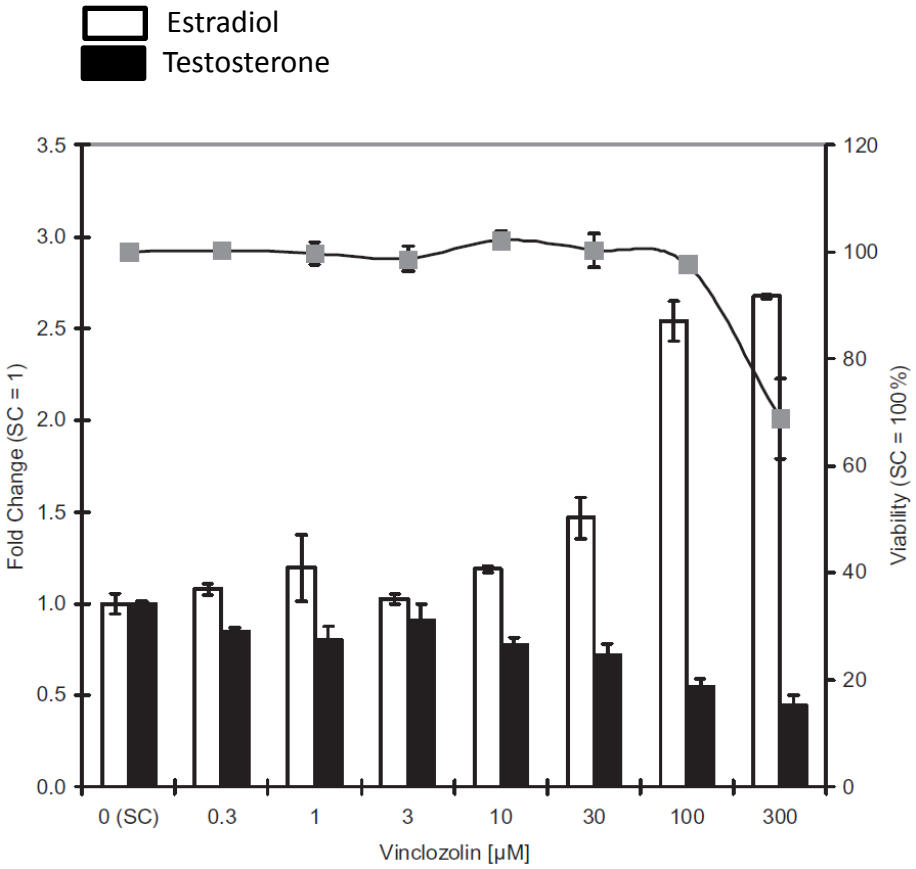
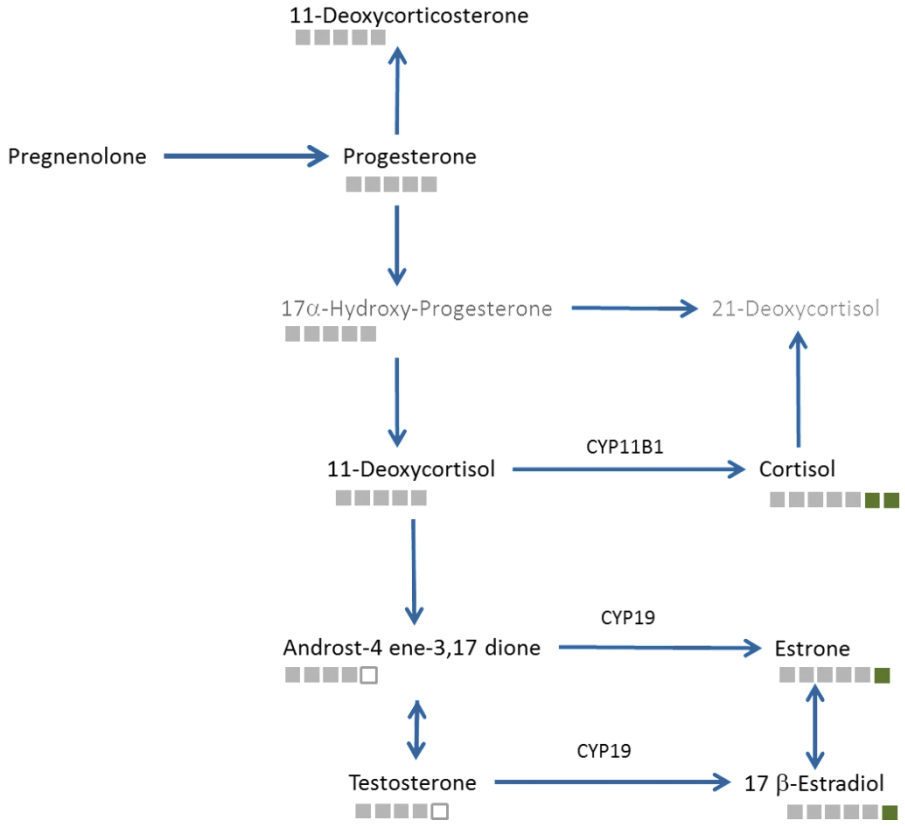


