EFSA / RIVM Symposium

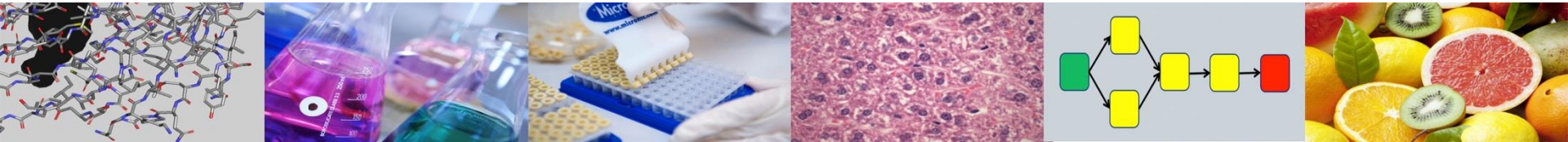
The future of risk assessment and toxicity testing for chemical mixtures

EuroMix

WP 3 Part: Endocrine Disruption

A testing approach for mixtures of endocrine disrupting chemicals

Toine Bovee, Leo v/d Ven, Jan van Benthem, Angelo Moretto, Ad Peijnenburg, Corinne Sprong, Mirjam Luijten, Alfonso Lampen and many, many more and Jacob van Klaveren



Before we start...

- I agree that in many situations it is better to use Endocrine Active Substance (EAS) instead of Endocrine Disruption Chemical (EDC), as many models measure the potential hormonal activity, but not an adverse effect...

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Introduction

- The risk of single compounds is rather well established
- Therefore, regulation is on single compounds
  - REACH Regulation
  - Water Framework Directive
- This is already a huge task...
- ...but, humans are exposed to mixtures

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- Too many possible mixtures to test → modelling is required
- Two predictive models:
  - Same mode of action
    Concentration/dose-addition model (dependent action)
  - Dissimilar mode of action
    Response-addition model (independent action)

- EFSA assumes that the concentration-addition model is sufficiently conservative to assess mixture risks...so far this seems okay...
- But, is the assumption also really valid...have the "appropriate" experiments been performed, does it hold for every mixture, or for every end point or for every adverse effect?

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WP 3 & 8 ED Focus:
AOPs involved in misbalance E-T (based on AOP WIKI's)

AOP29: ER agonism leading to reproductive dysfunction

AOP30: ER antagonism leading to reproductive dysfunction

AOP52: ER agonism leading to skewed sex ratios due to altered sexual differentiation in males

AOP24: AR antagonism leading to reproductive dysfunction

AOP19: AR antagonism leading to adverse effects in the male foetus (mammals)

AOP25: Aromatase inhibition leading to reproductive dysfunction (in fish)

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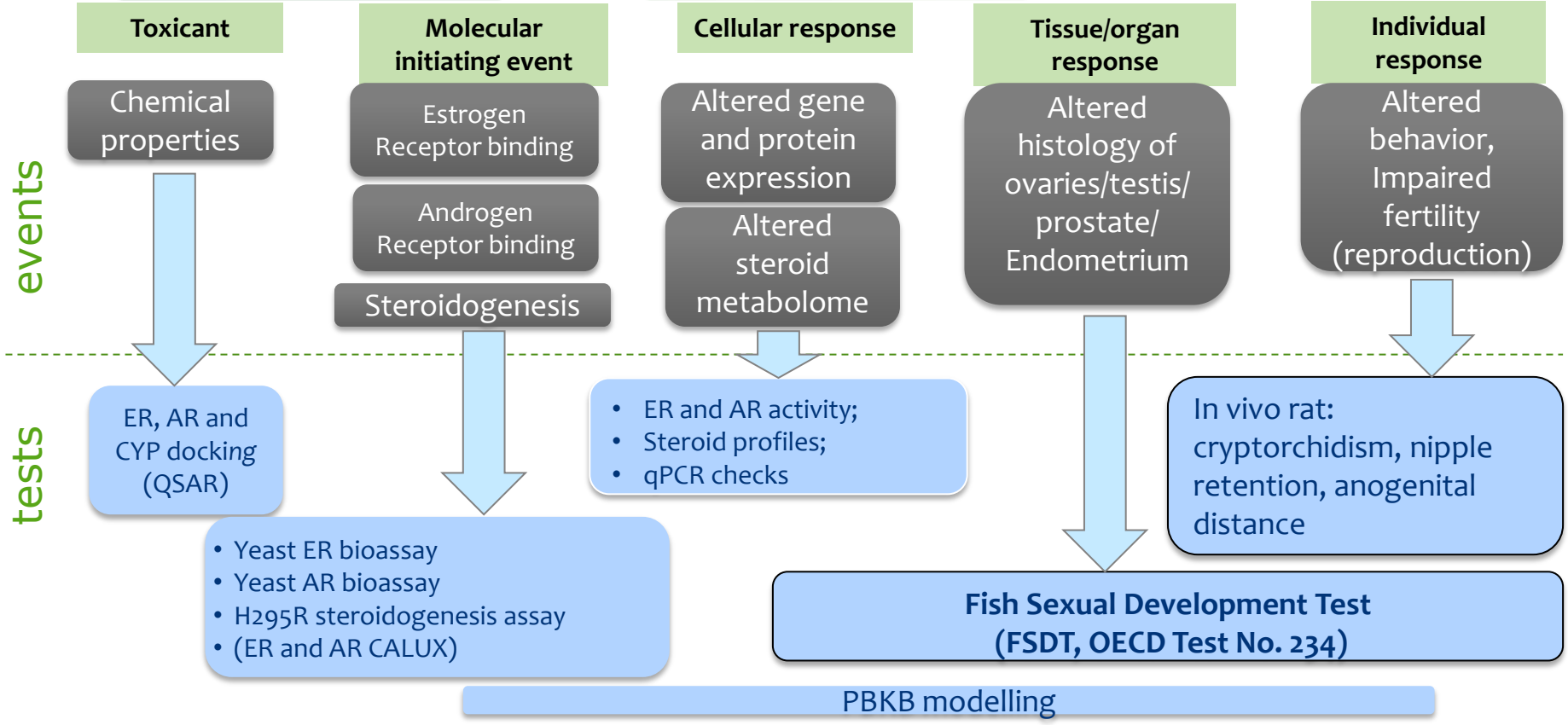
"Constructed AOP endocrine disruption" focused on E-T misbalance leading to reproductive dysfunction



EUROMIX Models and partners activities: WP 2 (left), WP 3 (centre) and WP 4 (right)

- ***In Silico:***
- ER docking/QSAR (UMIL/RIVM)
- AR docking/QSAR (UMIL/RIVM)
- CYP docking (UMIL)

- ***In Vitro***:
  - ER yeast estrogen bioassay (DLO-Rikilt)
  - AR yeast androgen bioassay (DLO-Rikilt)
  - H295R steroidogenesis assay (DLO-Rikilt) with qPCR on CYP genes (BPI)

- ***In Vivo***:
  - Rat – e.g. nipple retention (NR); cryptorchidism and anogenital distance (AGD) in male pups (URV)
  - FSDT (OECD 234) (RIVM)

Selection compounds and mixtures
(WP2, Wp5: Corinne Sprong, Amélie Crépet, Annika Hanberg, Anna Beronius, Bodil Jensen, Jiri Ruprich & many more)

Meeting _____ Date _____

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WP 3 & 8 ED Focus: AOPs involved in misbalance E-T (based on AOP WIKI's)

Reference compounds:

ER agonism: e.g. dienestrol , or 8-PN (a pure estrogen)

AR antagonism: e.g. flutamide (a pure anti-androgen)

Aromatase induction: e.g. BPA (there is no pure aromatase inducer !)

