Centrum pro výzkum toxických látek v prostředí

BIOMARKERS AND TOXICITY MECHANISMS

10 – Mechanisms
Nuclear Receptors

Luděk Bláha, PřF MU, RECETOX
www.recetox.cz

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

Various signalling types … now focus on nuclear receptors

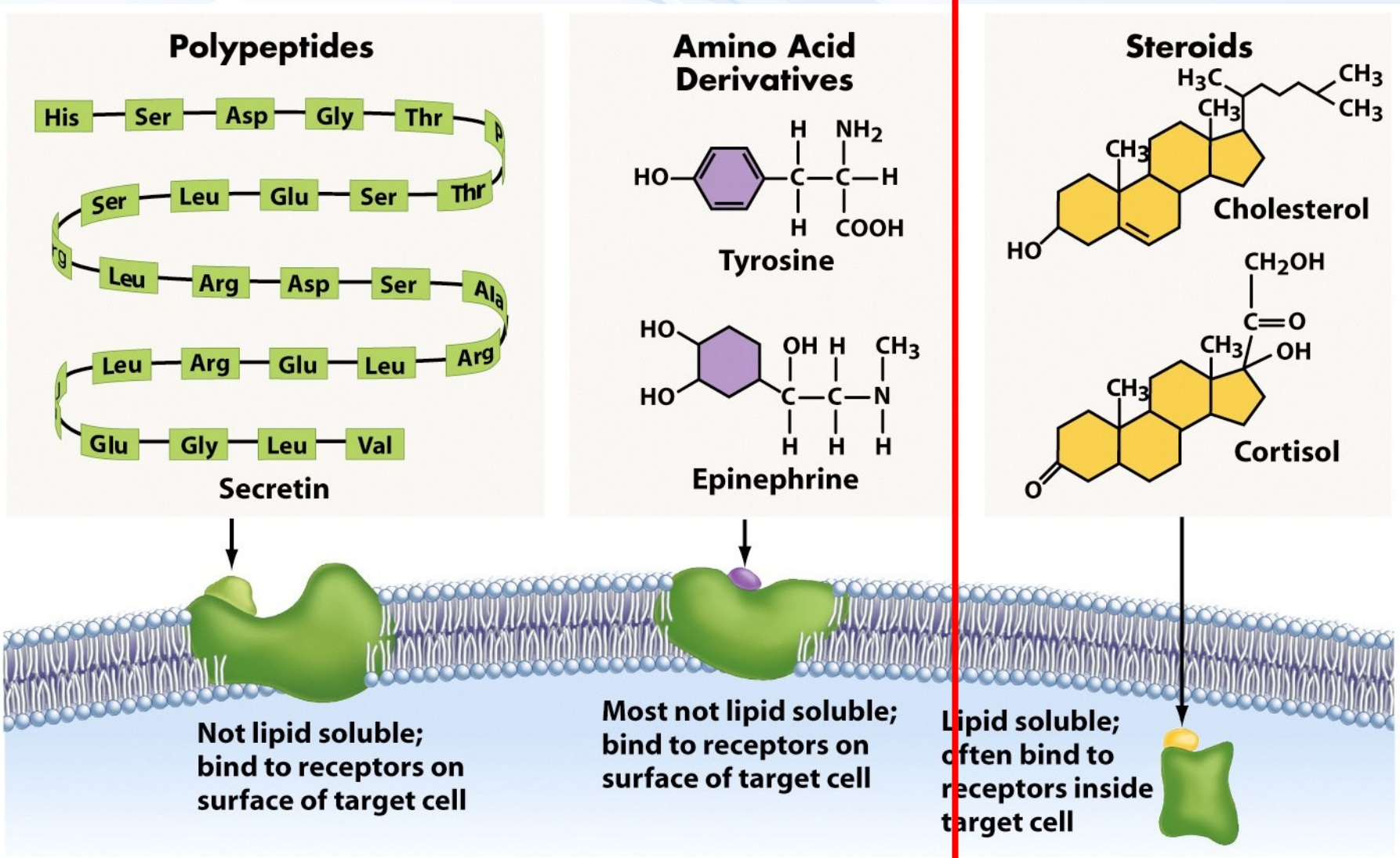


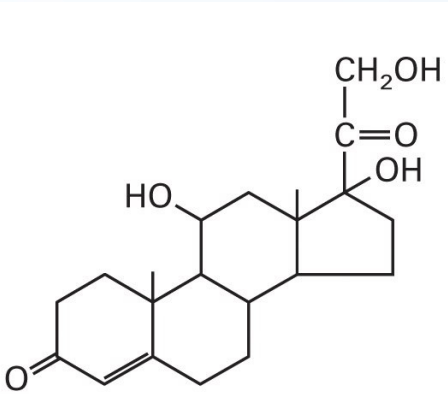
Figure 47-3 Biological Science, 2/e

NUCLEAR (Intracellular) RECEPTORS in summary

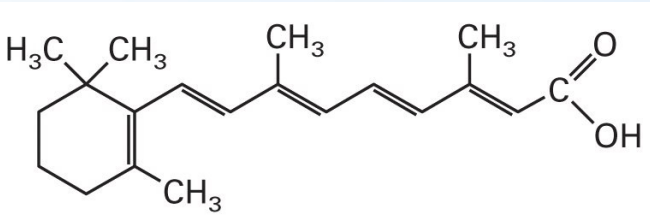
- Important physiological functions, and
- Important roles in pathologies and chemical toxicity
  - Endocrine disruption
  - Dioxin-like toxicity,etc.
- All NRs share similar structure and mechanisms of action
  - Act as direct transcription factors on DNA
- Natural ligands are small lipophilic hormones (steroids, thyroids, retinoids)
  - Role in toxicity – NR are modulated (activated/inhibited) by structurally close xenobiotics

Natural ligands of NR

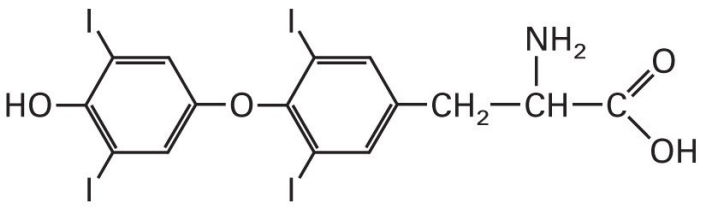
- **Small, lipid-soluble molecules**
  - Diffuse through plasma and nuclear membranes and interact directly with the transcription factors they control.
  - **STEROID HORMONES:**
    - sex steroids (estrogen, progesterone, testosterone)
    - corticosteroids (glucocorticoids and mineralcorticoids)
  - **OTHER HORMONES and ligands**
    Thyroid hormone, vitamin D3, retinoic acid, ligands of AhR
  - **Small molecules - gases**
    e.g. NO (signaling for immune reactions)



Cortisol



Retinoic acid



Thyroxine

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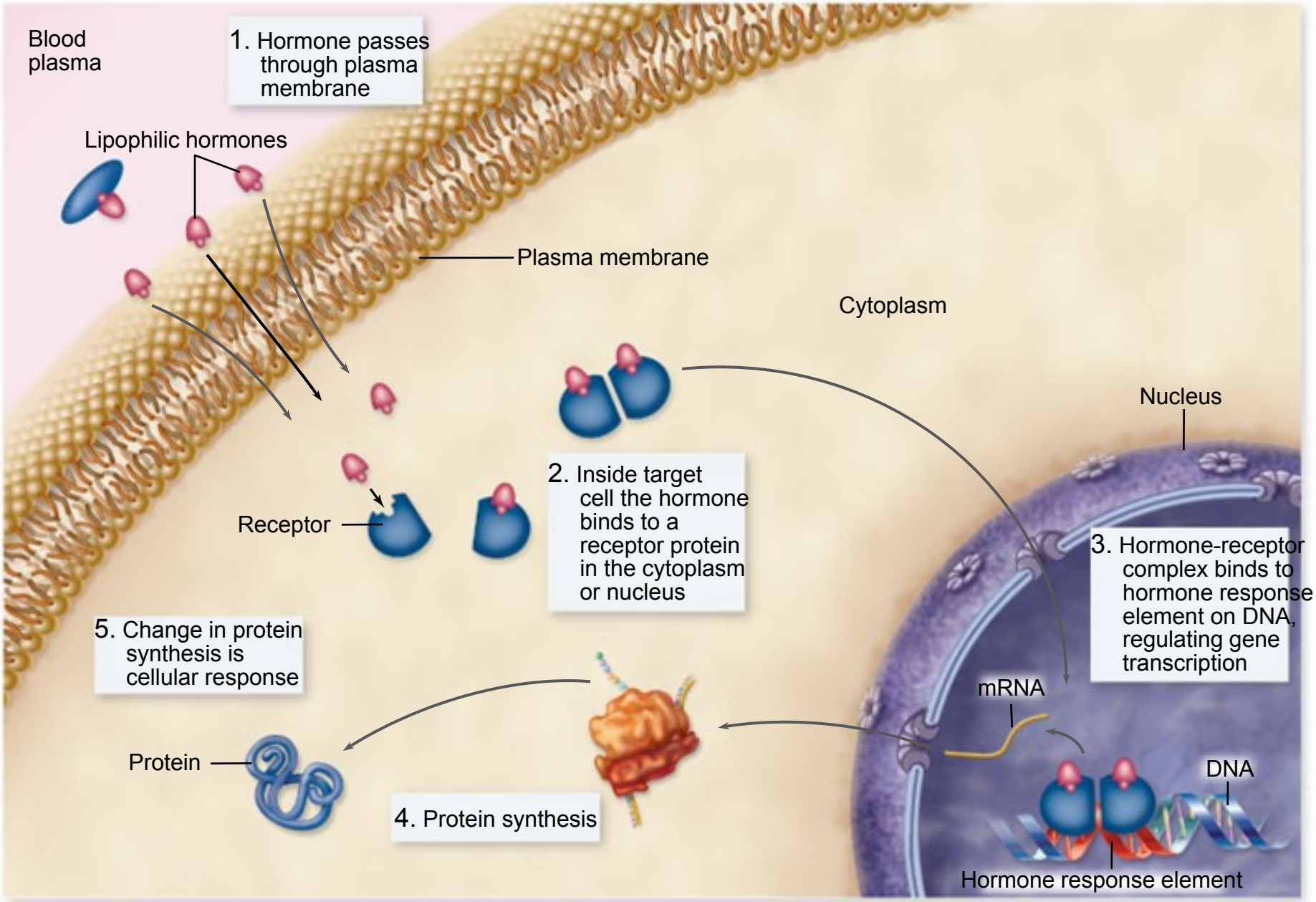


Fate and action of HORMONES activating NRs

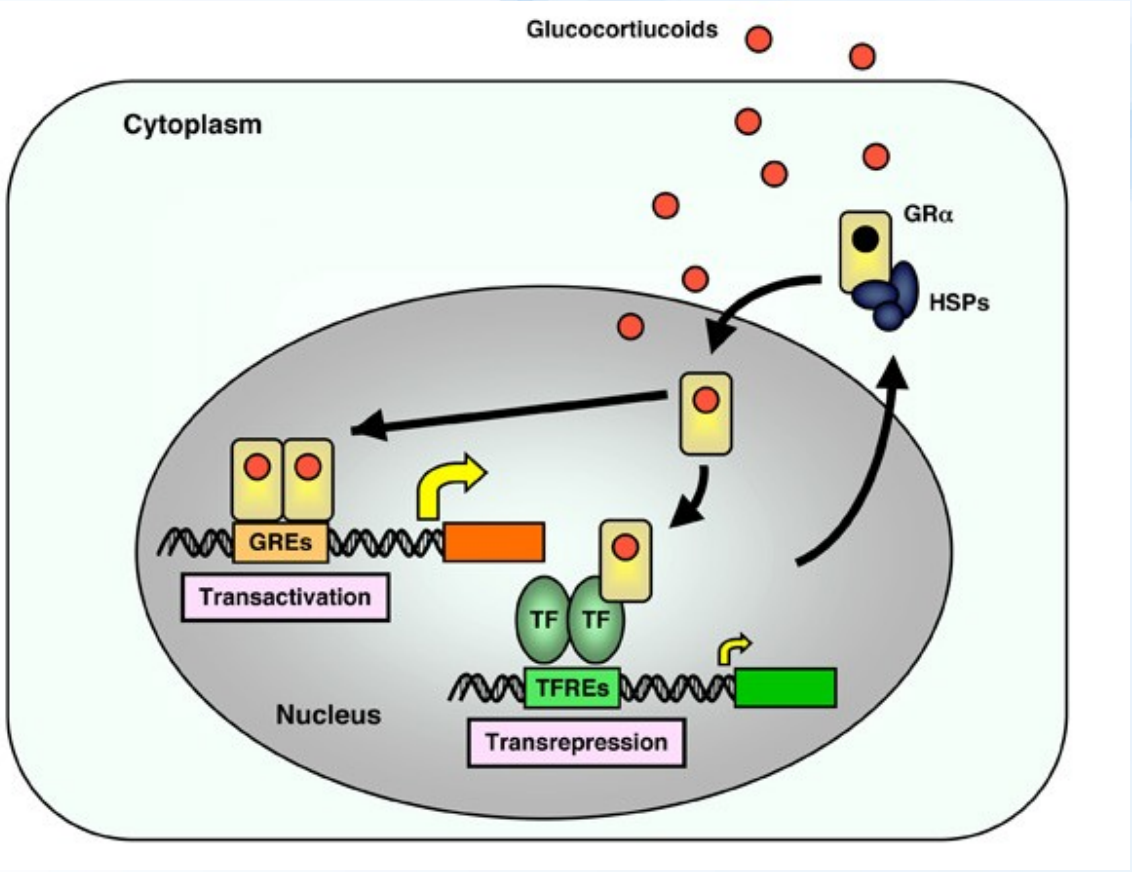
- Circulation in the blood bound to transport proteins
- Dissociation from carrier at target cells
- Passing through cell membrane
- Binding to an intracellular receptor (either in the cytoplasm or the nucleus)
- Hormone-receptor complex binds to hormone responsive elements in DNA

  → Regulation of gene expression

→ De-regulation at any level described above = TOXICITY

NR signalling is complex … examples of complexity (1)

1. Receptor activation is dependent not only on „ligand“ (**glucocorticoid**) but also on „inhibitor“ protein (**HSPs**)
2. Dimerization (after the activation) is often needed for proper action (binding to **GREs** – *glucocorticoid responsive elements)*
3. Receptor with ligand can activate its own targets (GREs) as well as „repress“ other binding sites (**TFREs**)



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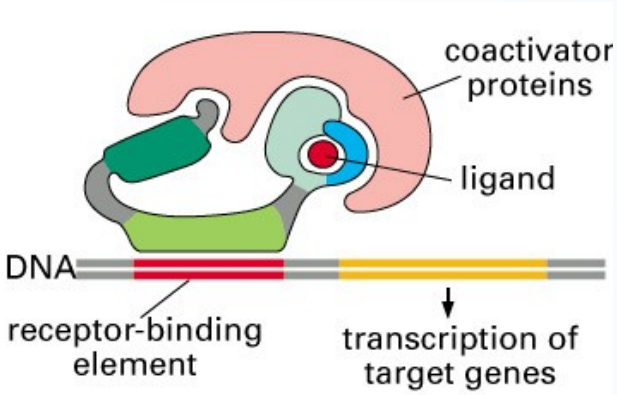
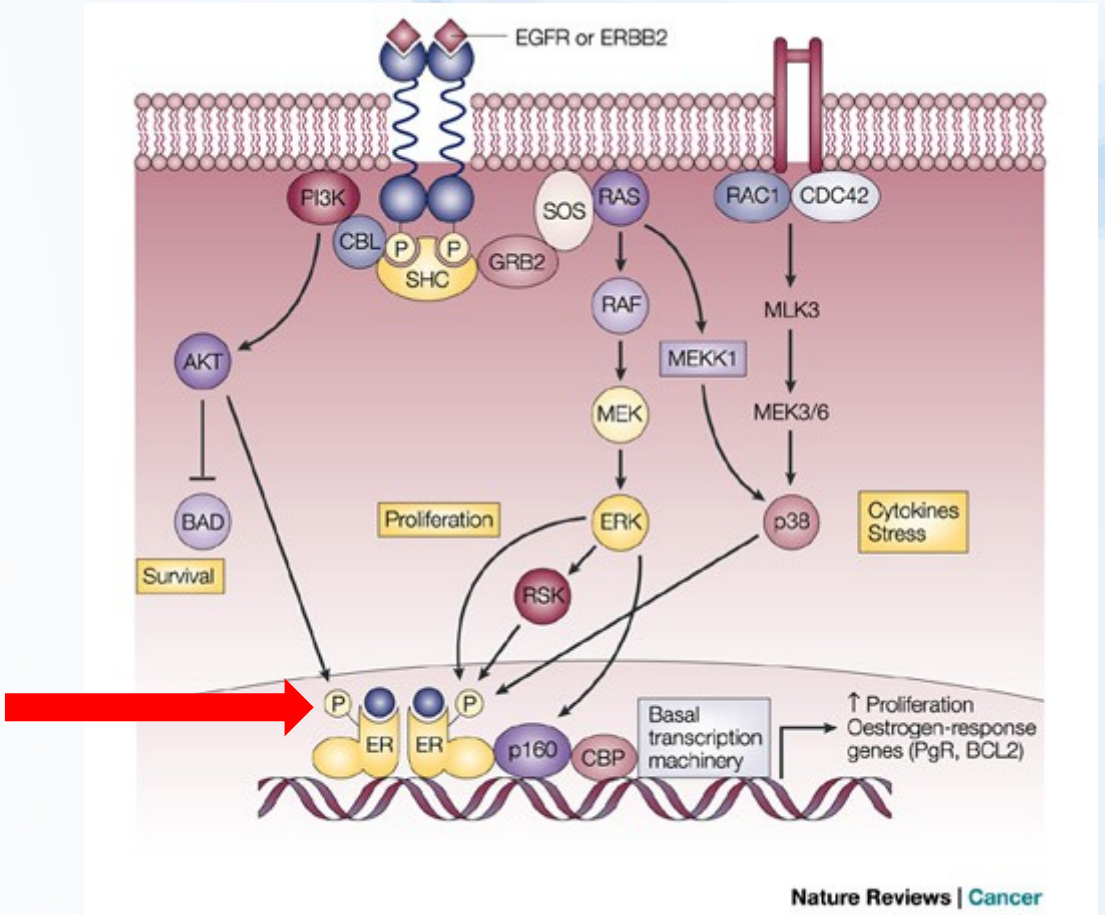
NR signalling is complex … examples of complexity 2