Figure 3.1: *Steroidogenic pathway in H295R cells. Enzymes are in italics, hormones are bolded and arrows indicate the direction of synthesis. Gray background indicates corticosteroid pathways/products. Sex steroid pathways/products are circled. CYP = cytochrome P450; HSD = hydroxysteroid hydrogenase.*

Summarized from EDSP, US-EPA homepage
OECD guideline 456

Tier 1:
Effects of vinclozolin on the levels of estradiol and testosterone





An integrated strategy for detection of Endocrine Modes of Action under 3Rs TIER 2

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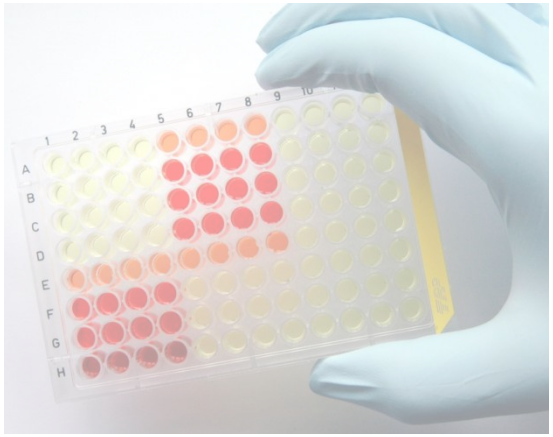
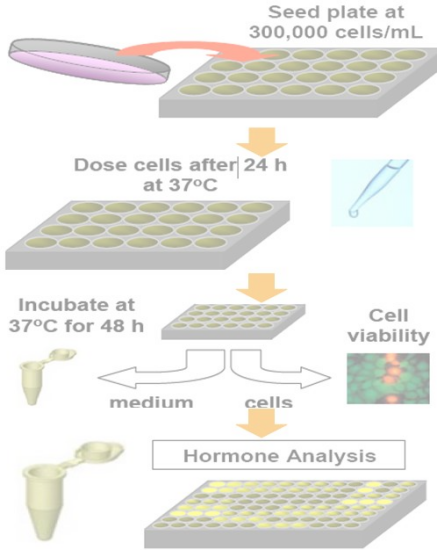
In vitro