Exposure driven (WP2/WP5): searching pesticides with effects on e.g. **AGD, cryptorchidism, NR**, puberty onset and behaviour (mammalians) or **feminisation and sex reversal (fish)**

In silico (WP2) and in vitro (WP3): pattern: ER-agonism, AR-antagonism and aromatase induction (Combination with EDCs in diet with same pattern, e.g. bisphenols, zearalenone and its derivatives? (known compounds with such activity pattern: e.g. EE2, DES and BPA)

Test mixtures in vivo (WP4)

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Okay...

- Nice, focused and simplified “constructed AOP endocrine disruption”: focused on E-T misbalance leading to reproductive dysfunction only, but...
- How good are the selected in silico and in vitro models/tools?

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ER, AR and CYP
WP2 Docking and QSAR

WP2 Ad Peijnenburg – **Ivano Eberini & Emiel Rorije**, Angelo Moretto, Jane Cotteril, and many more

Meeting

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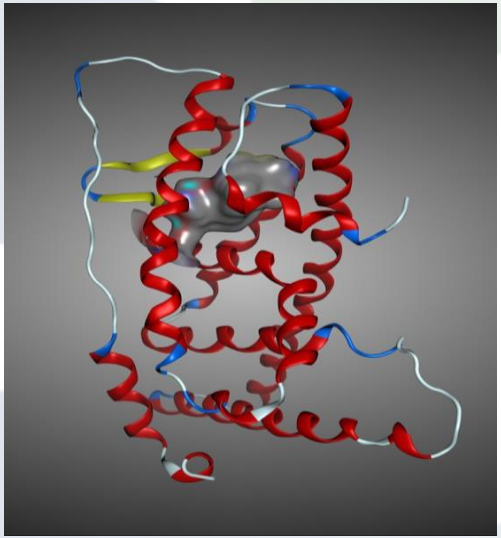
Molecular docking of compounds from EUROMIX databases on h-Estrogen Receptor alpha LBD (h-ERα)

All the compounds from several EUROMIX databases were docked into the LBD of h-ERα and complex binding free **energies** were computed. The following table reports just the ligands with an affinity for the tested receptor higher than the natural hormone one.

Stronger binders than estradiol.



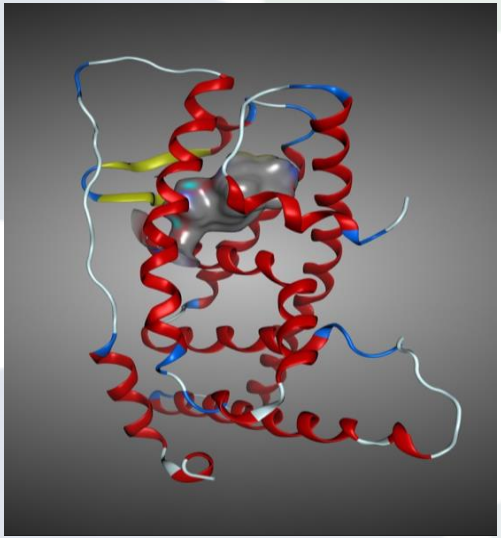
Compound	Binding free energy (kcal/mol)	dataset
Ethanol, 2-[2-(4-nonylphenoxy)ethoxy]-	-9.2	59_Easis.sdf
131860338 - Azoxystrobin	-8.4	271_PPP.sdf
17924924 - Zearalenone	-8.1	20_Mycotoxin.sdf
17 beta-estradiol	-8.1	Natural ligands

This project is funded by the Horizon 2020 Framework Programme of the European Union

Molecular docking of compounds from an initial EUROMIX WP 3 database on the h-ERα LBD

The following table reports the binding free energy values for selected compounds from an initial WP3 dataset dealing with **steatosis!**. Their affinities are lower than that of the natural hormone 17β-estradiol.

Weak, moderate to strong binders.



Compound ID	Binding free energy (kcal/mol)	dataset
17 beta-estradiol	-8.1	Natural ligands
67747095 - Prochloraz	-7.9	6_WP3
94361065 - Cyproconazole	-5.9	6_WP3
1948330 – tBHQ	-5.3	6_WP3
50892234 - Wy14643	-4.9	6_WP3
103902 - Acetaminophen	-4.4	6_WP3
10238218 - Glibenclamide	-4.2	6_WP3

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Molecular docking of selected training compounds: of interest for WP 3 ED (in vitro hormonal activities)

Among, specifically for hER and hAR: dienestrol, hexestrol, 17β-estradiol, DES, EE2, zearalenone, 8-prenylnaringenin, equol, BPA, flutamide, hydroxyflutamide, nilutamide, and flavone. Only zearalenone was included in the Euromix (mycotoxin) database.
Not everything is shown.

Compound ID	hPR	hAR	hER-α	hER-β
17 beta-estradiol	-2.6	-8.3	-8.1	-7.7
Dihydrotestosterone	-1.3	-9.2	-7.3	-5.3
Progesterone	-8.1	-4.4	-6.8	-3.8
Zearalenone - 17924924	-6.6	-5.4	-8.1	-7.9

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Molecular docking of selected pesticides: of interest for WP3 ED

Among: Deltamethrin, Dimethoate, Esfenvalerate, Triadimenol, Chlorpyrifos-methyl, Iprodione, Prochloraz, Linuron, Vinclozolin, Procymidone, p,p'-DDE, and DDT.
Not everything is shown.

Compound ID	hPR	hAR	hER-α	hER-β
17 beta-estradiol	-2.6	-8.3	-8.1	-7.7
Dihydrotestosterone	-1.3	-9.2	-7.3	-5.3
Progesterone	-8.1	-4.4	-6.8	-3.8
Deltamethrin - 52918635	-4.8	-5.8	-3.4	0.5
Dimethoate - 60515	-6.0	-5.4	-5.4	-5.6
Esfenvalerate - 66230044	-2.2	-3.4	-6.6	-3.2
Chlorpyrifos-methyl - 5598130	-6.6	-5.1	-5.2	-6.1
Triadimenol - 55219653	-7.7	-6.9	-7.3	-6.1
Prochloraz - 67747095	-6.7	-6.2	-7.9	-6.8
Linuron - 330552	-6.2	-5.9	-6.3	-6.3