4. **„Co-activator“ proteins** are needed for proper action on DNA



5. Nuclear receptor action are (also) controlled - stimulated / suppressed - by **other signalling pathways** (e.g. phosphorylation by protein kinases)



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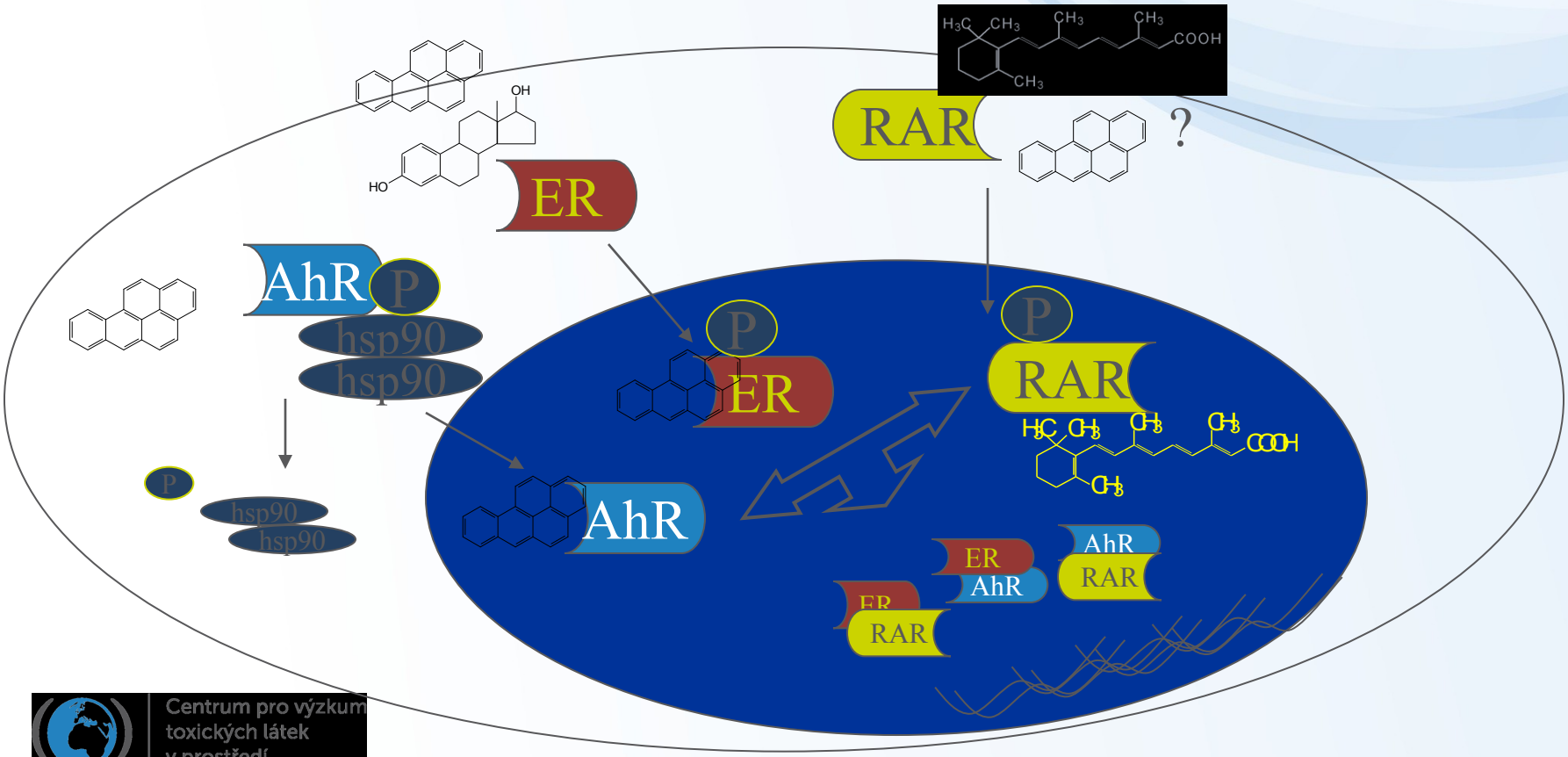
NR signalling is complex … examples of complexity 3

6. Interaction (crosstalk) among various NRs

- "antiestrogenicity" of AhR ligands
- fast clearance of retinoids after AhR activation
- Immunosuprresions after ER activations



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Details - specificities of NRs

- Regulation of transcription activity - mechanisms may vary
  - Steroid receptors often **dimerize** with a partner to activate gene transcription
  - Receptors for vitamin D, retinoic acid and thyroid hormone form **heterodimers** and then bind to responsive elements on DNA
    - Second component of the heterodimer is RXR monomer (i.e, RXR-RAR; RXR-VDR)

- **NR dimers**
  - Heterodimeric receptors - exclusively nuclear;
    - without ligand represses transcription (by binding to their cognate sites in DNA)
  - Homodimeric receptors
    - mostly cytoplasmic without ligands → hormone binding leads to nuclear translocation of receptors

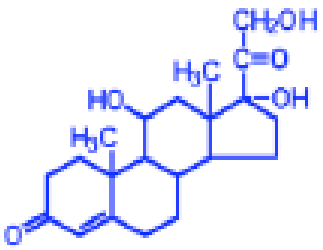
STEROIDs - most studied ligands detailed view

Steroid hormones - a review

Steroid hormones are derived from cholesterol metabolism in mitochondria

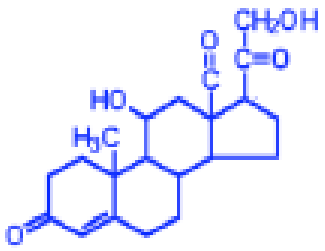
Cortisol

The dominant glucocorticoid in humans. Synthesized from progesterone in the *zona fasciculata* of the adrenal cortex. Involved in stress adaptation, elevates blood pressure and Na^+ uptake. Immunomodulation.



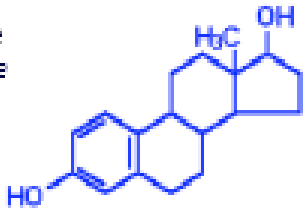
Aldosterone

Principal mineralocorticoid. Produced from progesterone in the *zona glomerulosa* of adrenal cortex, raises blood pressure and fluid volume, increases Na^+ uptake.



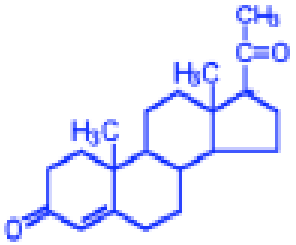
Estradiol

An estrogen, principal female sex hormone, produced in the ovary, responsible for secondary female sex characteristics. After menopause estrogen is produced from testosterone in the adrenal glands.



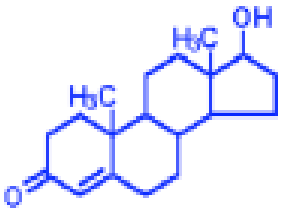
Progesterone

Produced from pregnenolone and secreted from the corpus luteum. Responsible for changes associated with luteral phase of the menstrual cycle, differentiation factor for mammary glands



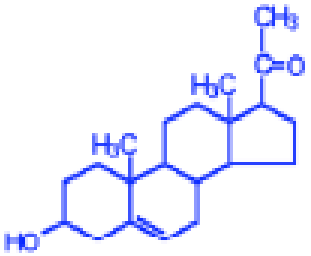
Testosterone

An androgen, male sex hormone synthesized in the testes from progesterone. Responsible for secondary male sex characteristics.

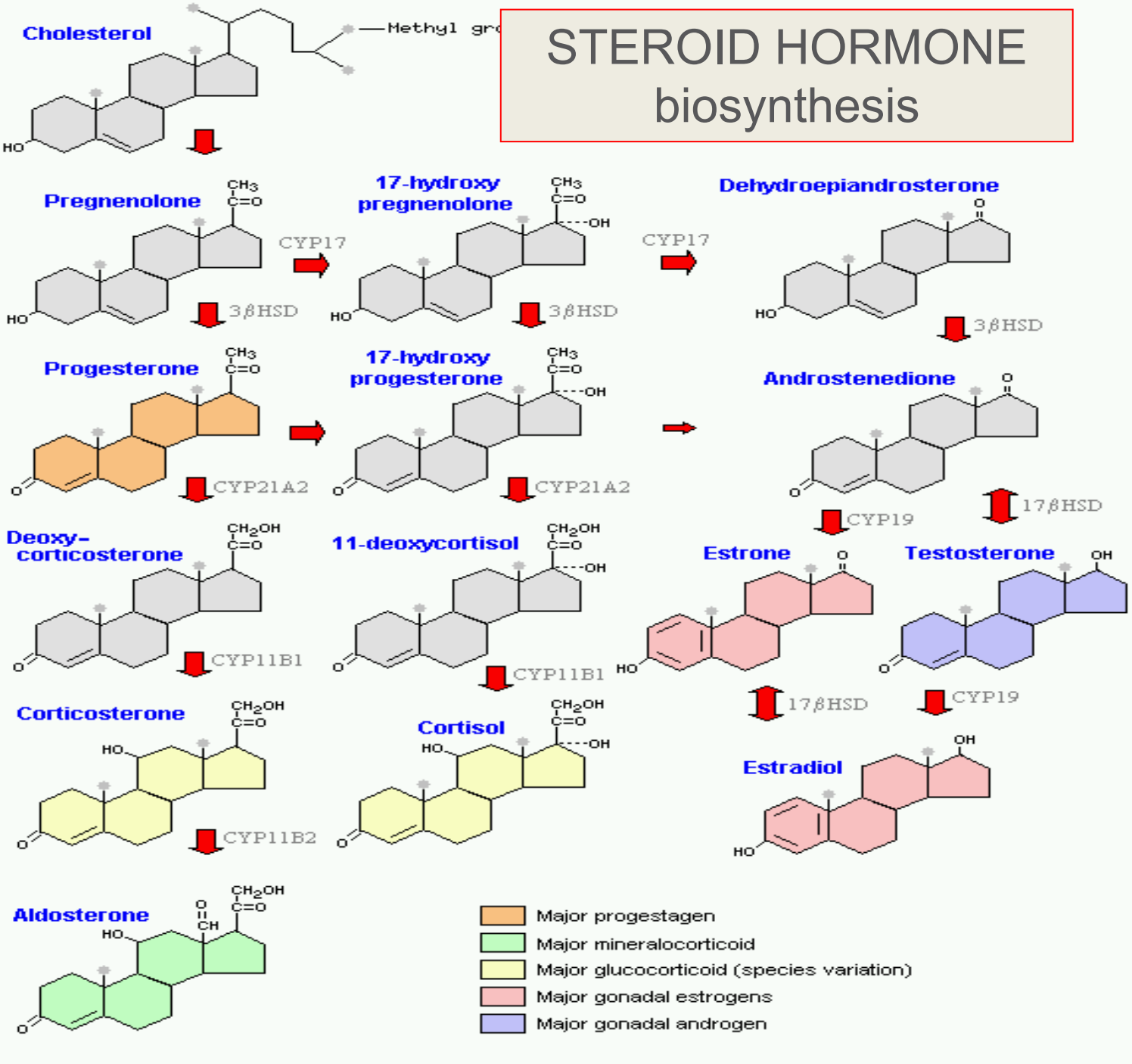


Pregnenolone

Made directly from cholesterol, the precusor molecule for all C_{18}, C_{19} and C_{21} steroids



STEROID HORMONE biosynthesis

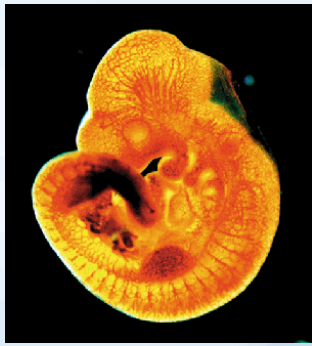


Why are NR important?

→ common mediators of Endocrine Disruption

Endocrine disruption

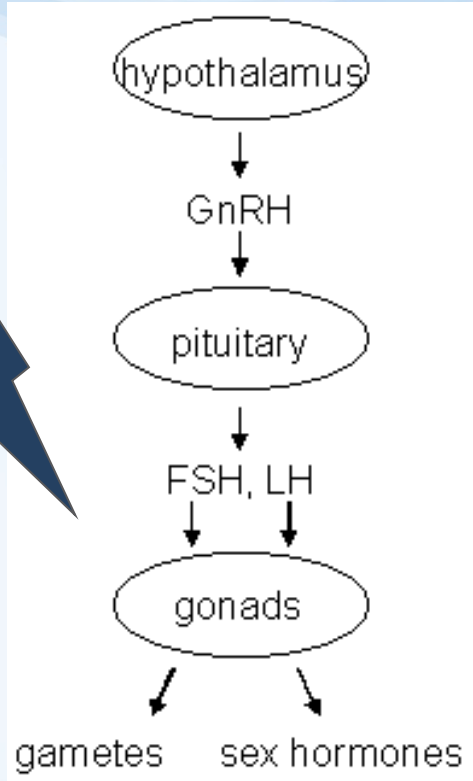
- **Interference of xenobiotics with normal functioning of hormonal system**



Known consequences

→ Disruption of homeostasis, reproduction, development, and/or behavior (and other hormone-controlled processes), such as

- Shift in sex ratio, defective sexual development
- Low fecundity/fertility
- Hypo-immunity, carcinogenesis
- Malformations
- etc.



Toxicants interact with hormonal system at different levels

Synthesis

Transport

Interaction with receptors

Metabolization

Consequences (both negative!)

Suppression

Stimulation

Possible mechanisms of endocrine disruption

- Disruption of the „master“ hormones (FSH/LH)
- Decrease of HR cellular levels
- Nonphysiological activation of hormone receptor (HR)
- Binding to HR without activation
- Changes in hormone metabolism (clearance)

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Mechanisms of toxicant effects in detail

→ various MoAs of endocrine disruption

biosynthesis and release of hormones

e.g. steroidogenesis

↓

e.g. modulation of CYP11A and/or CYP19 activities

binding to plasmatic transport proteins

↓

e.g. down-regulation of receptor levels

binding to nuclear hormonal receptor (HR)

↓

Direct interference (activation / inhibition)

activation of HR
(dissociation of associated heat shock proteins, formation of homodimers)

↓

e.g. modulation of other nuclear receptors
(PPAR/RXR, RXR/TR)

binding of the activated receptor complex to specific DNA motifs - HREs

↓

chromatin rearrangement and transcription of estrogen-inducible genes

↓

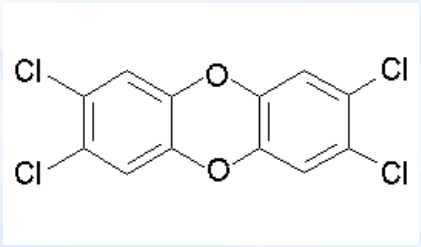
effects at the cellular, tissue, organ, organism, and/or population level

Endocrine disrupters in the environment?