Refined *in vivo*

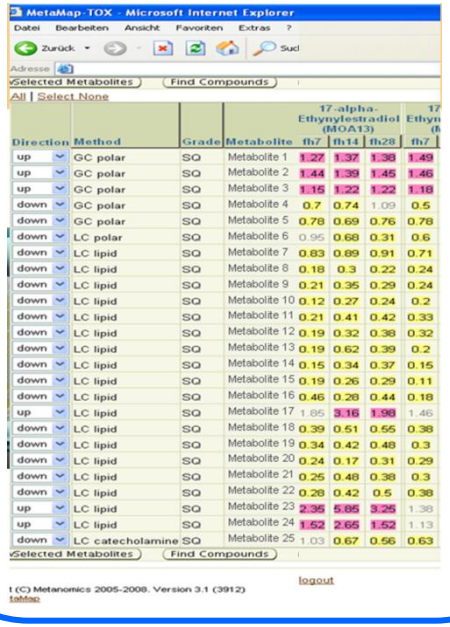
Receptor dependent



Hormone synthesis



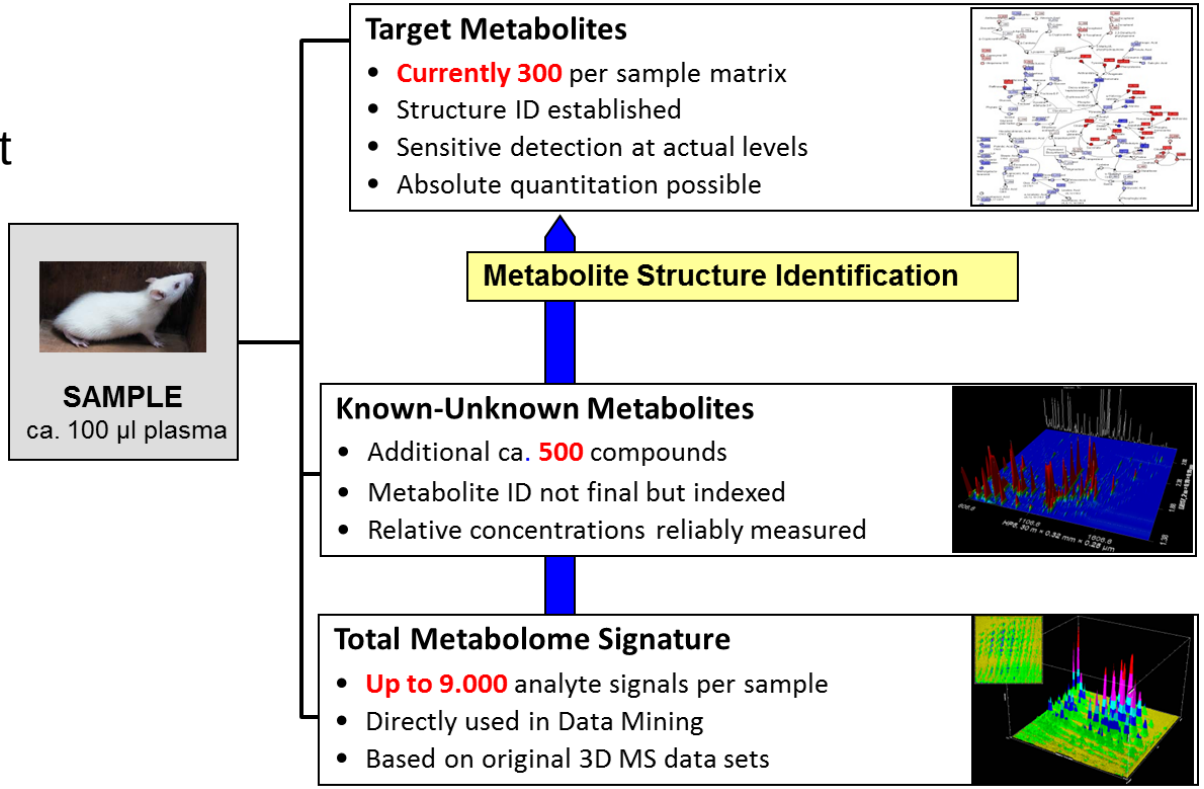
Metabolic profiling



Assessment for Mammalian Endocrine Activity

Tier 2:
In vivo 28 day study combined with metabolomics analysis

- Confirmation of *in vitro* findings (potency / NOAEL)
- Detection of compounds that only act in vivo (e.g. metabolism specific)
- OECD 407 (28 day rat study)
- Estrus cycle analysis
- Pathology of endocrine organs
- **Metabolome analysis**