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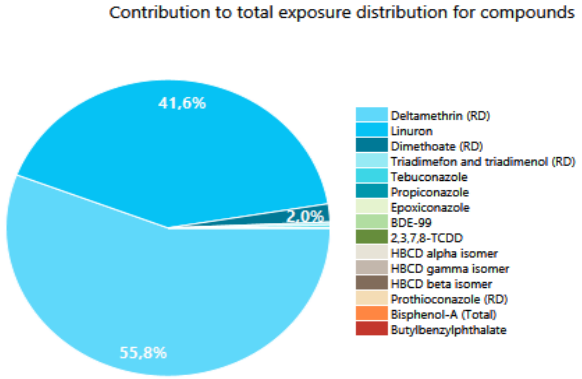
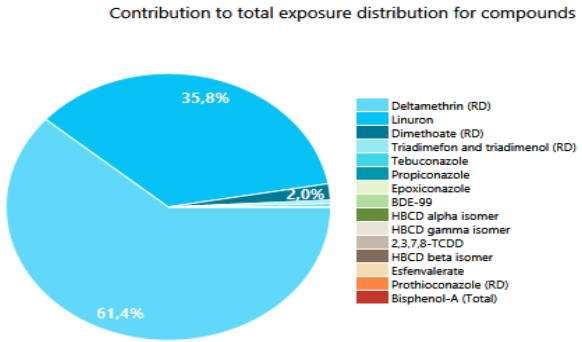
WP5 Exposure

CAG: Anogenital, all substances, women					
Chronic			Acute		
Variance explained by the mixture : 64%			Variance explained by the mixture : 98%		
Name	Code	NMF value	Name	Code	NMF value
Linuron	RF-0264-001-PPP	67%	Linuron	RF-0264-001-PPP	51%
Deltamethrin (RD)	RF-0120-001-PPP	33%	Deltamethrin (RD)	RF-0120-001-PPP	49%
Dimethoate (RD)	RF-0139-001-PPP	1%			

Selection compounds and mixtures
(WP2, Wp5: Corinne Sprong, Amélie Crépet, Annika Hanberg, Anna Beronius, Bodil Jensen, Jiri Ruprich & many more, role of Hilco and Waldo)





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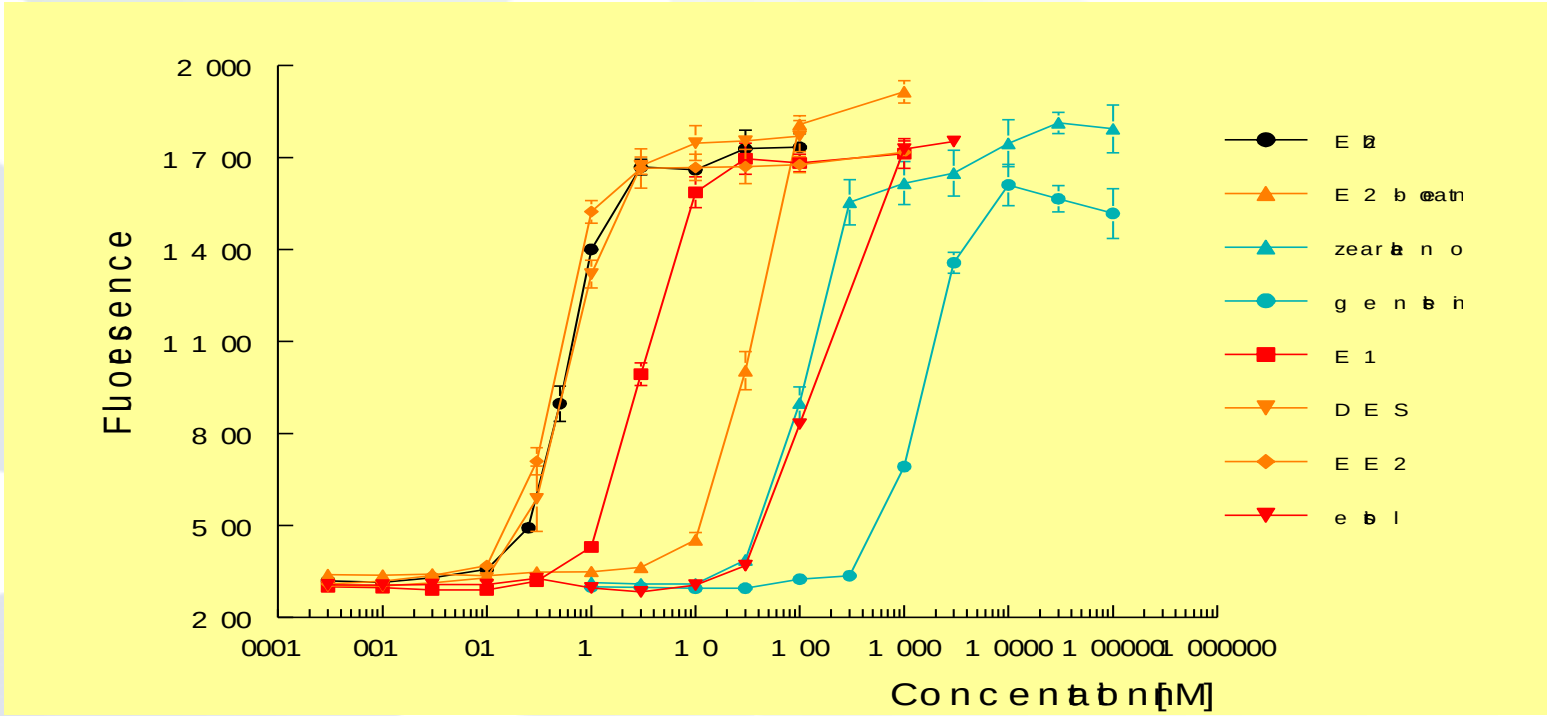
WP3 ER, AR and H295R (CYP) activities
The yeast estrogen bioassay (hERα)



Bovee et al., *Gene* **325** (2004) 187-200
Bovee et al., *JSBMB* **91** (2004) 99-109

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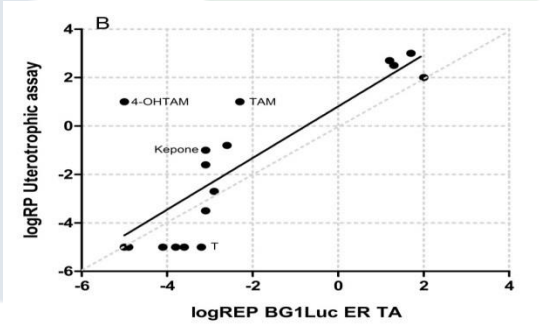
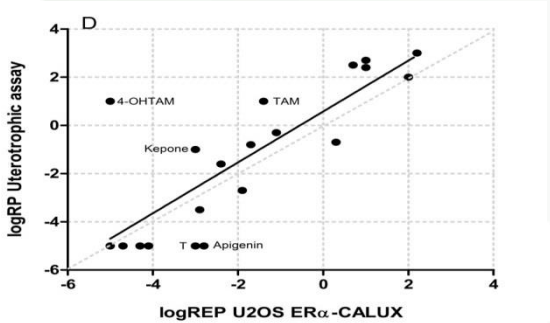
In vitro estrogenicity testing vs in vivo

R^2=0.62



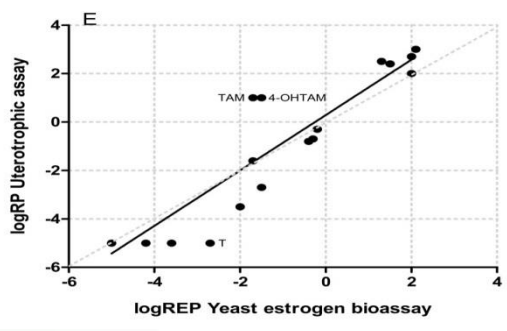
BG-1Luc ER TA
OECD TG457

R^2=0.68



ERα-CALUX TA
Me too test in OECD TG

R^2=0.87



Wang et al., *ALTEX* **30** (2013) 145-157
Wang et al., *J. Appl. Toxicol*. **34**: (2014) 1031-1040