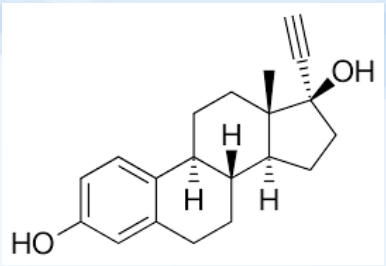
EDCs…

- Persistent Organic Compounds (POPs and their metabolites)
- steroid hormones and their derivatives from contraception pills
- alkylphenols
- organometallics (butyltins)
- pharmaceuticals
- Pesticides
- + number of unknowns …

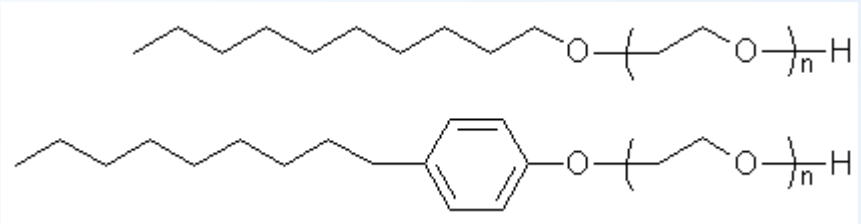
2,3,7,8-TCDD



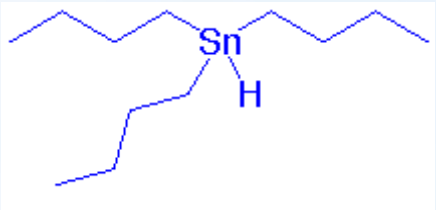
ethinylestradiol



alkylphenols



Tributyl-tin



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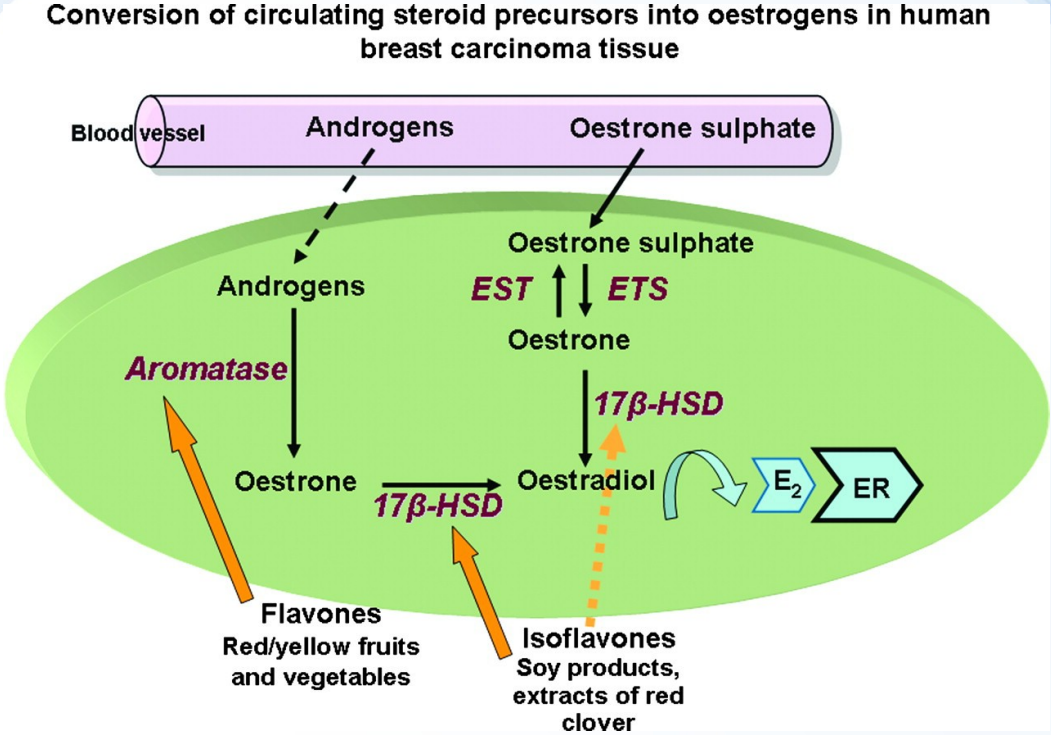
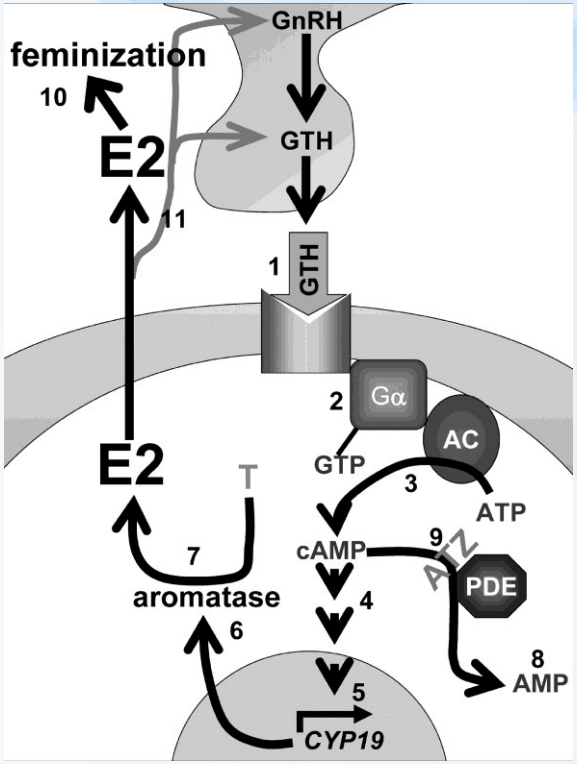
Examples – modulations of (synthetic) enzyme activities

Phytoestrogens promote **synthesis of estrogens**
→ **feminization**



Crosstalk with other signalling pathways (such as **cAMP**), which can be target to toxicants



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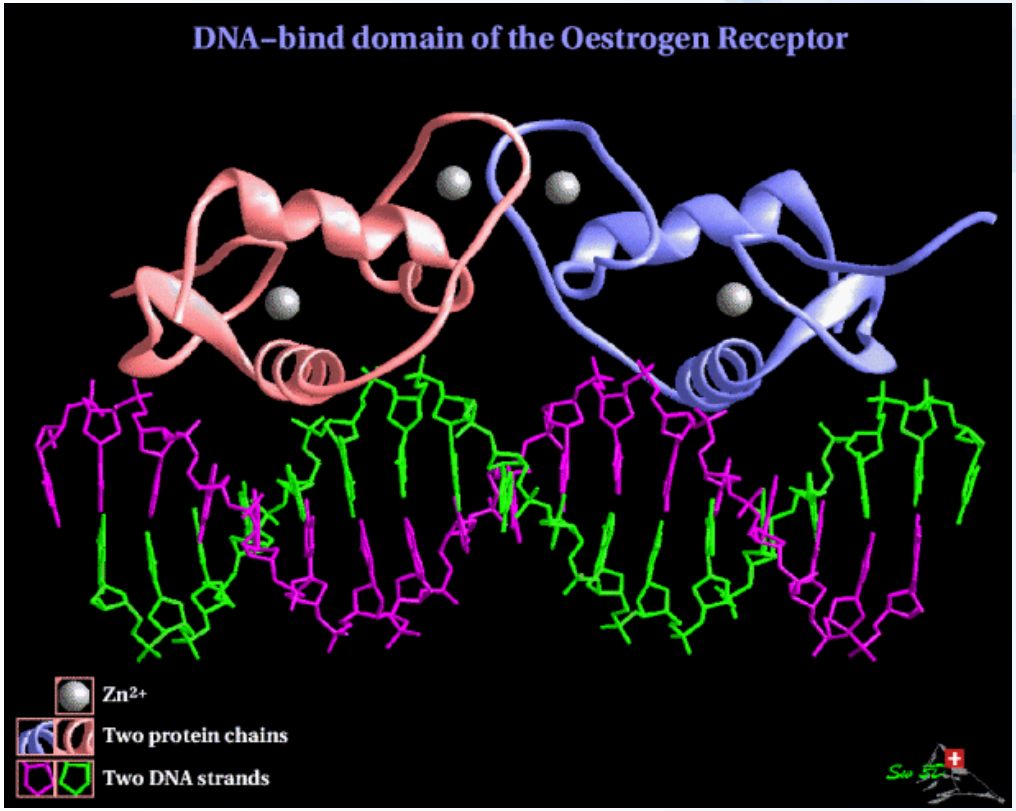
ESTROGEN RECEPTOR – ER

the most studied target of EDCs



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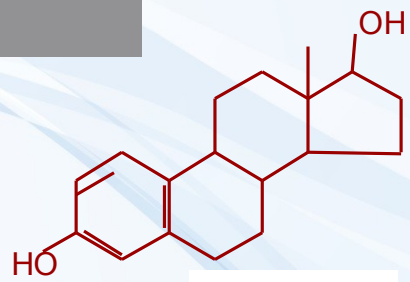
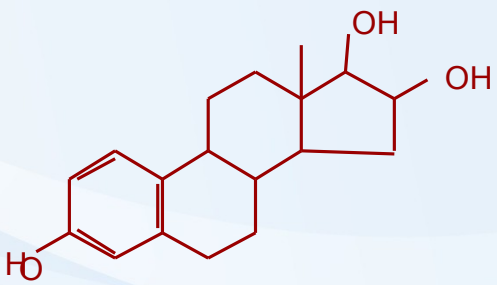
Estrogens



17-β-estradiol



estriol

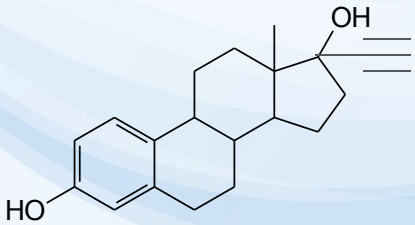
- **Synthesis in ovaries**

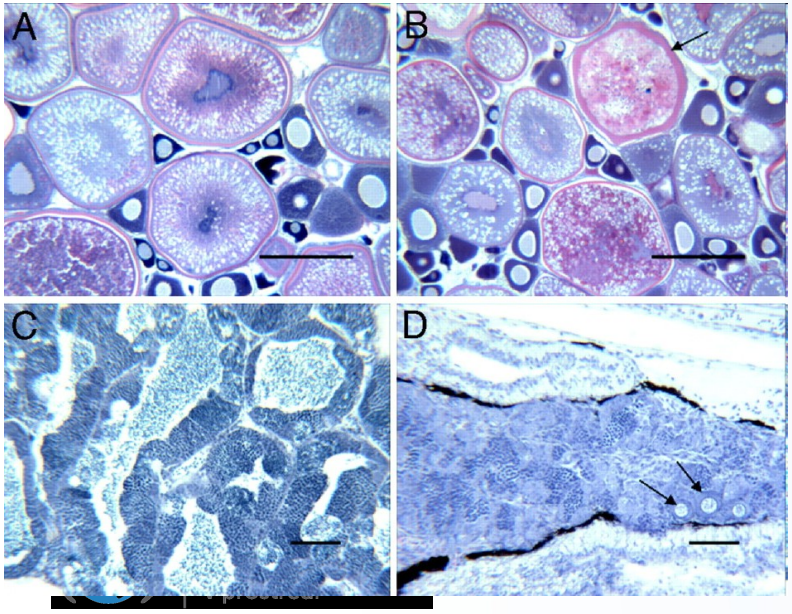
- **Functions**
  - key roles in female hormone regulation and signalling
  - responsible for metabolic, behavioural and morphologic changes occurring during stages of reproduction
  - involved in the growth, development and homeostasis in a number of tissues
  - control the bone formation, regulation of homeostasis, cardiovascular system and behaviour
  - regulate **production, transport and concentration of testicular liquid and anabolic activity of androgens** in males

- **DISRUPTION OF ESTROGEN SIGNALLING**
  → many documented effects in aquatic biota & laboratory organisms

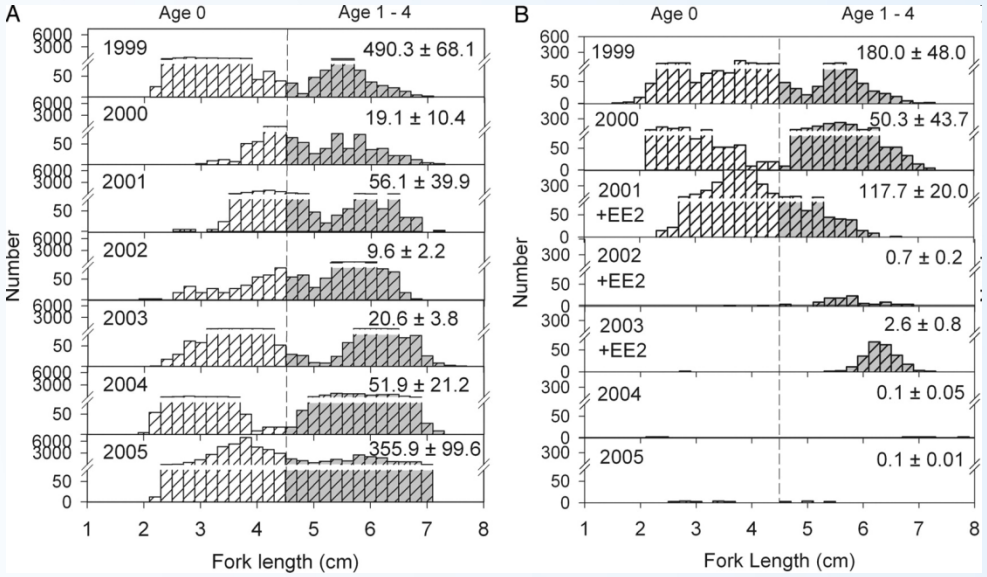
Kidd, K.A. et al. 2007. Collapse of a fish population following exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences* 104(21):8897-8901

5 ng/L (!)
7 years





Controls +Ethinylestradiol

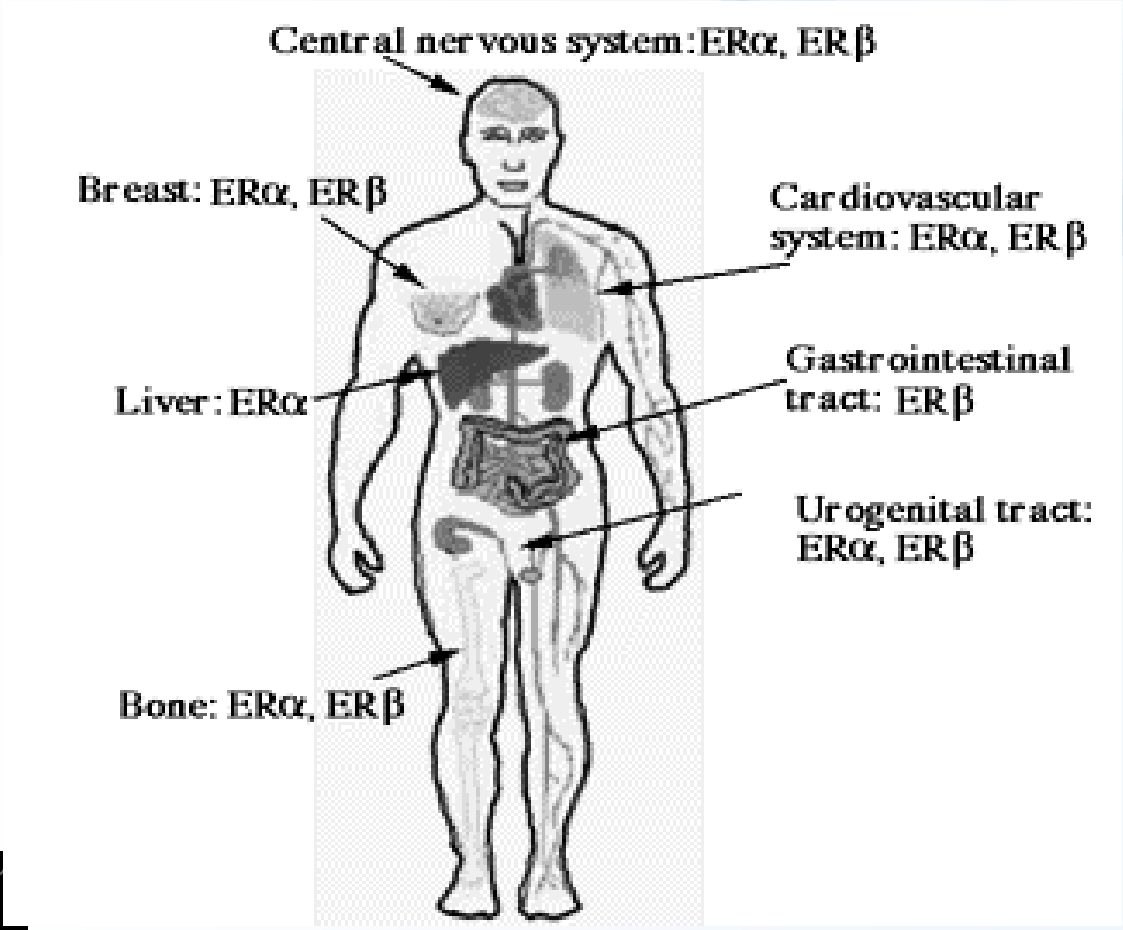


ESTROGEN RECEPTORS - subtypes

ER-α (in breast, ovary, brain, liver, bone and cardiovascular system, adrenals, testis and urogenital tract)

ER-β (in kidneys, prostate and gastrointestinal tract)

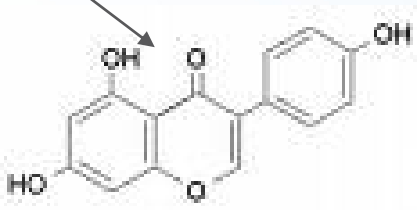
(ER-γ in fish)



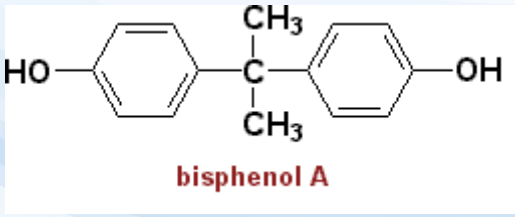
Environmental estrogens (xenoestrogens, exoestrogens)

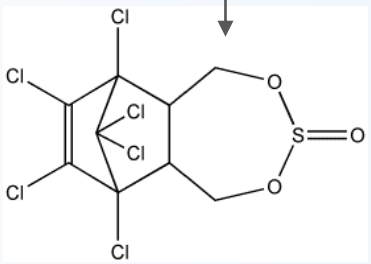
>> Highly diverse group of substances
>> Do not necessarily share structural similarity to the prototypical estrogen 17β-estradiol
>> may act as **AGONISTS** **and/or** **ANTAGONISTS** (depending on situation and concentration!)

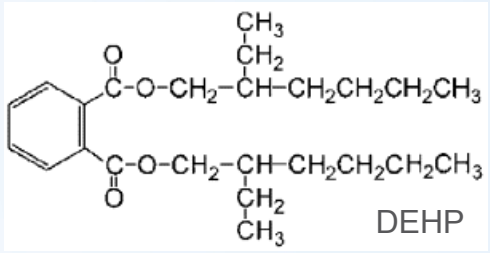
Natural products
genistein
naringenin
coumestrol
zearalenone



Industrial chemicals
Bisphenol A
Nonionic surfactants
Pthalate esters (eg. DEHP)
Endosulfan (pesticide)







Various POPs
DDT
kepone
PCBs/OH-PCBs
PAHs and dioxins

Pharmaceuticals
Ethinyl estradiol
Diethylstilbestrol
gestodene
norgestrel

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Exoestrogens - Relative Potencies to bind to ERa (REPs)

REP – a measure of toxic potency of a compound (similar also at other NRs)

Chemical group	Substance	REP
Endogenous hormones	Estradiol	1
	Estriol	$6{,}3.10^{-3}$
	Testosteron	$9{,}6.10^{-6}$
Phytoestrogens	Cuomestrol	$6{,}8.10^{-3}$
	Genistein	$4{,}9.10^{-4}$
Pesticides	o,p´-DDT	$1{,}1.10^{-6}$
PCBs	2,4,6-trichlorbiphenyl-4´-ol	1.10^{-2}
	2,5-dichlorobiphenyl-4´-ol	$6{,}2.10^{-3}$
	3,3´,5,5´tetrachlorobiphenyl-4,4´-diol	$1{,}6.10^{-4}$
alkylphenoles	4-tert-oktylphenol	$3{,}6.10^{-6}$
phthalates	butylbenzylphthalate	4.10^{-6}

REP (RElative Potencies) of selected compounds related to 17-β-estradiol derived from reporter yeast assay

How to assess ESTROGENICITY?