Analysis of effects of 14 compounds tested *in vitro* and *in vivo*

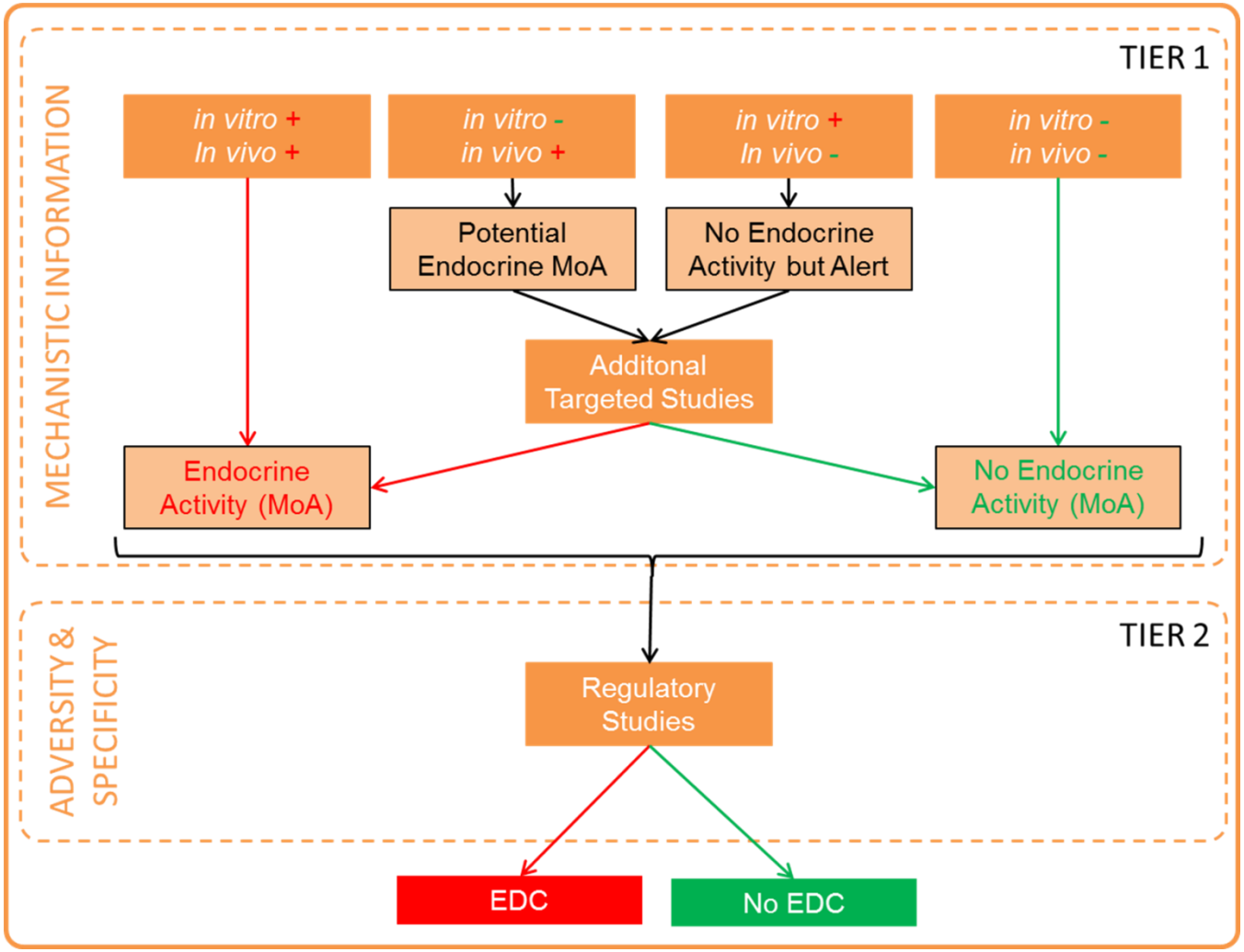
Substance	CAS No.	YES	YAS	H295R E_2 levels	H295R T levels	Metabolome analysis[e]
Bisphenol A	80-05-7	E -[a]	- AA	↑	↓	+/-
Caffeine	58-08-2	- -	- -	-[b]	-[b]	-
Colchicine	64-86-8	- -[a]	- -	-[b]	-[b]	-
		E	-			E