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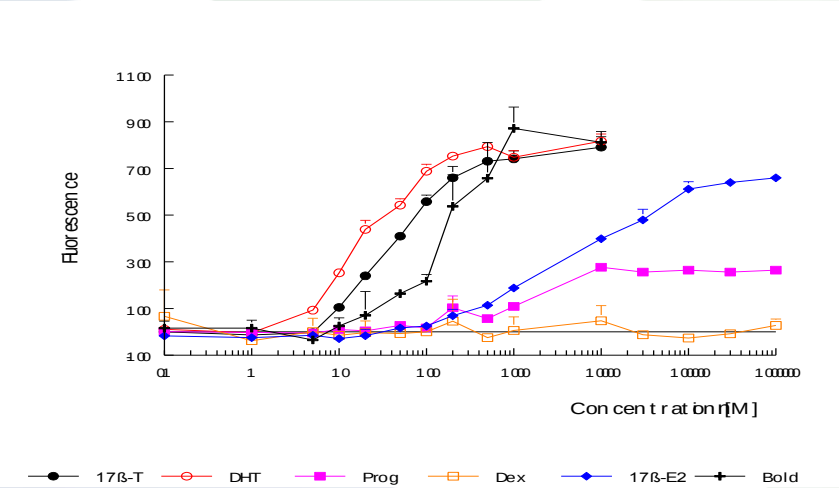
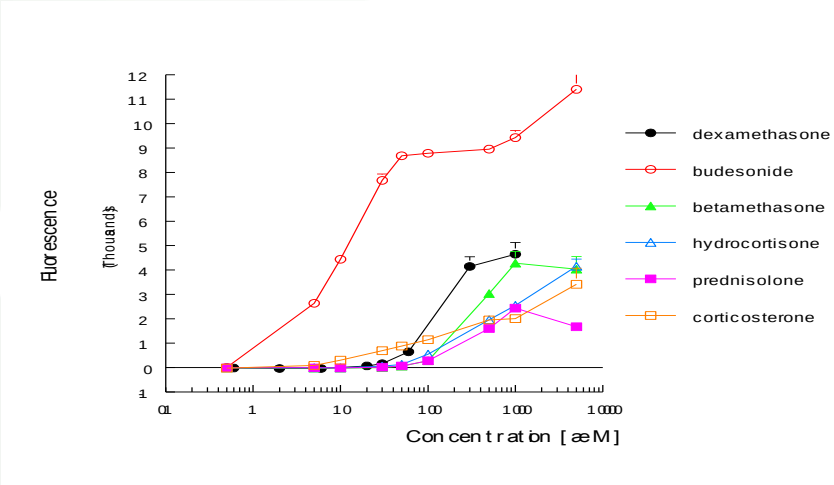
Similarly a yeast androgen bioassay and yeast corticoid bioassay were developed and validated (compounds and urine and feed samples)





Bovee et al., *ABC* **389** (2007) 1549-1558
Bovee et al., *ABC* **401** (2011) 873-882

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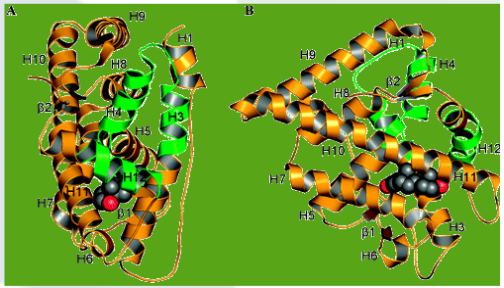
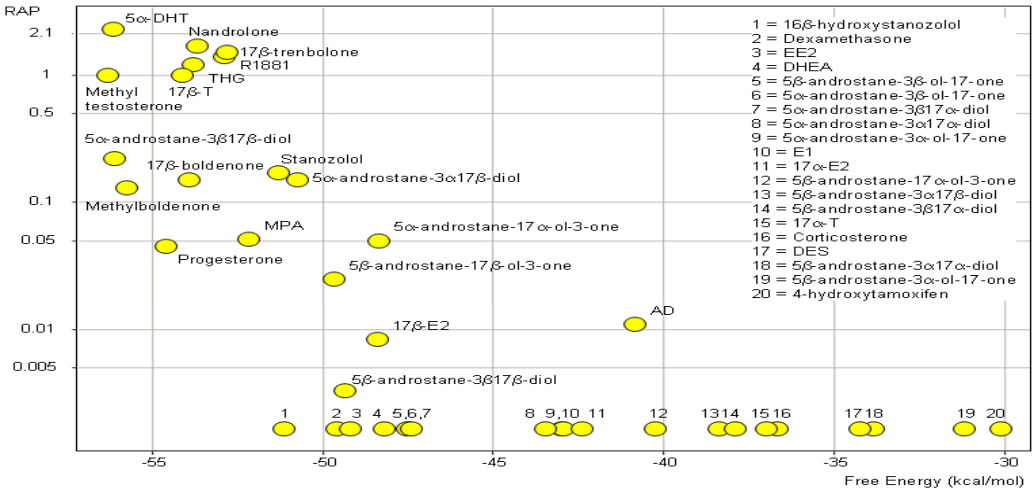
In vitro androgenicity vs in silico docking





*T.F.H. Bovee et al., JSBMB **108** (2008) 121-131*

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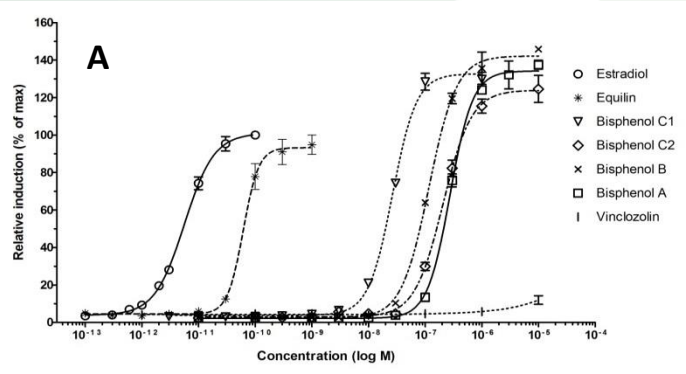
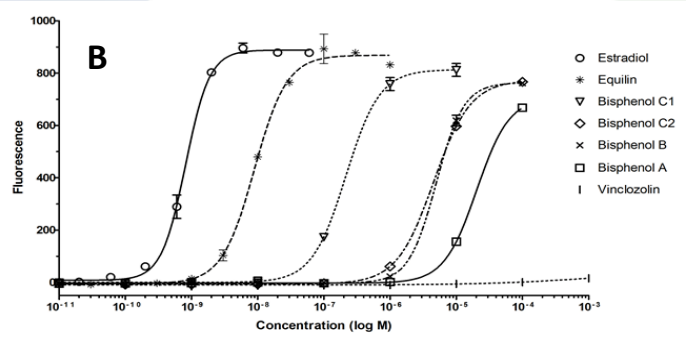
In vitro estrogenic potency of bisphenols