Number of in vivo and in vitro methods available

Assay (ref.)	Exposure type	Detects ER-dependent agents?	Detects non-ER-dependent agents?	Distinguishes agonist versus antagonist?	Pharmacokinetic and metabolism included?
Receptor-based assays					
Receptor binding assay (27)	Cell lysate	Yes	No	No	No
Receptor activation assay (32–34)	Cells in vitro	Yes	No	Yes[a]	No
In vitro estrogen-regulated response assays					
MCF-7 cell proliferation assay (41)	Cells in vitro	Yes	Limited	Yes[a]	No
Induction assays (46,48)	Cells in vitro	Yes	Limited	Yes[a]	No
DNA synthesis assays (47)	Cells in vitro	Yes	Limited	Yes[a]	No
In vivo estrogen-regulated response assays					
Uterotrophic response assay (49)	Whole animal	Yes	Limited	Yes[a]	Yes
Vaginal cornification assay (50)	Whole animal	Yes	Limited	Yes[a]	Yes
Vaginal opening (11)	Whole animal	Yes	Limited	Yes[a]	Yes
Uterine fluid imbibition (11)	Whole animal	Yes	Limited	Yes[a]	Yes
Uterine epithelial hypertrophy (51)	Whole animal	Yes	Limited	Yes[a]	Yes
Inhibition of steroid synthesis assays					
In vitro ovarian steroid assay (55)	Minced tissue	No	Yes	Yes	No
Ex vivo ovarian steroid assay (56)	Whole animal	No	Yes	Yes	Yes

[a]Detection of antagonists requires use of additional groups with test material + estradiol.

Janošek, J., Hilscherová, K., Bláha, L., and Holoubek, I. (2006). Environmental xenobiotics and nuclear receptors-Interactions, effects and in vitro assessment. *Toxicology in Vitro* 20, 18-37.

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IN VIVO ASSAYS FOR ESTROGENICITY

- uterotropic assay
- vaginal cornification assay

- production of estrogen-inducible proteins
  (e.g. **vitellogenin** and zona radiata protein)

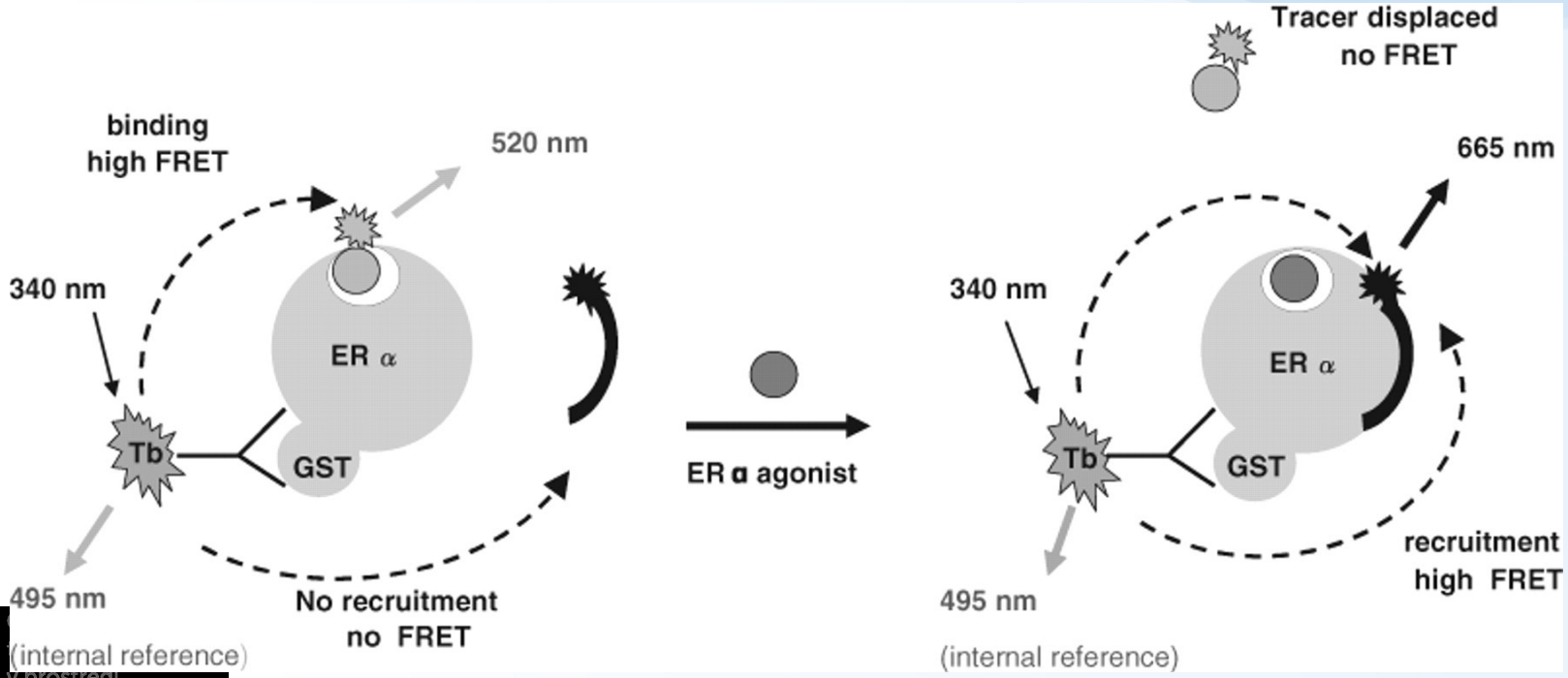
  → also discussed at “biomarkers” part

- standard (in vivo) test procedures for reproductive and developmental toxicity
  - using mice, rats, fish, amphibians etc.

In vitro assays for estrogenicity

- **Level 1 – interaction of toxicant with the protein (receptor)**
  - INTERACTION (BINDING) to the receptor
    - **competitive ligand binding assays**
      - Various variants (e.g. displacement of radioactive substrate, fluorescence resonance energy transfer (*FRET*) techniques etc.

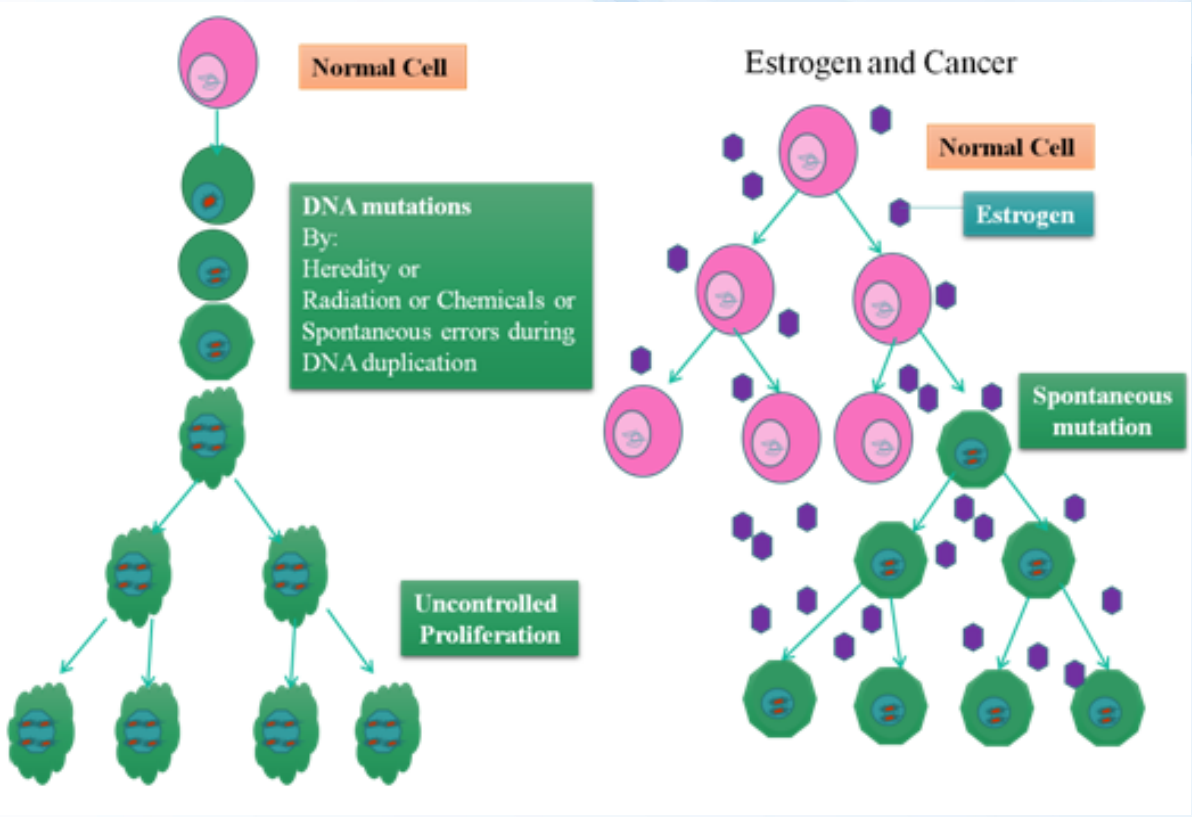
    → information only about "binding potency" but the effect remains unknown (? Activation / suppression / no effect ?)



In vitro assays for estrogenicity

- **Level 2 - effects at cellular level**
  → interference with receptor biological activity

- **Cell proliferation assays**
  – Estrogens induce proliferation



Centrum pro výzkum
toxických látek
v prostředí

In vitro assays for estrogenicity

- **Level 2 - effects at cellular level**
  → interference with receptor biological activity

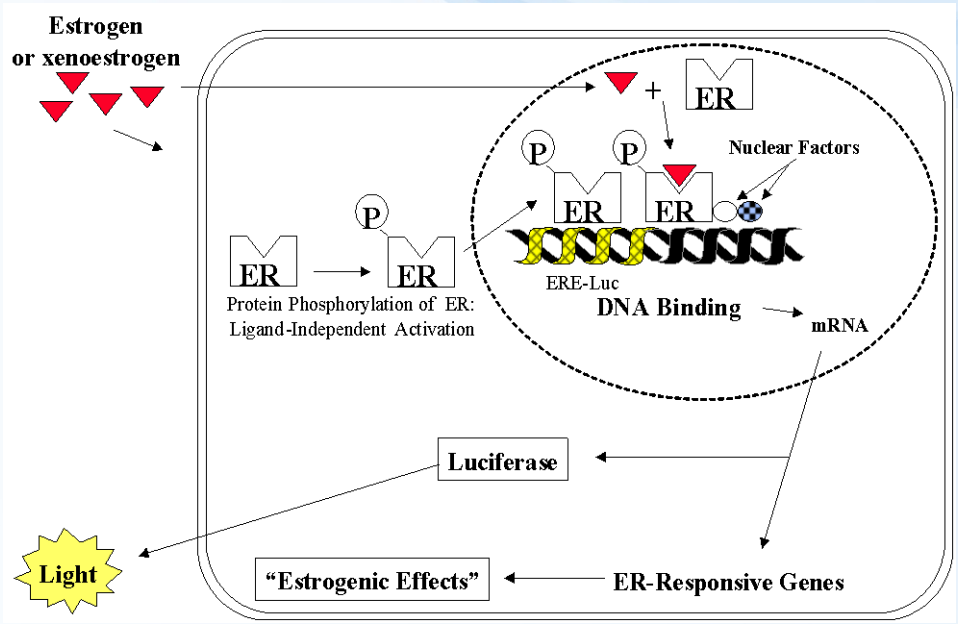
- **Endogenous protein expression** (or enzyme activity) assays
  - **reporter gene assays**

Cell assays *in vitro*

•Cells (e.g. breast carcinoma) naturally carrying functional ER.

•Genetic modification - stable transfection with firefly **luciferase gene**: under the control of ER

•Estrogens in media → light induction



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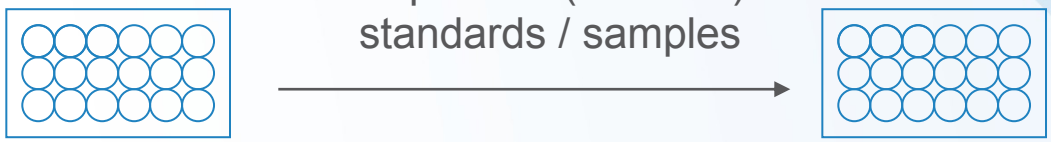
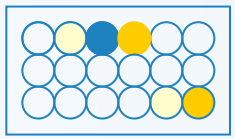
Luciferase reporter assay for estrogenicity in brief

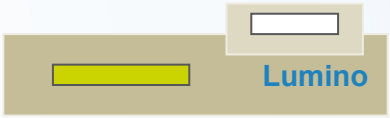
96 microwell plate
cultivation of transgenic cell lines

ER: breast carcinoma **MVLN cells**



Cell lysis
→ extraction of induced luciferase





Luminescence determination
(microplate luminescence reader)

Similar principle for other NRs activities

Mammalian cells
* AhR – H4IIE.luc cells (CALUX)
* AR – MDA.kb2 cells
* RAR/RXR - P19/A15 cells

Yeast models
* Luciferase based
* Also beta-galactosidase etc.

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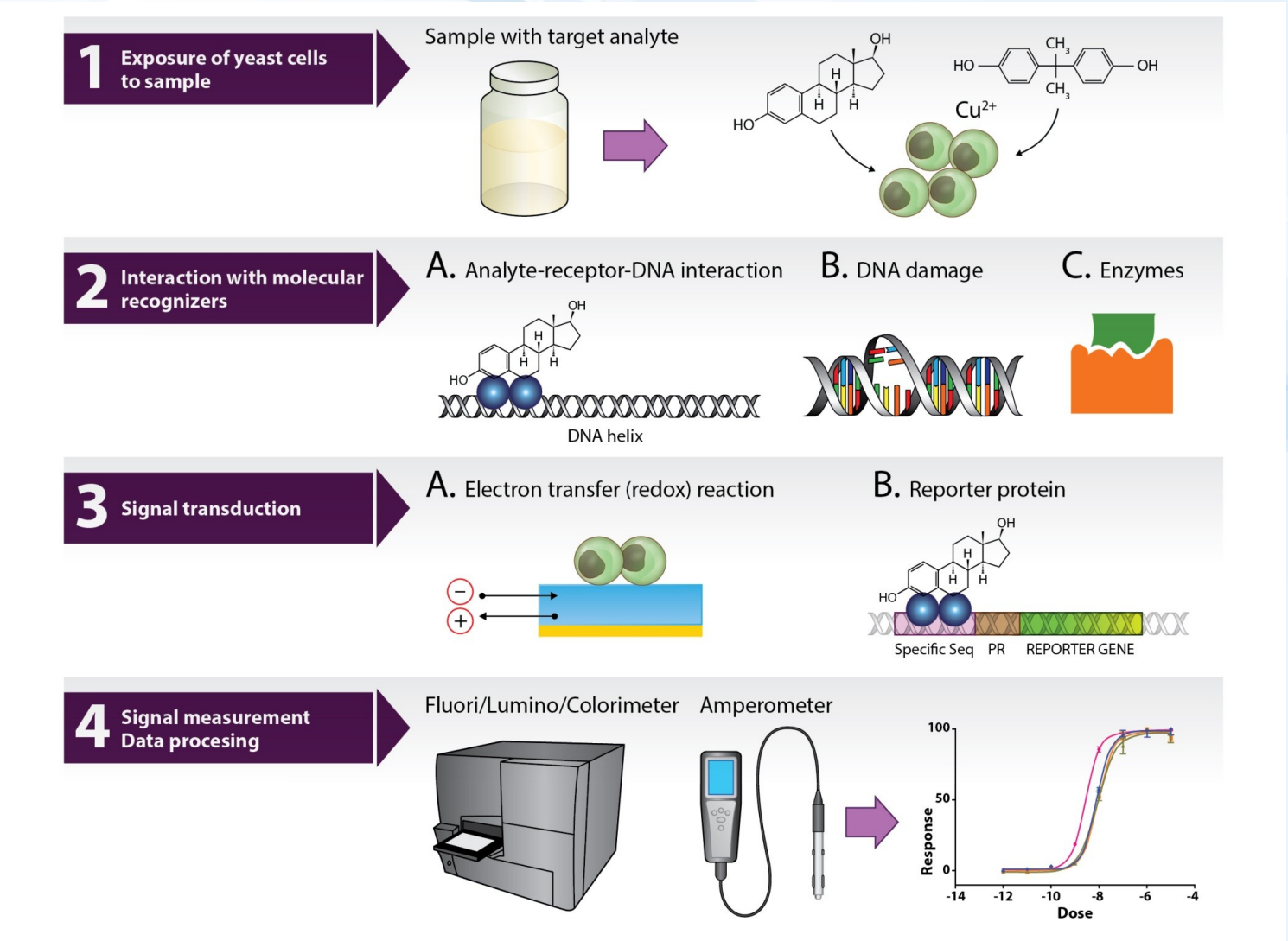
Bioassay (biosensor) for NR-modulator based on yeast cells



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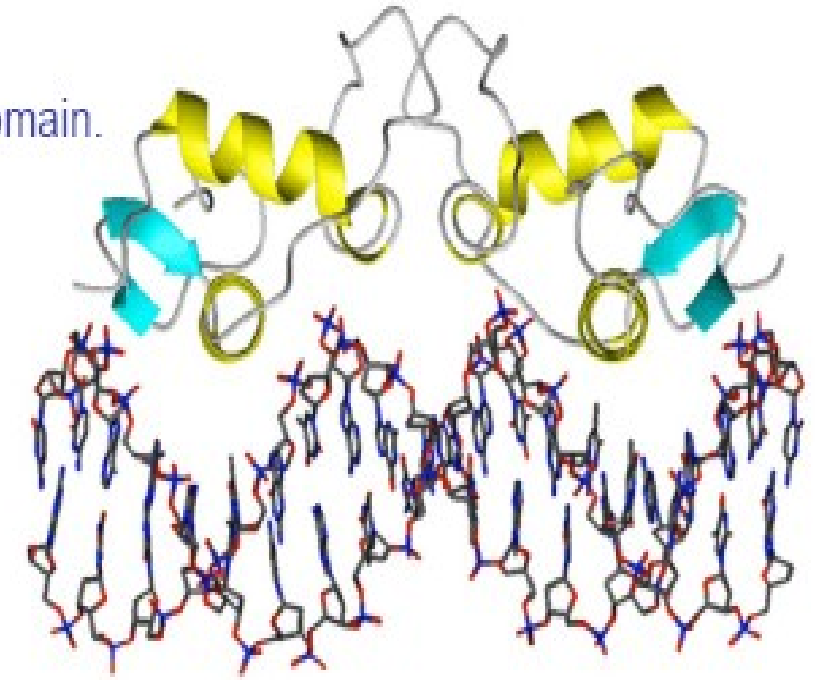
Jarque, S., M. Bittner, L. Bláha and K. Hilscherová (2016). "Yeast biosensors for detection of environmental pollutants: current state and limitations." Trends in Biotechnology 34(5): 408-419 (doi:10.1016/j.tibtech.2016.01.007).