- Of the nine known EDCs all substances were determined to exert receptor mediated and/or biosynthesis related mechanisms.
- **The effects were confirmed by *in vivo* metabolome analysis** in the OECD TG 407 study.

testosterone	[illegible]	AE	- a	↓	↑	A
Tamoxifen	10540-29-1	E AE	- (AA)	↓	↓	E A adrenal steroid synthesis inhibition[f]
Trenbolone	10161-33-8	E -	A -[a]	-[d]	-[d]	E A
Vinclozolin	50471-44-8	- -[a]	- AA	↑	↓	AA adrenal steroid synthesis inhibition[f]
Zearalenone	17924-92-4	E -[a]	- AA	↑	↑	E

(Kolle et al., 2012, RTP)

Assessment of mammalian endocrine effects using a combination of *in vitro* assays and *in vivo* assays



(Kolle et al., 2012, RTP)

Tiered Testing for Identification of Endocrine Disruption

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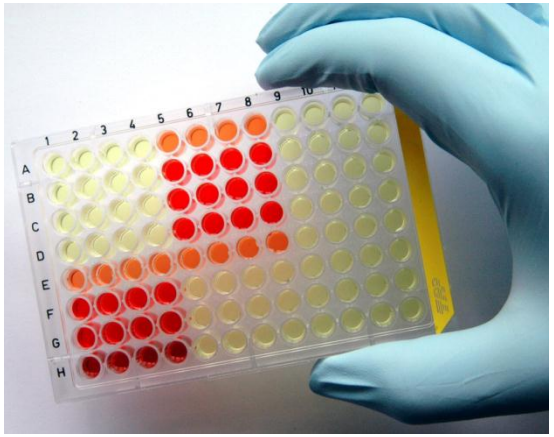
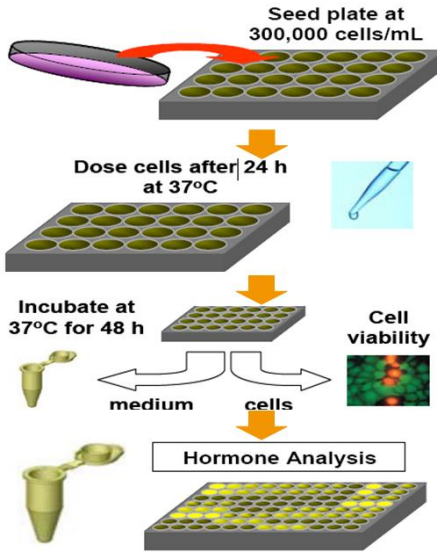
In vitro

Refined *in vivo*

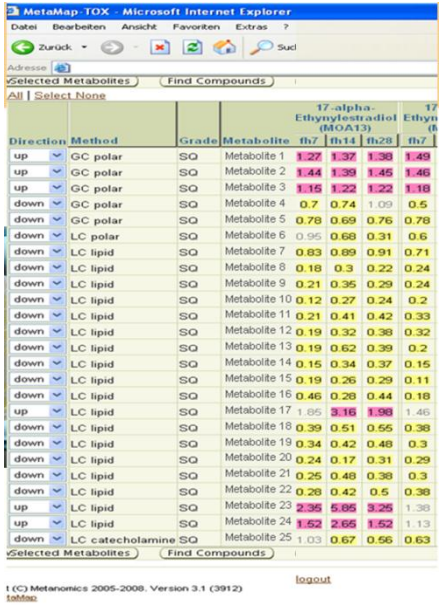
Receptor dependent



Hormone synthesis



Metabolic profiling



Retrospective analysis of in house data showed that with the proposed testing strategy additional *in vivo* studies **Hershberger assay** (OECD 441), **uterotrophic assay** (OECD 440), **pubertal assays** are not needed.

Thank you

- Laboratory of Development of Alternative Methods
- Laboratory of Applied Alternative Methods
- BASF Experimental Toxicology and Ecology
- Metanomics GmbH