A: BDS U2OS ERα-CALUX®



B: RIKILT Yeast Estrogen Bioassay

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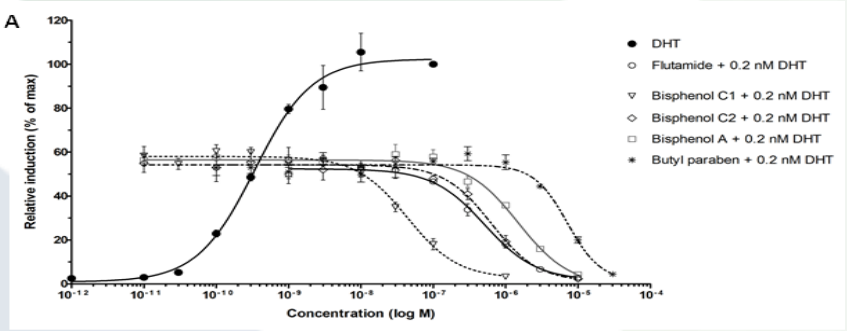
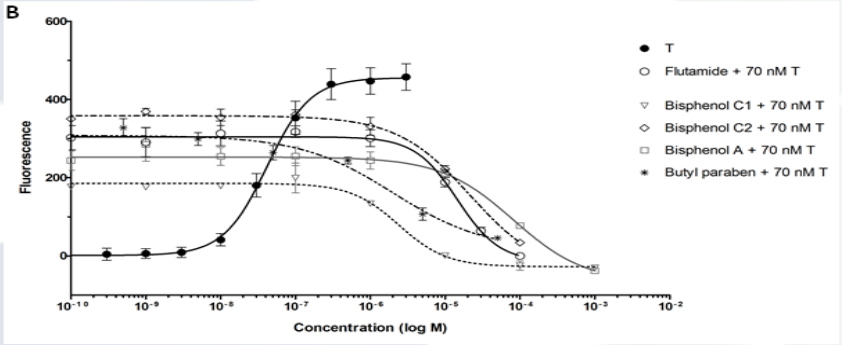
In vitro androgenic potencies of bisphenols



A: BDS U2OS AR-CALUX®



B: RIKILT Yeast Androgen Bioassay

Wang S, Rijk JC, Besselink HT, Houtman R, Peijnenburg AA, Brouwer A, Rietjens IM, Bovee TF (2014) Extending an in vitro panel for estrogenicity testing: the added value of bioassays for measuring antiandrogenic activities and effects on steroidogenesis. *Toxicol. Sci.* **141**: 78-89.

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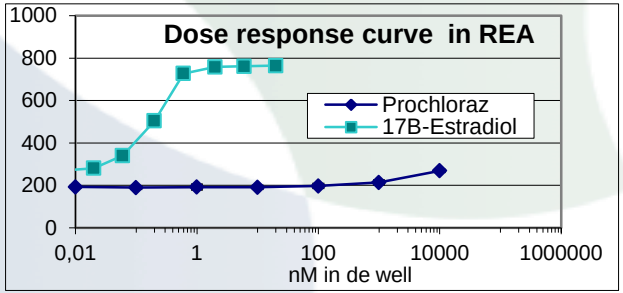
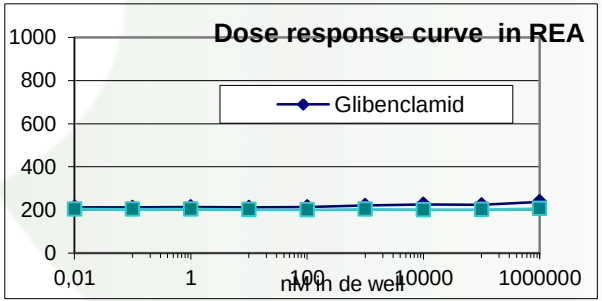
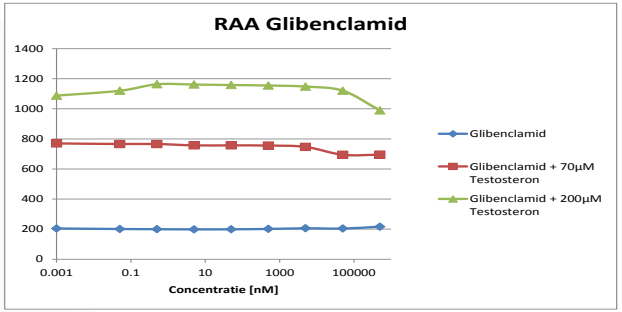
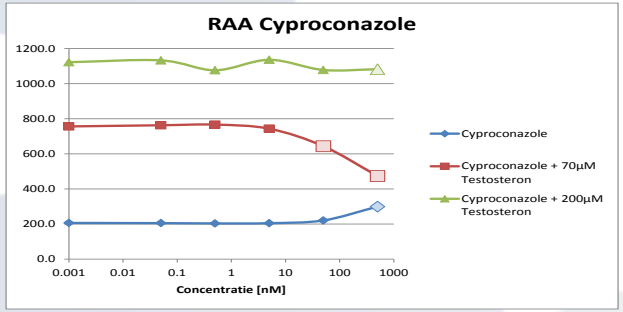
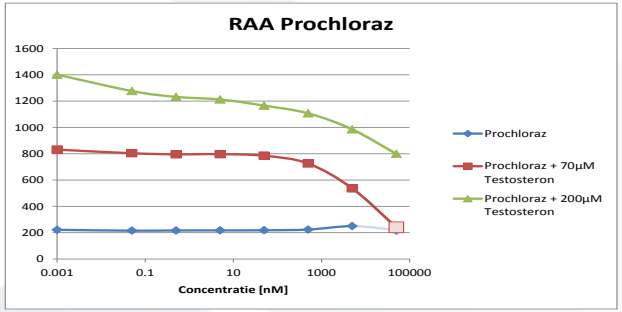
WP3 ER, AR and H295R (CYP) activities reference compounds Steatosis!

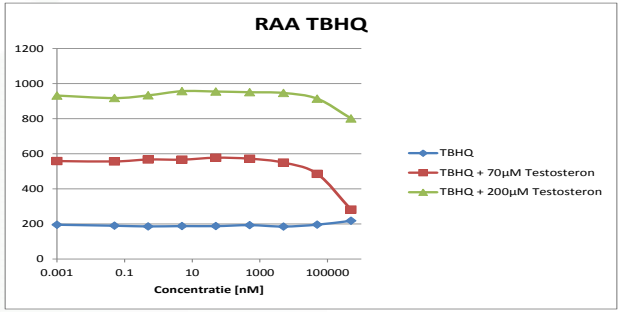












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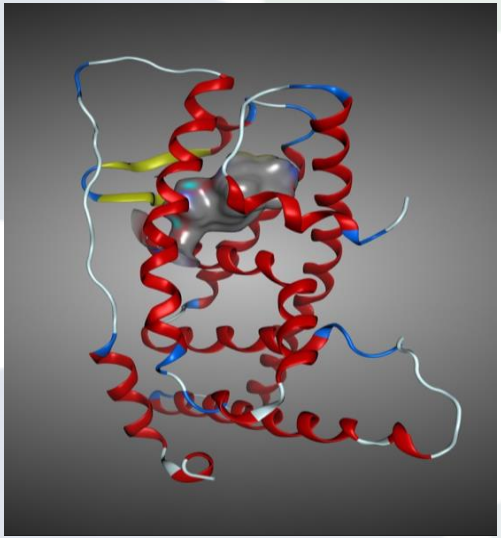
Remember this previous slide…

The following table reports the binding free energy values for selected compounds from an initial WP3 dataset dealing with **steatosis!**. Their affinities are lower than that of the natural hormone 17β-estradiol.