ANDROGEN RECEPTOR (AR)

role in toxicity confirmed ... but less explored than ER

Androgen receptor DNA binding domain.



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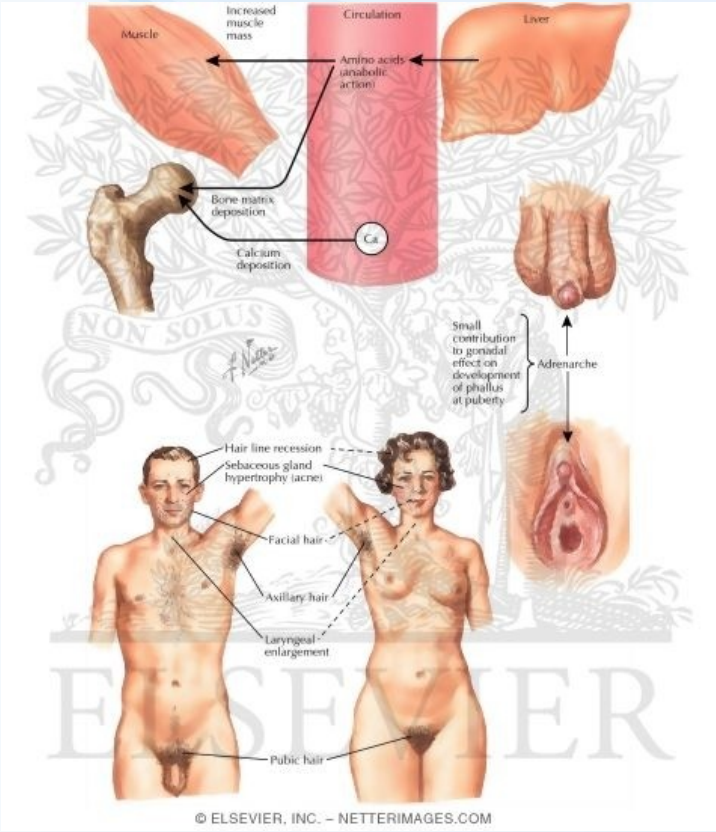
Androgens

- **Role in males similar to the of estrogens in females**
  - development of male sexual characteristics
  - stimulating protein synthesis, growth of bones
  - cell differenciation, spermatogenesis
  - male type of behaviour



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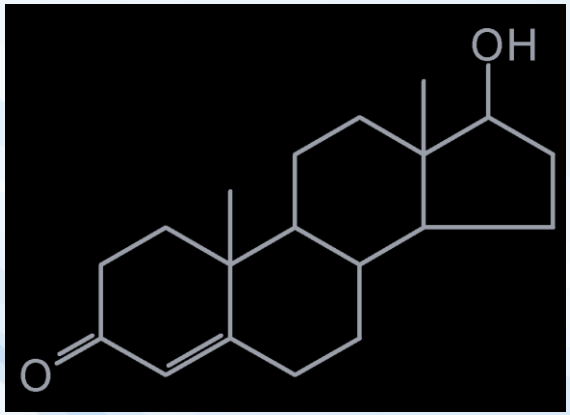
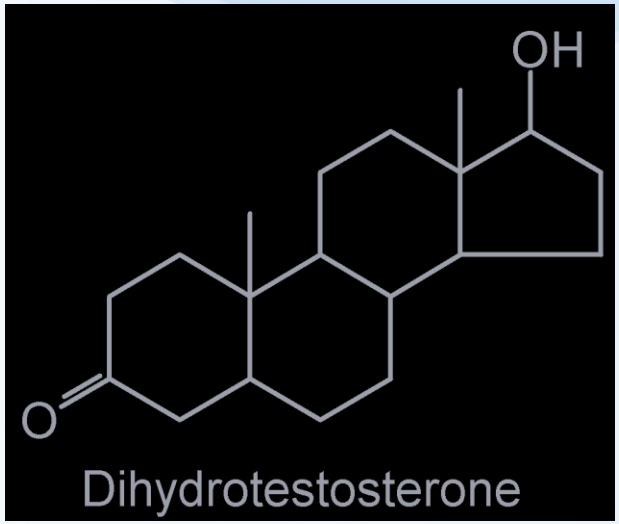
Androgens

- **Endogenous ligands – androgen hormones**
  - **Two key androgens**
    - **testosterone (T)**
    - **dihydrotestosterone (DHT)**
  - Other androgens – androstanediol, dehydroepiandrosterone, androstenedione



Testosterone



Dihydrotestosterone

- **T: synthesis in testis (Leydig cells)**
  - in lesser extent in adrenals
- **DHT**: Formed **extratesticulary** from T
  - In several tissues (seminal vesicles, prostate, skin) higher affinity to androgen receptor than T
  - Daily production 5-10% of testosterone

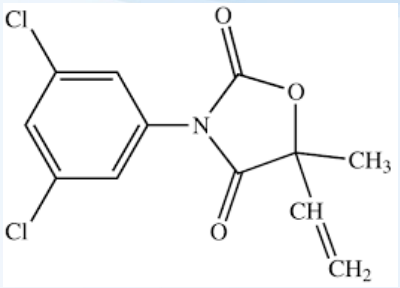
Mechanisms of androgen signalling disruption

1) Binding to AR

- Mostly competitive inhibition
- Xenobiotics mostly DO NOT activate AR-dependent transcription

- Only few compounds able to activate AR in the absence of androgen hormones but they are **anti-androgenic in the presence of strong androgens like T or DHT**
  - metabolites of **fungicide vinclozoline**, some PAHs

vinclozoline



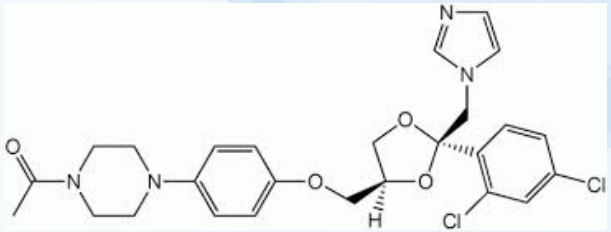
2) FSH/LH (gonadotropins) signalling disruption – less explored

- FSH/LH expression - regulation via negative feedback by testosterone
- Suppression → alterations of spermatogenesis

Mechanisms of androgen signalling disruption

3) Alterations of testosterone synthesis

- Inhibition of P450scc needed for side chain cleavage of cholesterol or inhibitions of 17-beta-hydroxylase and other CYPs
  - **fungicide ketoconazol**



4) Testosterone metabolic clearance

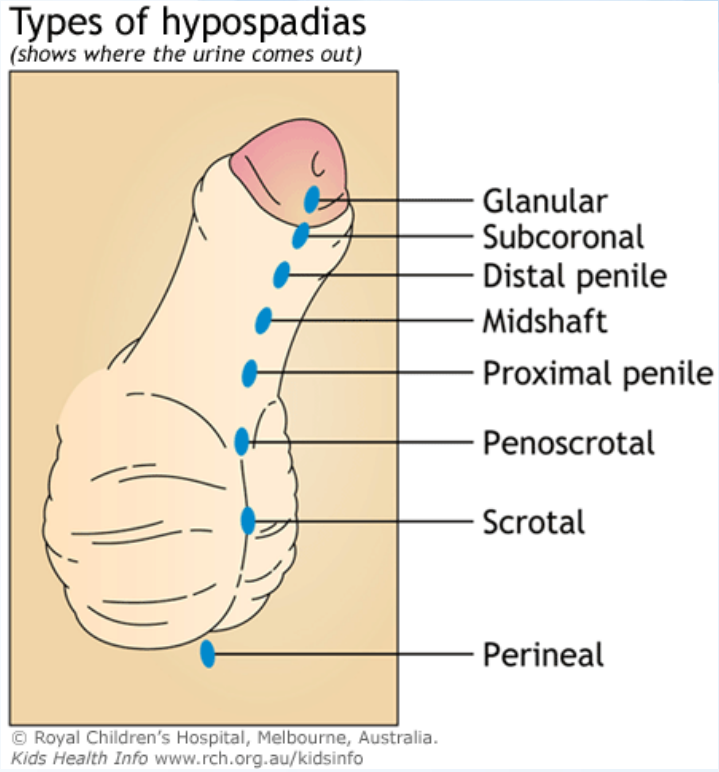
- Induction of detoxification enzymes (UDP-glucuronosyltransferase or monooxygenases CYP1A, 1B)
  - Pesticides endosulfan, mirex, o-p´-DDT

Effects of **male** exposure to antiandrogens

- **Exposure during prenatal development:**
  - malformations of the reproductive tract
    - reduced anogenital distance
    - **hypospadias** (abnormal position of the urethral opening on the penis)
    - vagina development
    - undescendent ectopic testes
    - atrophy of seminal vesicles and prostate gland

- **Exposure in prepubertal age:**
  - delayed puberty
  - reduced seminal vesicles
  - reduced prostate

- **Exposure in adult age:**
  - oligospermia
  - azoospermia
  - loss of sexual libido



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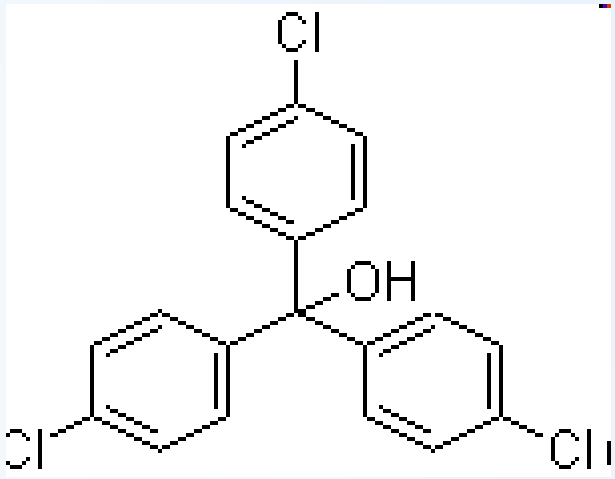
AR-binding – potencies - reference **DHT: EC50 ~ 0.1 μM**)

Compound	IC_{50} (μM)
Benz[a]anthracene	3.2
Benzo[a]pyrene	3.9
Dimethylbenz[a]anthracene	10.4
Chrysene	10.3
Dibenzo[a,h]anthracene	activation in range 0.1-10μM
Bisphenol A	5
vinclozolin metabolites	9.7
hydroxyflutamide	5
Aroclor typical values	0.25-1.11
Individual PCBs typical values	64 - 87
tris-(4-chlorophenyl)-methanol	0.2

Antiandrogenic compound

- **tris-(4-chlorophenyl)-methanol**
  - Ubiquitous contaminant of uncertain origin
  - Probable metabolite of DDT-mixtures
  - Levels in human blood serum cca. 50nM
  - antiAR potency - EC50 – cca. 200nM



Centrum pro výzkum toxických látek v prostředí

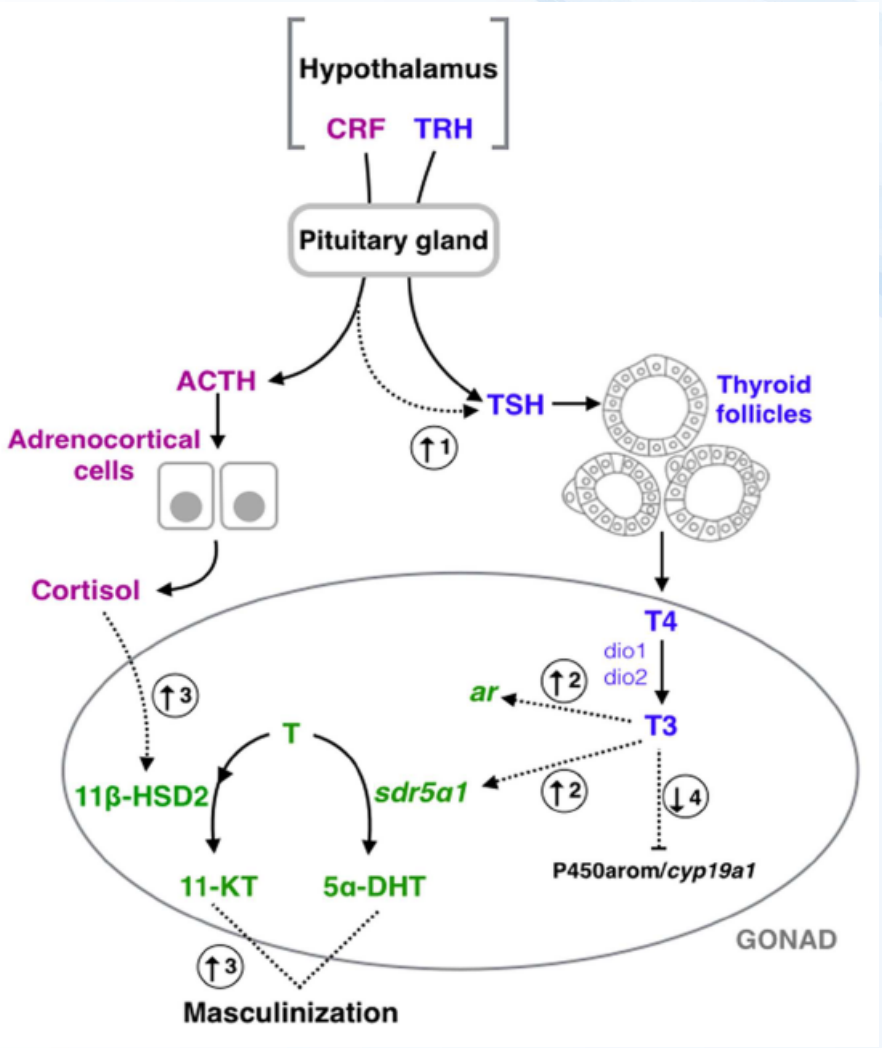
*(Anti)*androgenicity assessment

- **In vivo Hershberger assay**
  - castrated rats treated with examined substance
  - Endpoint – after 4-7 days – seminal vesicles and ventral prostate weight
- **In vivo measurement of testosterone blood levels**

- **In vitro cell proliferation assays**
  - cells with androgen-dependent growth: mammary carcinoma cell lines
  - prostatic carcinoma cell lines

- **Receptor-reporter assays**
  - Gene for luciferase (or GFP) under control of AR
    - AR-CALUX (human breast carcinoma T47D)
    - PALM (human prostatic carcinoma PC-3)
    - CHO515 (Chinese hamster ovary CHO)
  - Yeast transfected cells
    - beta-galactosidase reporter

THYROID SIGNALLING

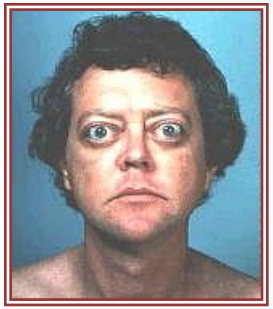


Thyroid hormones

- Crucial roles in metabolism, development and maturation
  - Regulation of metabolism
    - increasing oxygen consumption
    - modulating levels of other hormones (insulin, glucagon, somatotropin, adrenalin)
  - Important in cell differenciation
  - Crucial role in development of CNS, gonads and bones

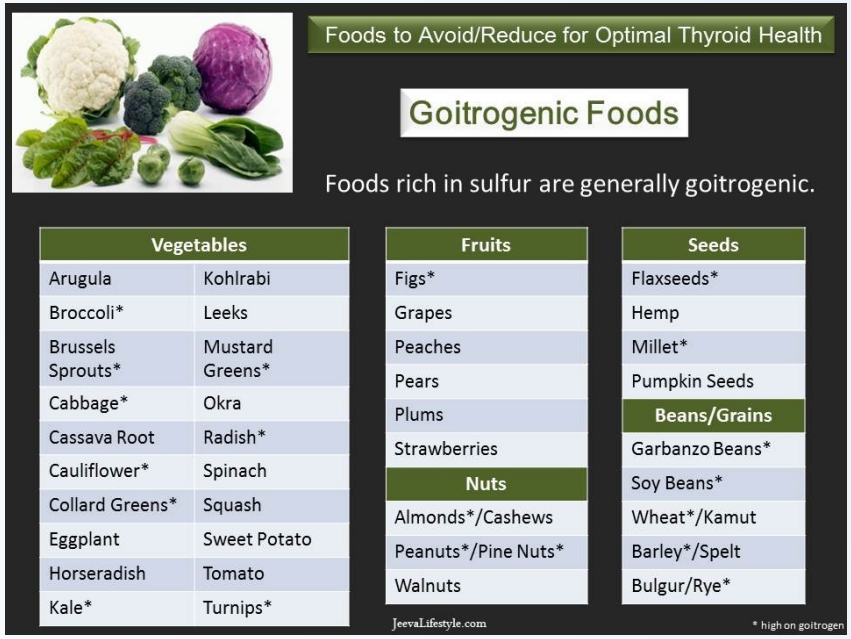
- EDC compounds interfering with thyroid signalling **"GOITROGENS"**
- Many food (vegetables) contain goitrogens