Weak, moderate to strong binders.



Compound ID	Binding free energy (kcal/mol)	dataset
17 beta-estradiol	-8.1	Natural ligands
67747095 - Prochloraz	-7.9	6_WP3
94361065 - Cyproconazole	-5.9	6_WP3
1948330 – tBHQ	-5.3	6_WP3
50892234 - Wy14643	-4.9	6_WP3
103902 - Acetaminophen	-4.4	6_WP3
10238218 - Glibenclamide	-4.2	6_WP3

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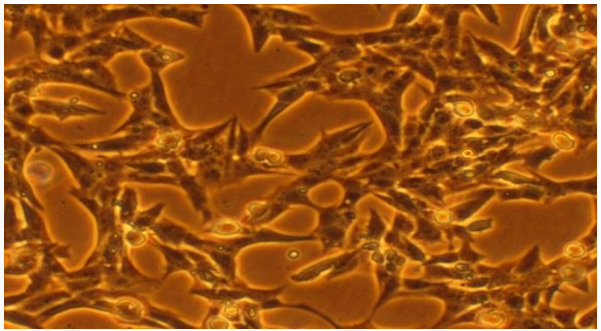
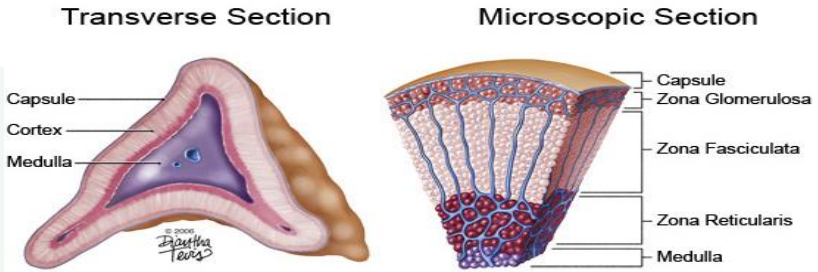
The extended H295R steroidogenesis assay

- Zonally undifferentiated foetal adrenal cells originating from a human adenocarcinoma



- Express all genes and enzymes involved in steroidogenesis



- OECD validated (TG 456)

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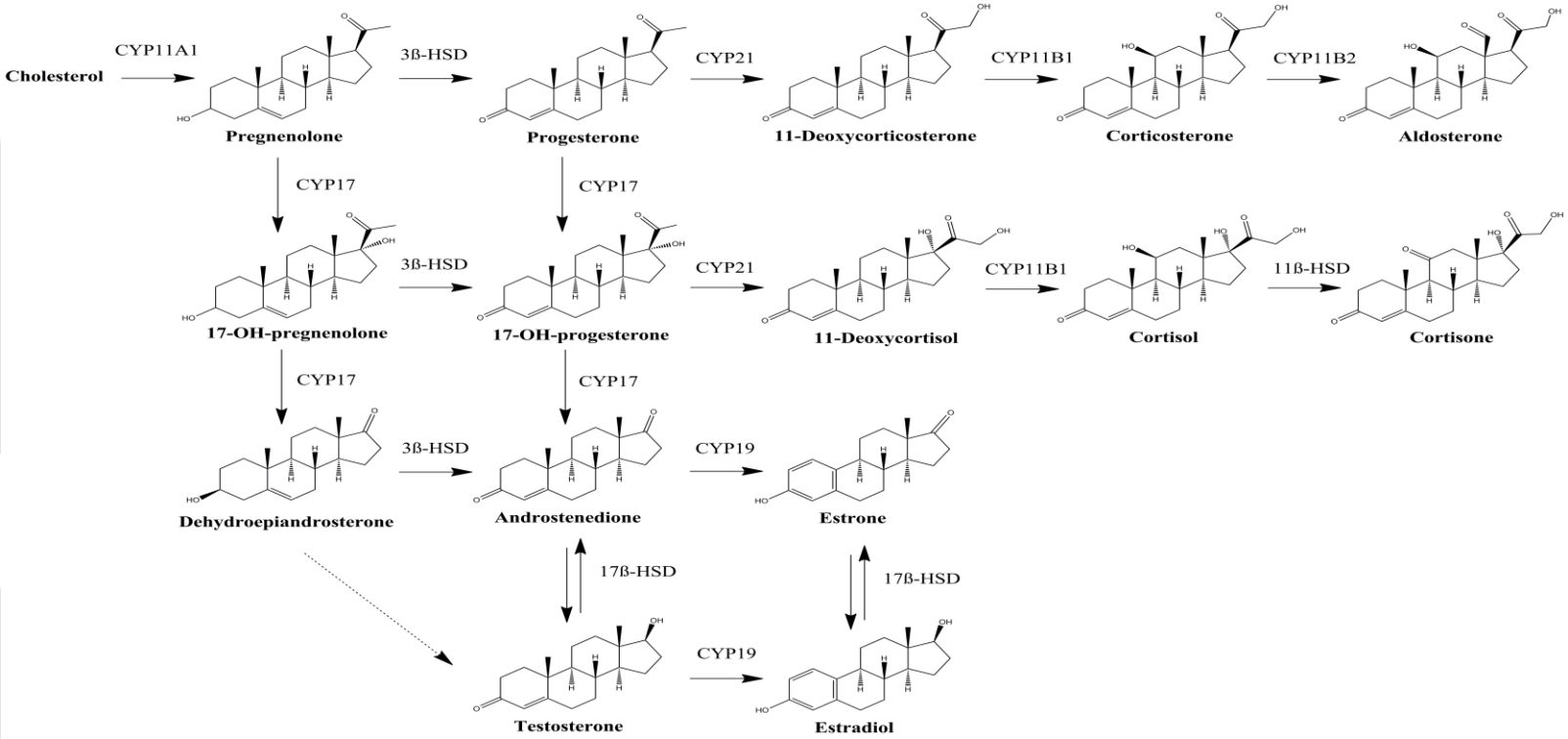
The extended H295R steroidogenesis assay



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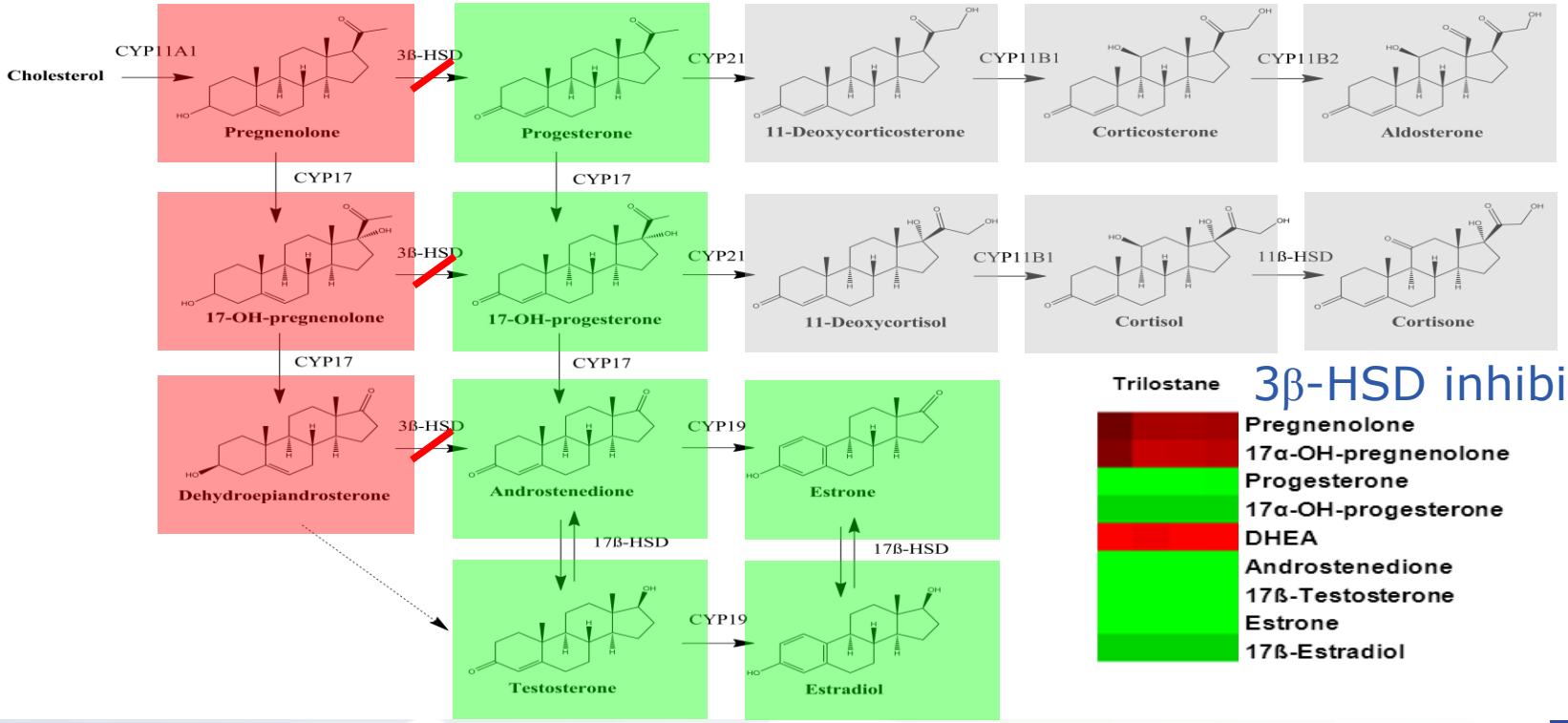
Trilostane effects in the H295R assay
GC-MS/MS analysis of exposed cells



Meeting

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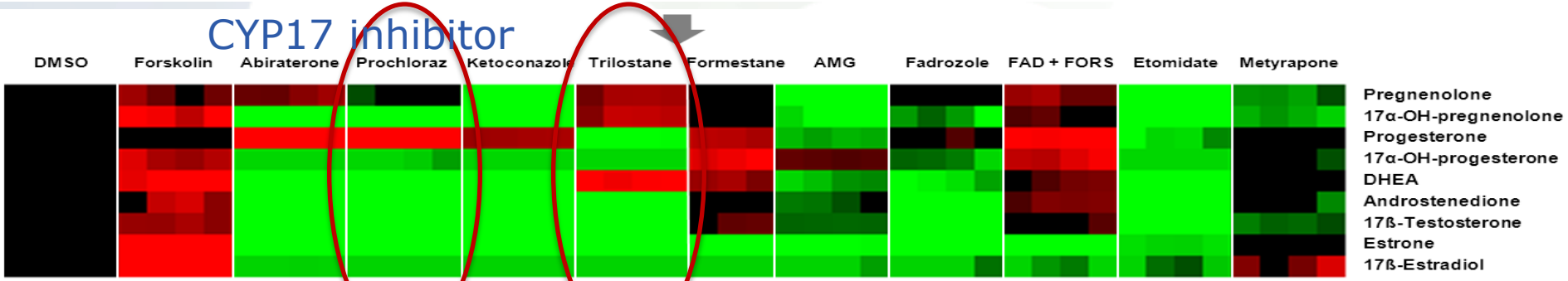
Effects in the H295R assay – GC-MS/MS

DMSO	Forskolin	Abiraterone	Prochloraz	Ketoconazole	Trilostane	Formestane	AMG	Fadrozole	FORS + FAD	Etomidate	Metyrapone	Hormone
1.00±0.14	1.79±0.45*	1.97±0.31**	0.87±0.39	0.1±0.00***	1.15±0.16	0.78±0.27	0.17±0.03***	1.00±0.12	2.06±0.37**	0.07±0.02***	0.49±0.11**	Pregnenolone
1.00±0.11	4.53±2.02*	>0.01***	0.04±0.03***	>0.01***	2.65±0.41***	0.85±0.33	0.15±0.05***	0.40±0.17**	1.52±0.13**	0.05±0.00***	0.38±0.05***	17α-OH-pregnenolone
1.00±0.10	1.20±0.19	135±17.2***	6.12±0.34***	2.41±0.09***	0.22±0.04***	2.30±0.71*	0.38±0.03***	1.45±0.10**	4.32±0.31***	0.33±0.10***	0.97±0.06	Progesterone
1.00±0.09	2.70±0.47***	>0.07***	0.34±0.06***	>0.07***	>0.07***	3.21±1.00**	1.62±0.07***	0.49±0.00**	3.20±0.54**	0.07±0.00***	0.77±0.14	17α-OH-progesterone
1.00±0.10	5.15±1.30**	>0.05***	>0.05***	>0.05***	3.97±0.21***	1.98±0.57**	0.40±0.08***	0.30±0.07***	1.68±0.28*	0.10±0.01***	0.95±0.14	DHEA
1.00±0.15	2.40±0.80***	0.01±0.00***	0.01±0.00***	0.00±0.00***	0.01±0.00***	0.82±0.30	0.61±0.12*	0.11±0.03***	1.86±0.25**	0.04±0.01***	0.79±0.23	Androstenedione
1.00±0.06	2.29±0.16***	0.08±0.01***	0.07±0.01***	0.05±0.00***	0.07±0.00***	1.35±0.44	0.57±0.01***	0.19±0.03***	1.40±0.14*	0.05±0.01***	0.58±0.07***	17ß-Testosterone
1.00±0.15	21.9±2.74***	0.09±0.01***	>0.08***	>0.08***	>0.08***	>0.08***	0.22±0.03***	>0.08***	>0.08***	0.30±0.01***	0.09±0.25	Estrone
1.00±0.34	39.8±2.20***	>0.30*	>0.30*	>0.30**	>0.30***	>0.30***	>0.30*	>0.30*	>0.30*	0.35±0.29	2.07±0.74	17ß-Estradiol

CYP17 inhibitor



Rijk et al., *Chem. Res. Toxicol.* **25** (2012) 1710-1731

Meeting

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WP3 ER, AR and H295R (CYP) activities (reference) compounds