HYPOTHYROIDISM



HYPERTHYROIDISM

Centrum pro výzkum toxických látek v prostředí



Thyroid hormones

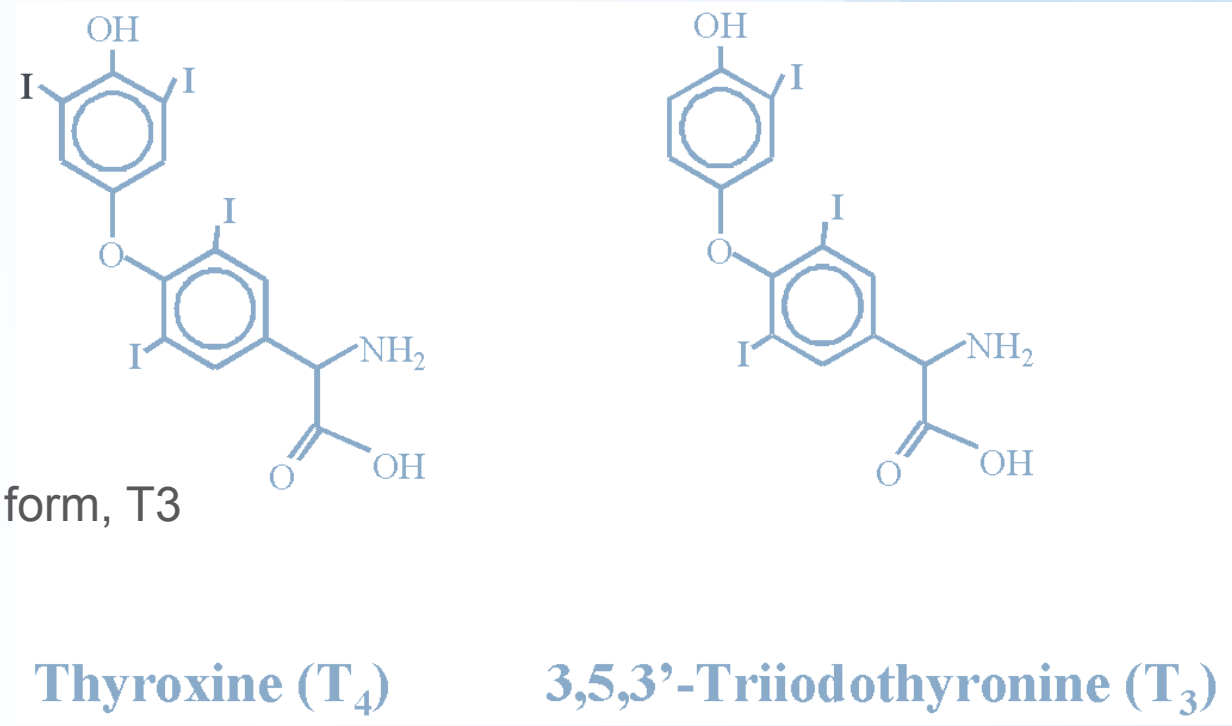
Thyroxine (T4)

Also called tetraiodothyronine
Contains 4 iodide ions

Triiodothyronine (T3)

Contains 3 iodide ions

-Most T3 produced
by deiodination
in target tissues (deiodinases)

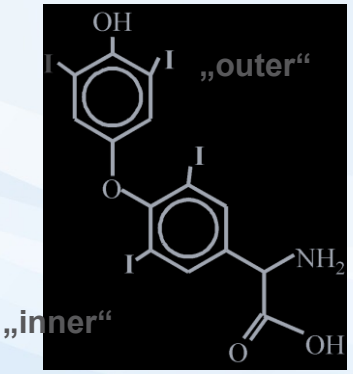


T4 – prohormone
5 -deiodination → active form, T3

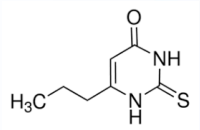
Centrum pro výzkum toxických látek v prostředí

Disruption of enzymes involved in Thyroid metabolism

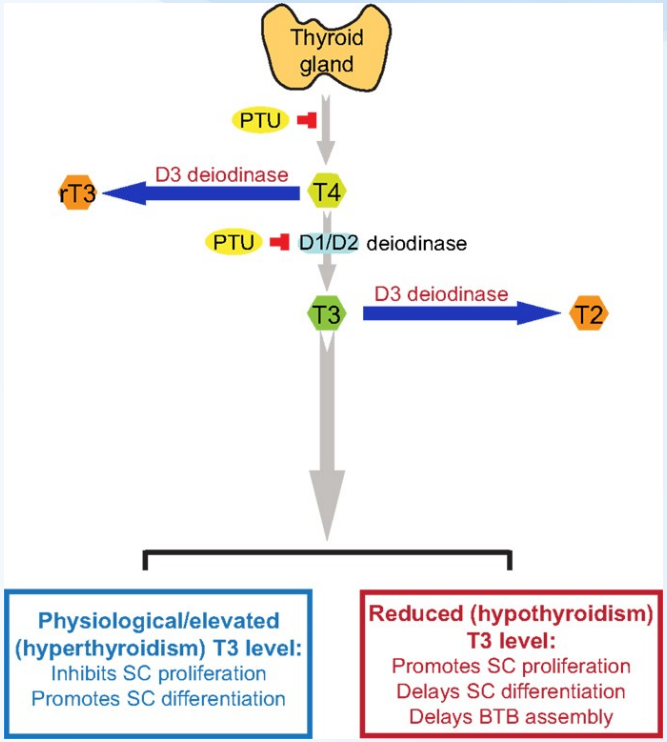
- **Thyroid peroxidases**
  - iodination of tyrosyl residues
  - coupling of iodinated tyrosyl residues
- **Thyroid deiodinases**
  - D1, D2 - activation of T4 into T3 via deiodination on „outer“ ring
  - D3 - deactivation into rT3 via deiodination on „inner“ ring



- **Many goitrogens** affect expression, activities and outcomes of these key enzymes
  - **PTU – propylthiouracil**
    →effect deiodinases



  - **Thiocyanate ([SCN]⁻) or perchlorate ($NaClO_4$)**
    →effect on iodine uptake



Centrum pro výzkum toxických látek v prostředí

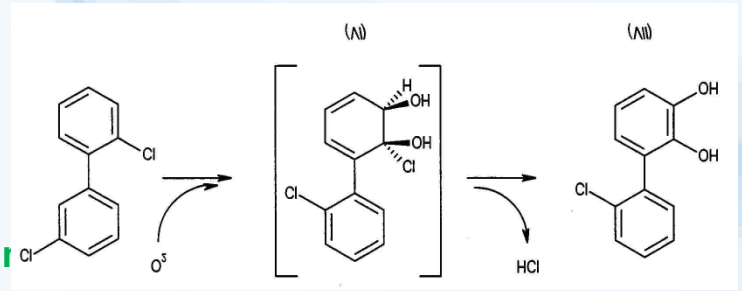
Disruption of transport of thyroid hormones in blood

- SPECIFIC TRANSPORTERS in blood
  - regulating free T4 and T3 levels
  - 3 types :
    - Thyroid-binding prealbunin (transthyretin) (20-25%)
    - Albumin (5-10%)
    - **Thyroid binding globulin (TBP, 75%)**

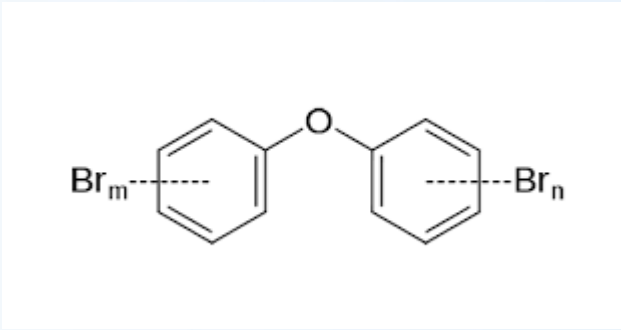
- **NUMBER OF EDCs → act on transport proteins**
  - OH-PCBs, **brominated and chlorinated flame retardar**
    DDT, dieldrin
  - **OH-PCBs** – equal affinity to **TBP** as T4 and T3 (!!!)

- Increased levels of "free T4" in blood
  - negative feedback to TSH release
    → increased depletion
    → increased weight, changes in thyroid gland
  - Documented after exposures to POPs in vertebrates

Hydroxylated PCB formation



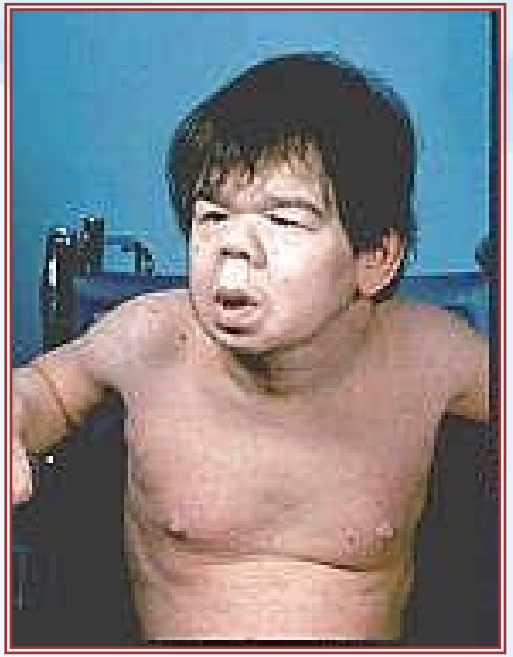
Polybrominated diphenyl ethers (PBDEs) – flame retardants



Centrum pro výzkum toxických látek v prostředí

Effects of thyroid disruption

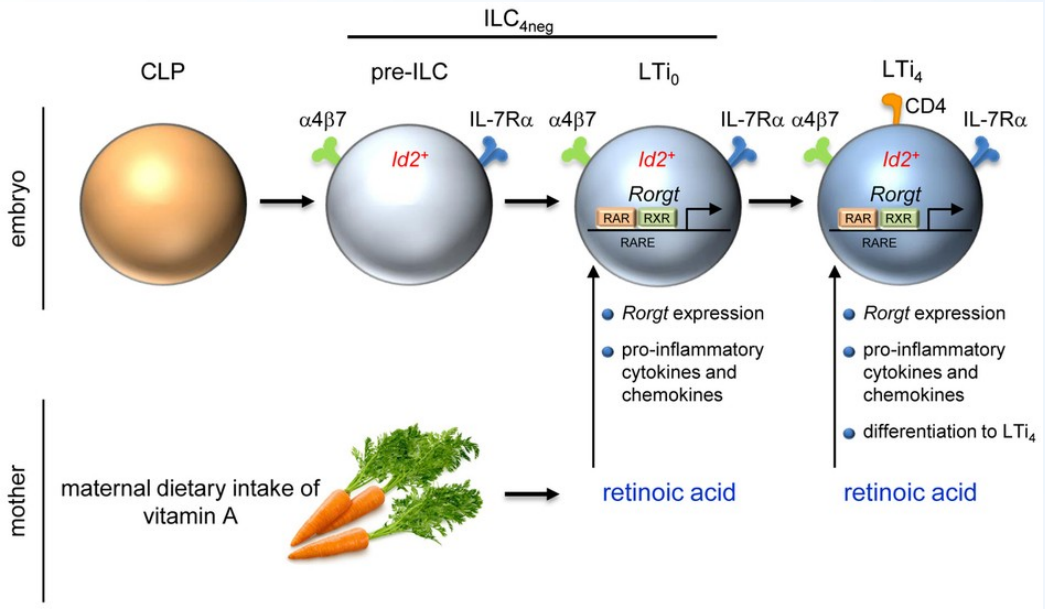
- **Exposures during prenatal stages**
  - severe damage of CNS (cretenism, delayed eye opening, cognition)
  - Megalotestis
  - Histological changes in thyroid gland (goitre)



- **Exposures during development**
  - nervous system fails to develop normally
  - mental retardation
  - skeletal development

RAR/RXR receptors
- vitamin A and its derivatives: RETINOIDS -

& their role in toxicity



Centrum pro výz
toxických látek
v prostředí

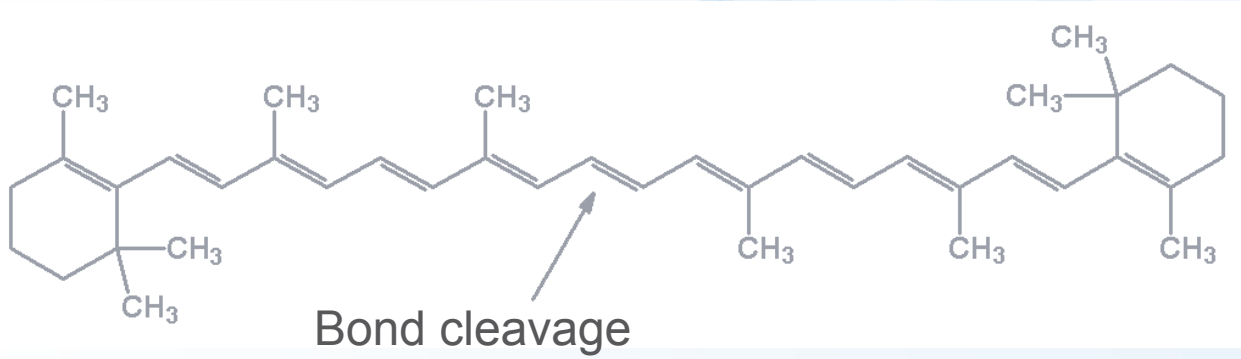
RETINOIDS

Sources: from diet - dietary hormones

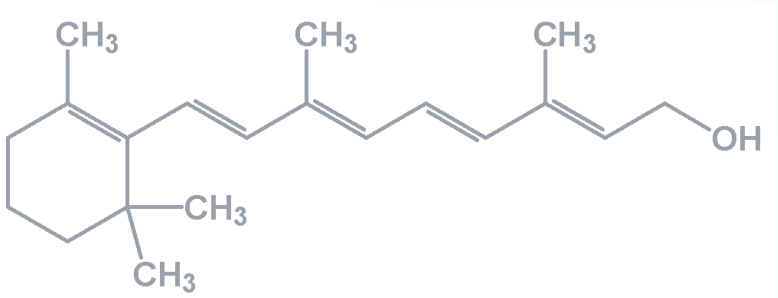
Retinyl esters – animal sources

Plant carotenoids

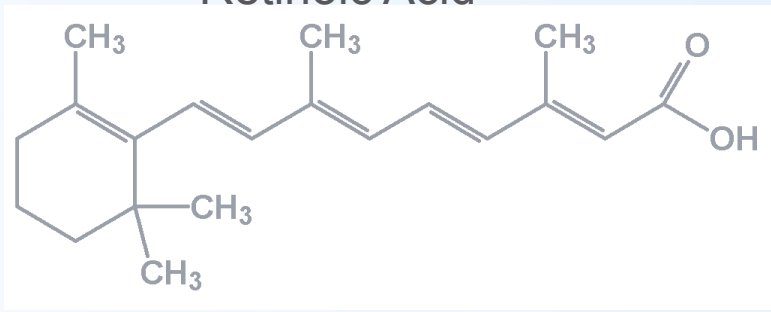
β-karoten



Retinol (vitamin A)



atRA – all trans - Retinoic Acid



Centrum pro výzkum toxických látek v prostředí

Retinoids and their functions

- Regulation of development and homeostasis in tissues of vertebrates and invertebrates
- Development of embryonic, epithelial cells (gastrointestinal tract, skin, bones)
- Necessary for vision
- Suppressive effects in cancer development
- Important for cell growth, apoptosis and differenciation
- Antioxidative agent
- Affect nervous and immune function

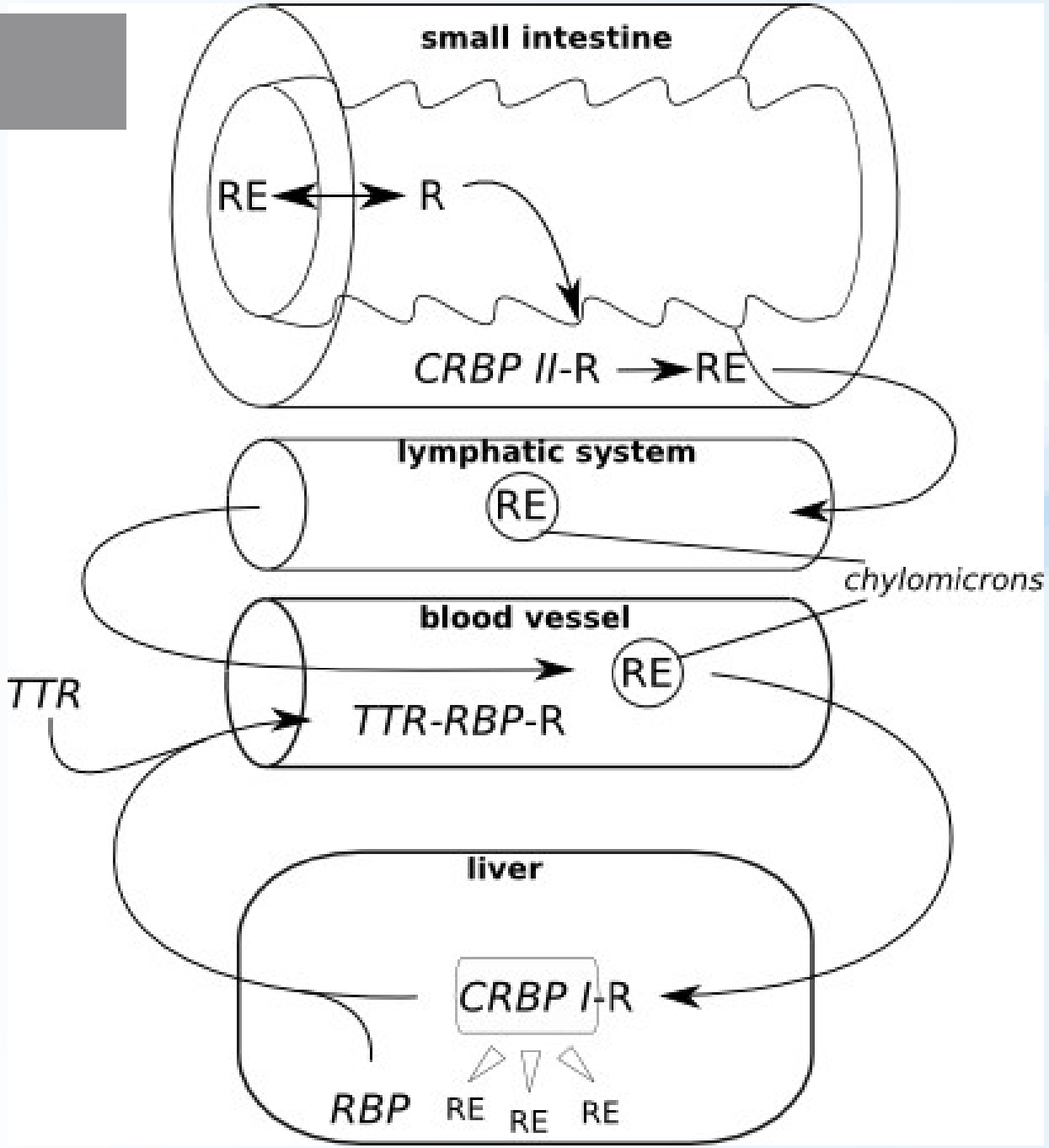
Retinoid transport

RE: Retinol-Ester

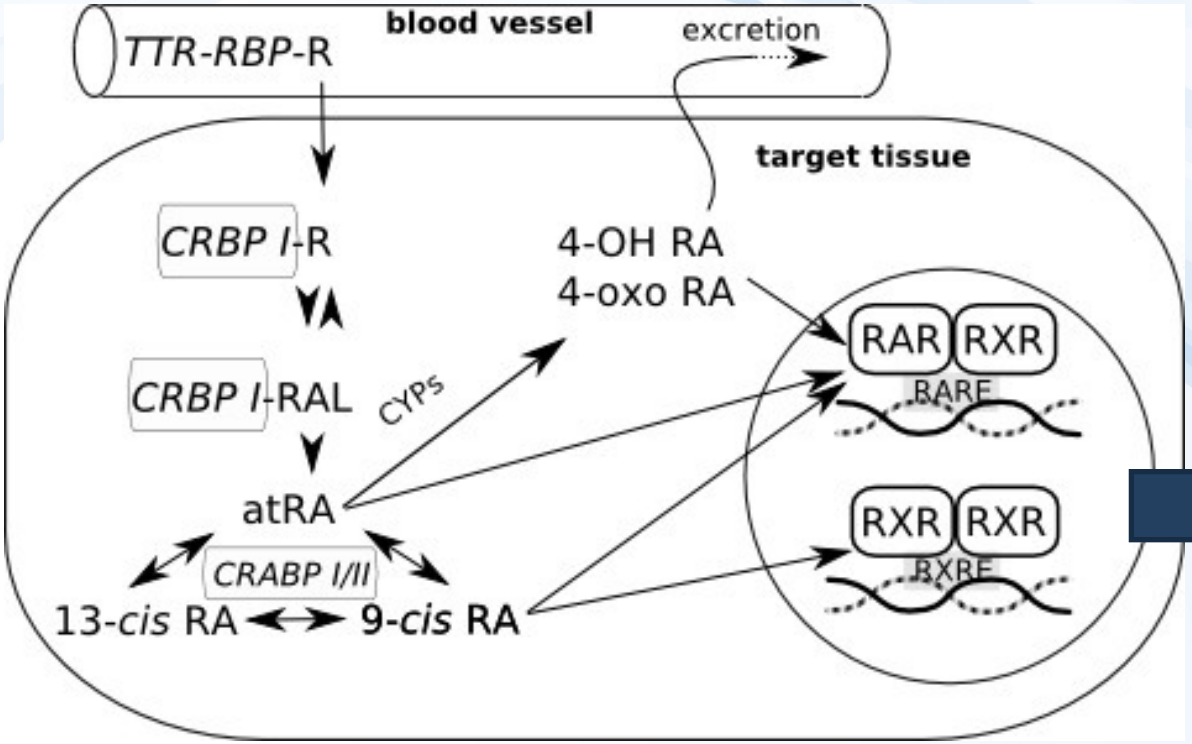
R: Retinol

RBP: Retinol Binding Protein (*LMW*)

TTR: Transthyrethin (*HMW*)



Retinoid fate in the cells



Gene expression

Retinoid binding proteins

CRBP – cellular retinol binding protein
- binding of retinol, immediate decrease of retinol concentration

CRBAP – cellular retinoic acid binding protein
- Controlling the ratio free retinol/free retinoic acid

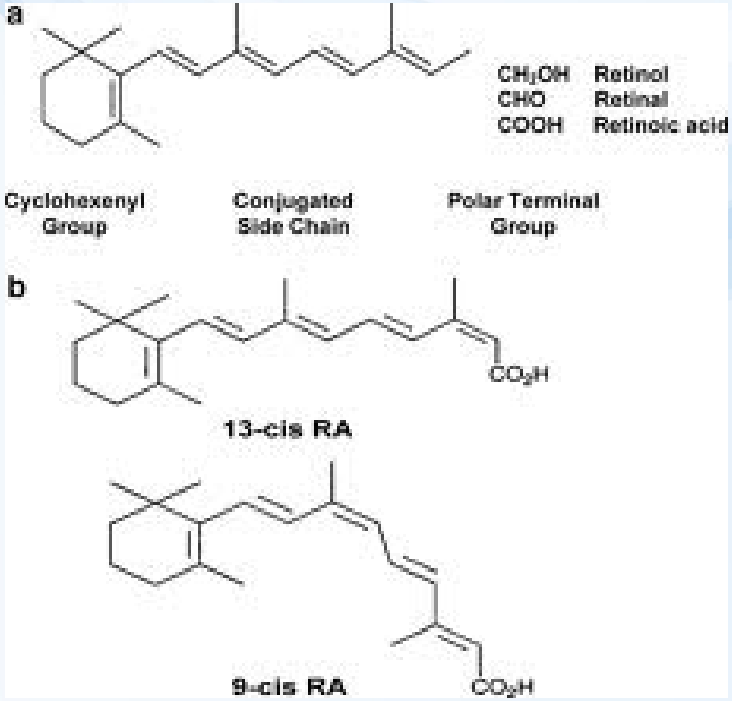
Centrum pro výzkum toxických látek v prostředí

RAR/RXR and RA

- Isoforms of RAR a RXR
  - Formation of homo- and heterodimers
  - 48 possible RAR-RXR heterodimers

  → sensitive regulation of gene expression
- RXR – heterodimers with other receptors
  - VDR, TR, PPAR ... → see crosstalk

- **RETINOIC ACID (RA)**
- 3 basic subtypes
  - all-trans- (ATRA)
  - 9-cis- and 13-cis-retinoic acid
- All-trans RA (ATRA) binds selectively to RAR
- Cis RA bind to both receptor types



Centrum pro výzkum toxických látek v prostředí

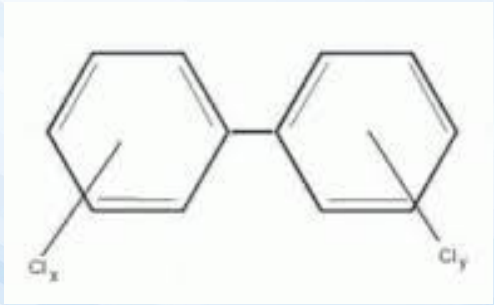
Disruption of retinoid signalling by xenobiotics

- **Possible modes of action – disruption of retinoid signalling:**
  - **Metabolization** of retinoids by detoxication enzymes
  - Disruption of binding retinoids **to transport proteins**
  - Retinoids as antioxidants may be **consumed by oxidative stress** induced by xenobiotics
  - Interference during **binding to RAR/RXR**

- **Effects**
  - **Decreased retinoid levels in organisms**
    - Downregulation of growth factors
    - Xerophtalmia, night blindness
    - Embryotoxicity, developmental abnormalities
  - **Increased ATRA concentration**
    - teratogenic effects

Disruption of retinoid signalling by xenobiotics

- **Polluted areas**
  - **mostly decrease of retinoid levels**
    - Documented in aquatic birds, mammals and fish



- **Disruption of retinoid transport:** PCBs

- **Effects on retinoid receptors:**
  - RAR, RXR binding and/or transactivation
    - pesticides (chlordane, dieldrin, methoprene, tributyltin…)
    - Effect on ATRA mediated response – TCDD, PAHs

- **Disruption of retinoid metabolism:**
  - **PCDD/Fs,** PAHs, PCBs, pesticides
  - changes of serum concentrations of retinol and RA
  - mobilization of hepatic storage forms

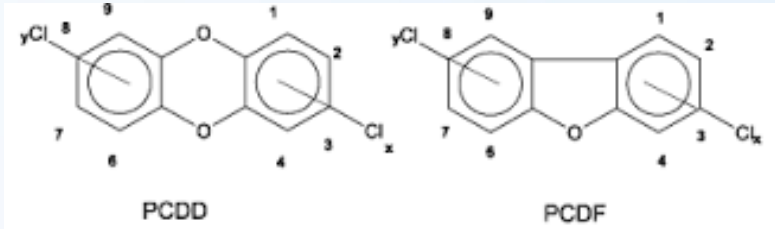
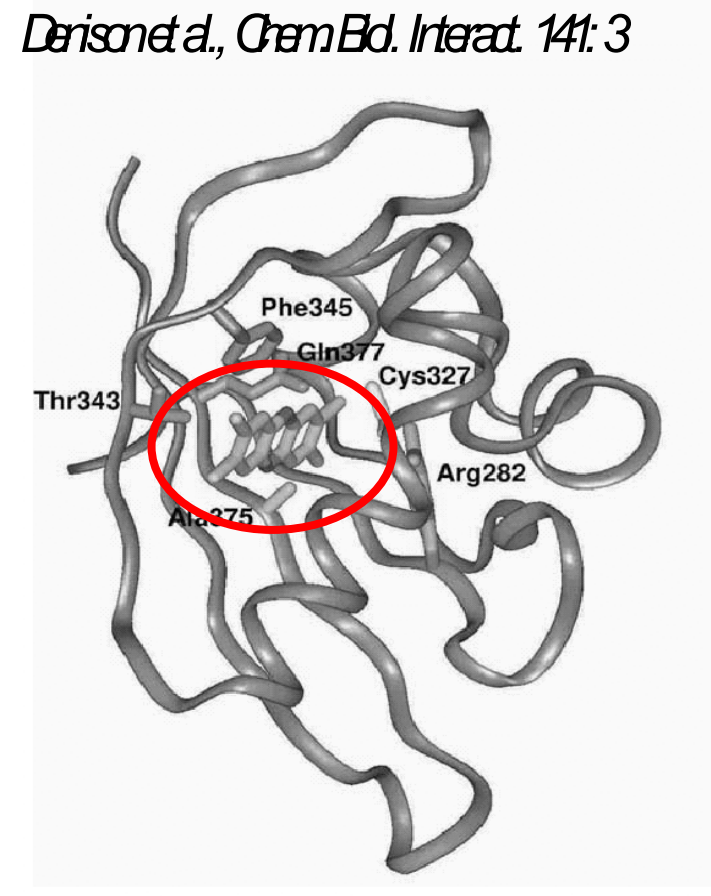


Figure 1. General molecular structure of polychlorinated dibenzo-p-dioxin (PCDD) and dibenzofurans (PCDF)

Centrum pro výzkum toxických látek v prostředí

AhR (Arylhydrocarbon receptor)

AhR structure



2,3,7,8-TCDD
(dioxin) bound to AhR

AhR

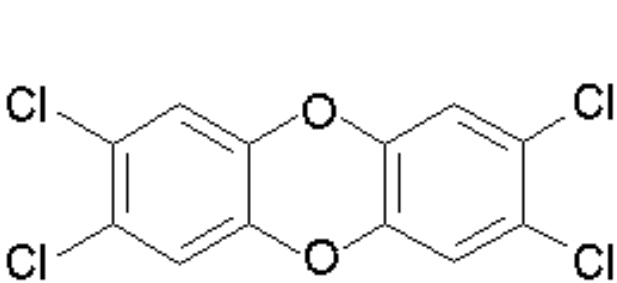
- Also known as „dioxin-receptor“ (and its modulation leads to so called „dioxin-like“ activity or toxicity)

- Ligand-activated transcription factor
  - Similar to all NRs
- AhR has effects on many different genes

- important mediator of toxicity of POPs – primary target of **planar aromatic substances**
  - regulator of xenobiotic metabolism and activation of promutagens

- Crossactivation/crosstalk with other NRs

- **Strongest known ligand - TCDD**
  - (not endogeneous !)



AhR regulated genes

- Many genes contain xenobiotic response elements (XRE) or dioxin responsive elements (DRE) in their promoter region:
  - **Detoxification genes** phase I enzymes (CYP 1A1, CYP 1A2, CYP 1B1) and phase II enzymes (UDP-glucuronosyltransferase, GST-Ya, NADP(H):oxidoreductase)
    → **Detoxification after toxicant exposure**
    … also with possible toxic consequences (oxidative stress, activation of promutagens accelerated clearance of hormones)
  - **Other genes** - regulation of cell cycle and apoptosis
    - Bax (apoptosis control), p27Kip1, Jun B (MAP-kinase), TGF-b (tumor growth factor)
      → Various adverse toxic effects

Physiological role of AhR

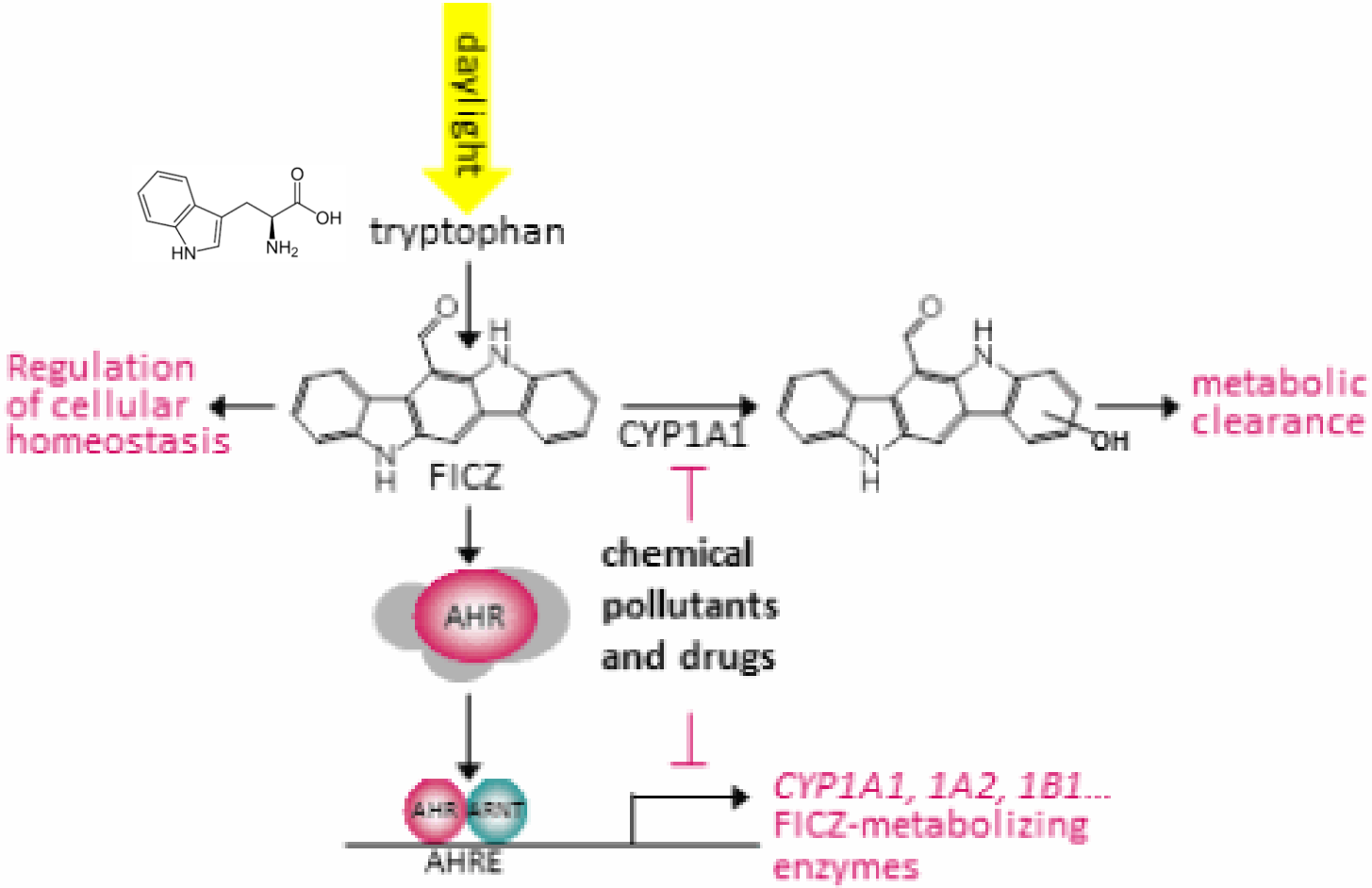
- **Physiological role for AhR still not known completely (?)**
  - Most likely – "protection" against toxicants → induction of detoxification

- Many adverse effects documented in **AhR-deficient** mice
  - significant growth retardation;
  - defective development of liver and immune system;
  - retinoid accumulation in liver;
  - abnormal kidney and hepatic vascular structures.
  - resistant to BaP-induced carcinogenesis and TCDD-induced teratogenesis;
  - no inducible expression of CYP 1A1 and 2.