	REA;RAA and H295R testing					
compound name	**ER**	**ant-ER**	**AR**	**ant-AR**	**H295R**	**comment**
17beta-estradiol	x	na	x	x		ER-agonist; AR-agonist; AR-antagonist
17alpha-ethynylestradiol (EE2)	x		n	x		ER-agonist; AR-antagonist
dienestrol	x		?	?		ER-agonist
hexestrol	x		n	x		ER-agonist
DES	x		n	x		ER-agonist; AR-antagonist
zearalenone	x		n	?		ER-agonist
8-prenylnaringenin	x	n	n	n		ER-agonist
equol	x	n	n	x		ER-agonist; AR-antagonist
flutamide	n		nd	x		AR-antagonist
hydroxyflutamide			?	x		AR-agonist: AR-antagonist
nilutamide			n	x		AR-antagonist
flavone	n	x	n	x		ER-antagonist; AR-antagonist
bisphenol A (BPA)	x		n	x	x	ER-agonist; AR-antagonist; CYP19 inducer
atrazine	n		n			CYP19 inducer (mRNA)
forskolin	nd	nd	nd	nd	x	adenylyl cyclase activator
trilostane	nd	nd	nd	nd	x	3beta-HSD inhibitor
prochloraz	**?**		n	x	x	
glibenclamide	n		n	n		
TBHQ	n		n	x		
acetaminophen	n		n	n		inhibitor of prostaglandin synth.: Christ. 2012
Wy 14643	n		n	n		PPARalpha agonist
AM 580	n		n	n		
CD 3254	n		n	n		RXRalpha agonist
cyproconazole	n		?	x		
vinclozolin	n	nd	n	nd		AR-antag.: Christiansen et al 2012
procymidone	n	x	n	nd		AR-antag.: Christiansen et al 2012
linuron	n	nd	n	nd		AR-agonist; AR-antag.: Christiansen et al 2012
p,p'-DDE	n	nd	n	nd		AR-agonist; AR-antag.: Christiansen et al 2012
DDT	x	nd	n	nd		ER-agonist; ER-antagonist; AR-antagonist

Meeting Date

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WP4 in vivo
Fish Sexual Development Test (OECD Test No. 234)

Test No. 234: Fish Sexual Development test

This Test Guideline describes an assay that assesses early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (e.g. oestrogens, androgens and steroidogenesis inhibitors) on fish sexual development. In the test, **fish are exposed, from newly fertilized egg until the completion of sexual differentiation at about 60 days post hatch**, to at least three concentrations of the **test substance dissolved in water**. In each treatment level and control(s) group(s), a minimum of four replicates is recommended. At termination of the test, **two core endpoints are measured in each fish**: **vitellogenin concentration** from head and tail or from blood sampling, and **proportion of males, females, intersex and undifferentiated fish** through gonadal histology. In fish species possessing a genetic sex marker, the genetic sex is identified to determine sex reversal in individual fish. The combination of the two core endocrine endpoints, vitellogenin concentration and phenotypic (and possibly genotypic) sex ratio, enable the test to indicate the mode of action of the test chemical.

This project is funded by the Horizon 2020 Framework Programme of the European Union

Experimental testing

	in vitro models	(in vivo) zebrafish	in vivo rat
as single exposures			
mode of action	✓		✓
relative potencies	✓	✓	✓
compound selection	✓		
as binary mixtures			
similar mode of action	✓	✓	✓
dissimilar mode of action	👎	✓	✓
as complex mixtures			
human relevance (exposure)	👎	✓	✓

Rat in vivo test

WP4 – Angelo Moretto, Jan van Benthem, Leo van de Ven, Tania Garcia, Margarita Torrente, Marti Nadal

URV: Rat - AGD and cryptorchidism (optional nipple retention (NR), we should include this)

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Euromix complementary with...
Exchange data, mixtures...

EDC-MixRisk

prenatal EDC exposure

epidemiological studies

mixture: 55 EDC

(phthalates, alkylphenols, PFAS)

>>> bad actors (e.g. triclosan !)

Analysis serum and blood focus on

Vitamin D and Thyroid hormones

Reproduction and sexual development

- AGD
- Puberty onset
- Play behavior

Meeting

Date

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Euromix complementary with... Additional tests

JRC

- E and T production/synthesis in Leydig cells?
- ER-CALUX?
- AR-CALUX?
- ?

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EuroMix participants

22 beneficiaries from 16 countries and 4 third parties including WHO and US-EPA.

EuroMix is coordinated by RIVM.

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport

WAGENINGEN UR
For quality of life

folkehelseinstituttet

BfR
Bundesinstitut für Risikobewertung

anses
French agency for food, environmental and occupational health & safety

INERIS
controlling risks for sustainable development

UNIVERSITEIT GENT

freshfel
EUROPEAN FRESH PRODUCE ASSOCIATION

Karolinska Institutet

SGL

Imperial College London

NIJZ Nacionalni inštitut za javno zdravje

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BENAKI PHYTOPATHOLOGICAL INSTITUTE

INRA
SCIENCE & IMPACT

UNIVERSITAT ROVIRA i VIRGILI

UNIVERSITÀ DEGLI STUDI DI MILANO

DTU Food
National Food Institute
DTU

ETH zürich

fera

matís

QSARs: CAG Endocrine Disruption

Model	Sensitivity (True positive rate: TP/TP+FN)	Specificity (True n	Accuracy (TN+TP)/(TN+FP+FN+TP)
DEREK (equivocal and above positive)	0.31		0.74
Toolbox ER-binding profiler	0.63		0.69
Toolbox rtER Expert system profiler	0.26		0.63
Toolbox DART alert	0.28		0.63
VEGA RBA model	0.78	0.88	0.84
COSMOS Nuclear receptor model		0.40	0.55
Majority Consensus using 6 models - Positive if 3 or more models positi		**0.82**	**0.78**

QSAR – Ask others e.g. Emiel Rorije RIVM

Meeting

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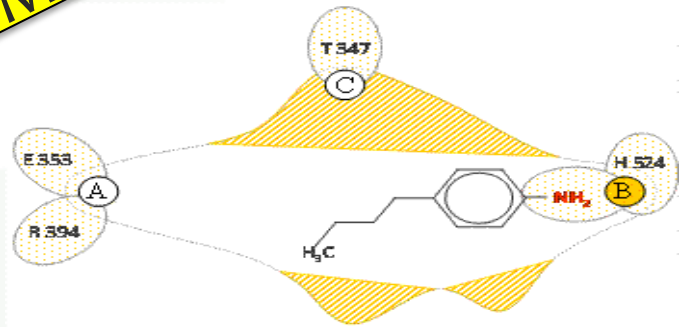
QSARs alert models – estrogen binding

OECD Toolbox: Chemicals with a single 5-or 6-member carbon ring structure with an unhindered amino-group ($-NH_2$) (e… the para- or meta-position on the ring) (5) are ER binders… …tency is related to the size and shape of non-amino-su… …aspect of the molecule

- **Non-binder**
- **Weak binder**
- **Moderate bin…**
- **Strong binder**
- **Very strong binder**

QSAR – Ask others e.g. Emiel Rorije RIVM



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