→ this implies presence of **natural endogeneous ligand(s)** (not only exogeneous toxicants can bind AhR)

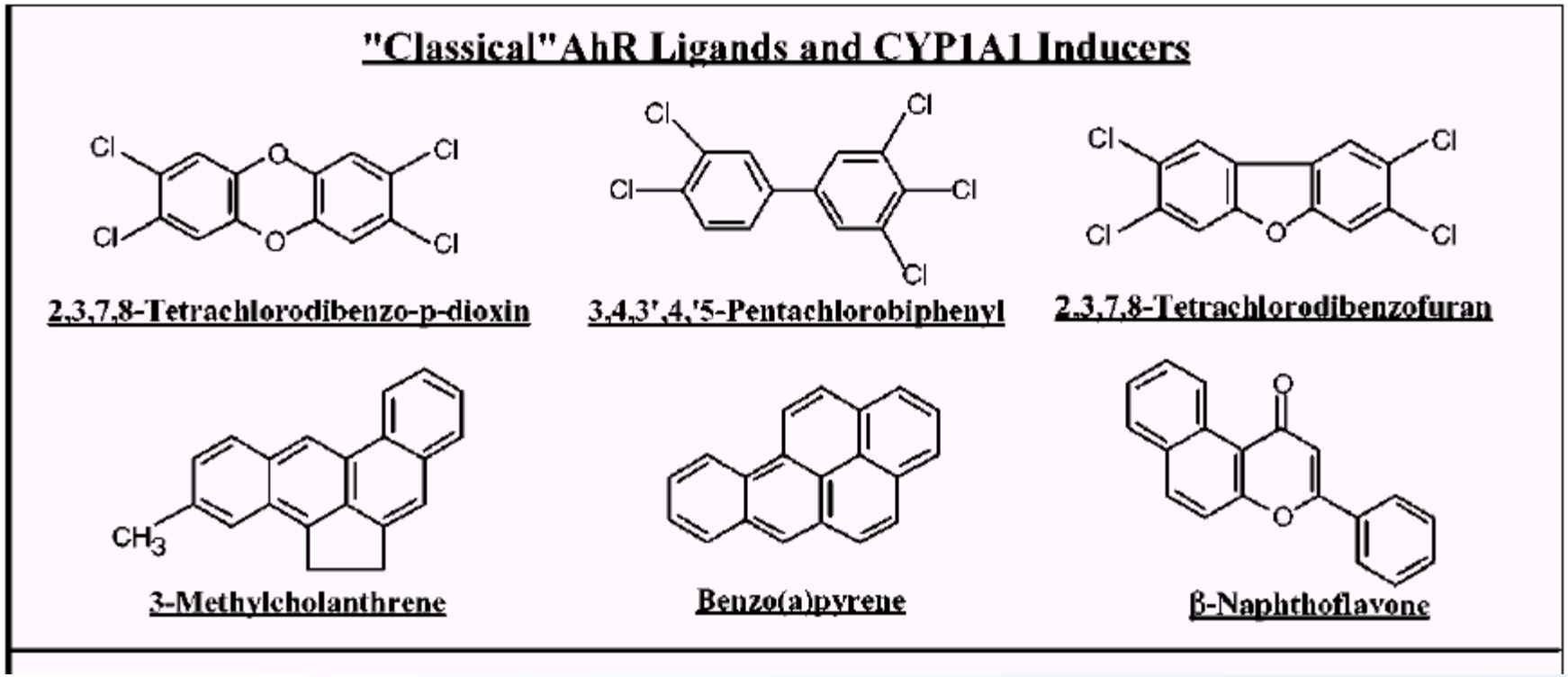
What is the natural (endogenous) physiological ligand of AhR ?

Potential candidate: 6-formylindolo[3,2-b]carbazole (FICZ)



Classical and “non-classical” AhR ligands

Classical = planar structures → direct binding to AhR

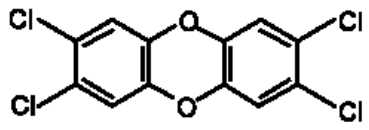


Denison & Nagy, Annu. Rev. Pharmacol. Toxicol. 43:309

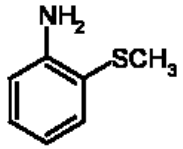
„Non-classical“ AhR ligands – various structures

M.S. Denison et al. / Chemico-Biological Interactions 141 (2002) 3–24

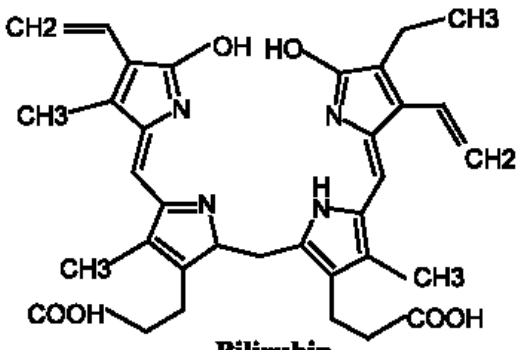
“Classical” ligand



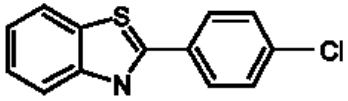
2,3,7,8-Tetrachlorodibenzo-p-dioxin



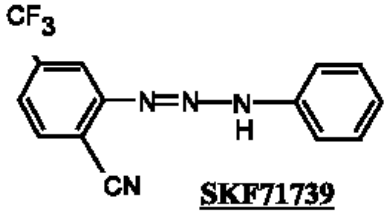
2-(Methylmercapto)aniline



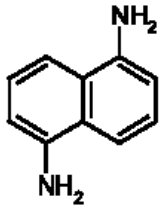
Bilirubin



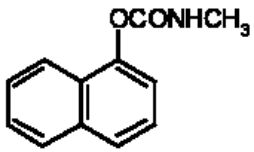
2-(4'-Chlorophenyl)benzothiazole



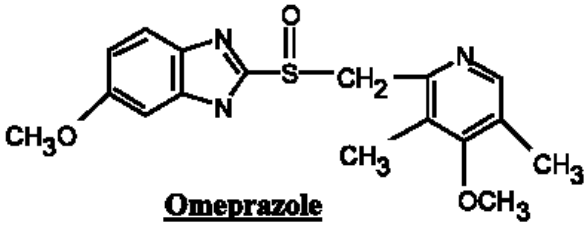
SKF71739



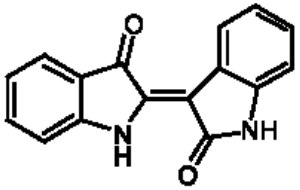
1,5-Diaminonaphthalene



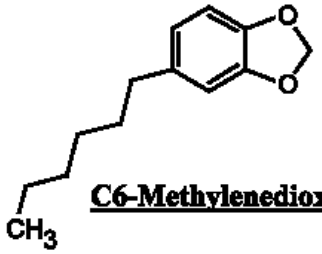
Carbaryl



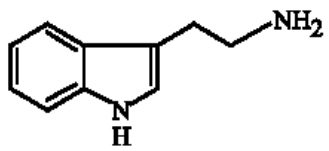
Omeprazole



Indirubin

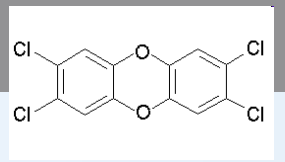


C6-Methylenedioxybenzene



Tryptamine

Biological responses to TCDD & AhR ligands



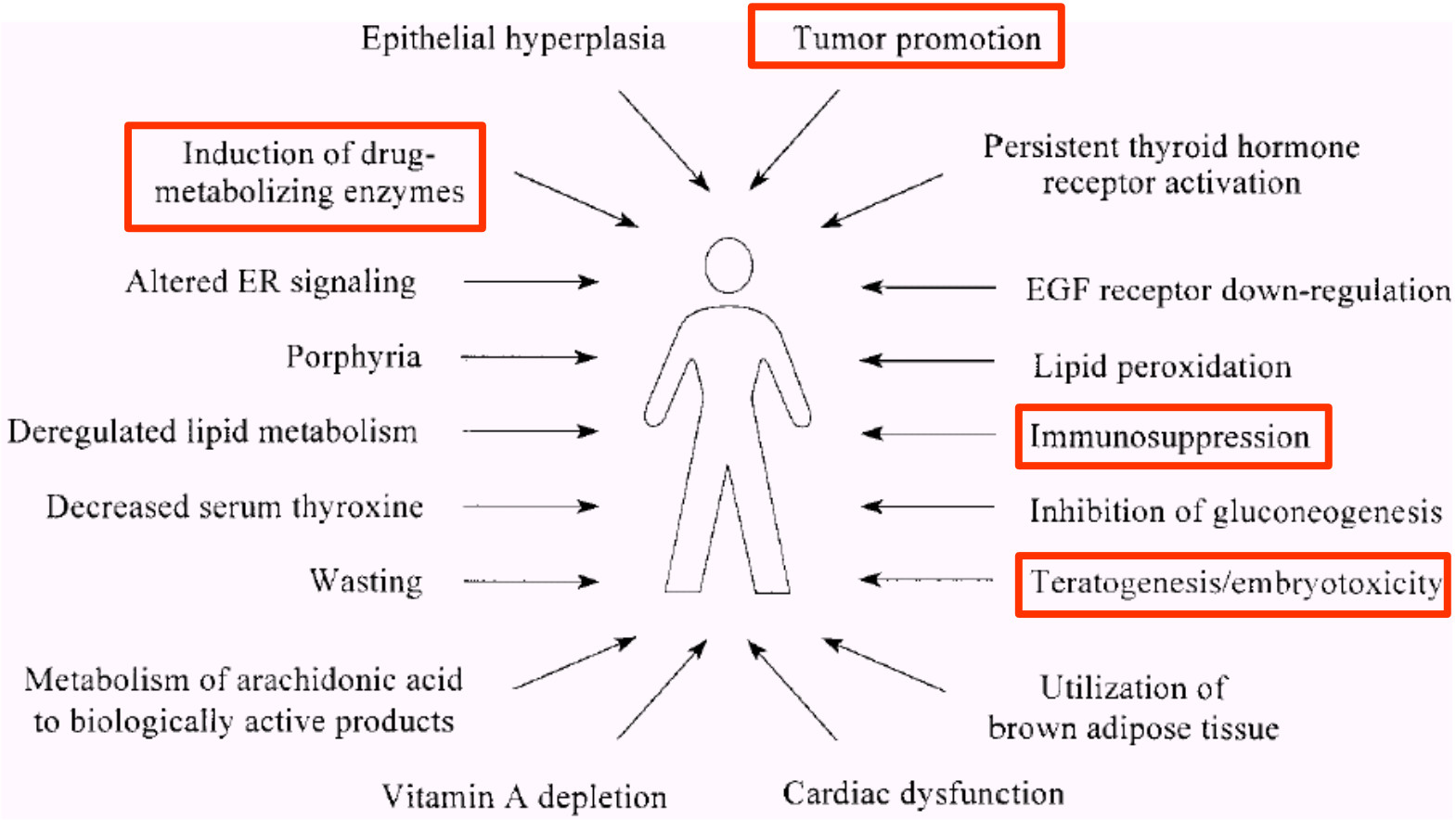


Figure 1 Biological responses to TCDD. A wide variety of cellular processes have been shown to be affected by TCDD.

Schmidt & Bradfield, Annu. Rev. Cell Dev. Biol. 12:55

Toxic equivalency factors (TEF)/TEQ concept

- Toxicity of compounds with similar toxicological properties as TCDD (activating AhR) may be evaluated by TEF/TEQ concept
  - TEF = Toxic Equivalency Factor ("characteristic" of the Chemical)
  - TEQ = Toxic Equivalent (sum of TEFs x concentrations)

- **TEFs are consensus values based on REPs (relative potencies)** across multiple species and/or endpoints.
  - TEFs are based upon a number of endpoints, from chronic in vivo toxicity to in vitro toxicity with the former having the greatest importance in determining overall TEF.

- **TEQs provide a simple**, single number that is indicative of **overall toxicity of a sample** (water, sediment, food) containing a mixture of dioxins and dioxin-like compounds.

- The total potency of a mixture can be expressed in TCDD TEQ concentration
  - i.e. TEQ = concentration corresponding to the effect that would be induced by TCDD

$$\mathrm{TEQ} = \Sigma\{\mathrm{compound}_1 \times \mathrm{TEF}_1 + \ldots$$

$$+ \mathrm{compound}_n \times \mathrm{TEF}_n\}$$

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Toxic equivalency factors for PCDDs, PCDFs and PCBs:

Table 4. Toxic Equivalent Factors established by the WHO (WHO-TEFs) for dioxins and dioxin-like PCBs [4]

PCDD Congener	WHO-TEF	PCDF Congener	WHO-TEF	PCB Congener	WHO-TEF
2,3,7,8-TCDD	1	2,3,7,8-TCDF	0.1	*Non-ortho*	
12,3,7,8-PeCDD	1	12,3,7,8-PeCDF	0.05	PCB#81	0.0005
123478-HxCDD	0.1	23478-PeCDF	0.5	PCB#77	0.0005
123678-HxCDD	0.1	123478-HxCDF	0.01	PCB#126	0.1
12,3,7,89-HxCDD	0.1	123678-HxCDF	0.1	PCB#169	0.01
1234678-HpCDD	0.01	234678-HxCDF	0.1	*Mono-ortho*	
OCDD	0.0001	12,3,7,89-HxCDF	0.1	PCB#105	0.0001
		1234678-HpCDF	0.01	PCB#114	0.0005
		1234789-HpCDF	0.01	PCB#118	0.0001
		OCDF	0.0001	PCB#123	0.0001
				PCB#156	0.0005
				PCB#157	0.0005
				PCB#167	0.00001
				PCB#189	0.0001

Eljarrat & Barceló, Trends Anal. Chem.22: 655

Final concentration is expressed as „Equivalents of TCDD“
(e.g. ng TEQ / kg = ng TCDD / kg)

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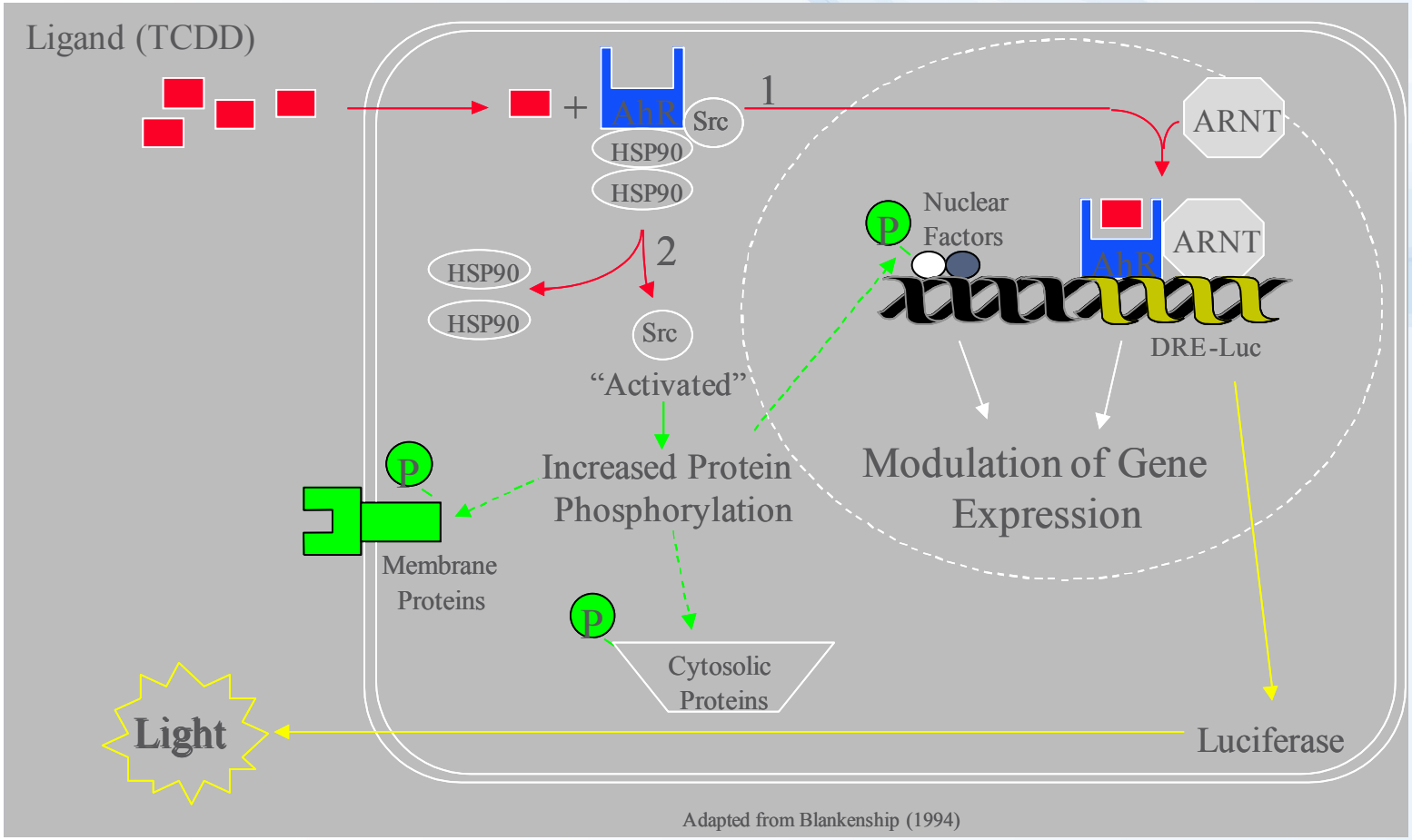
Biomarkers/bioanalytical methods for AhR toxicity

- In vivo studies
  - liver enlargement, reduction of thymus weight, wasting syndrome, reproductive and developmental disorders
- In vivo biomarkers
  - **EROD** activity, CYP 1A1 and 1B1 expression (discussed in biomarker section)

- in vitro assessment of chemical potencies
  - EROD (ethoxyresorufin-O-deethylase activity) in cell cultures;
  - **CALUX/CAFLUX assays** (luciferase expression – reporter gene assays)
  - GRAB assay (AhR-DNA binding)
  - yeast bioassay;
  - immunoassays;
  - detection of CYP1A mRNA (qPCR) or AhR protein (western blotting)

In vitro CALUX/CAFLUX assays

CALUX – Chemical Assisted Luciferase Expression
DR-CALUX (Dioxin Responsive CALUX)
(i.e. Luciferase Reporter Gene Assay with H4IIE.luc cells)



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DETECTION of EROD activity - example

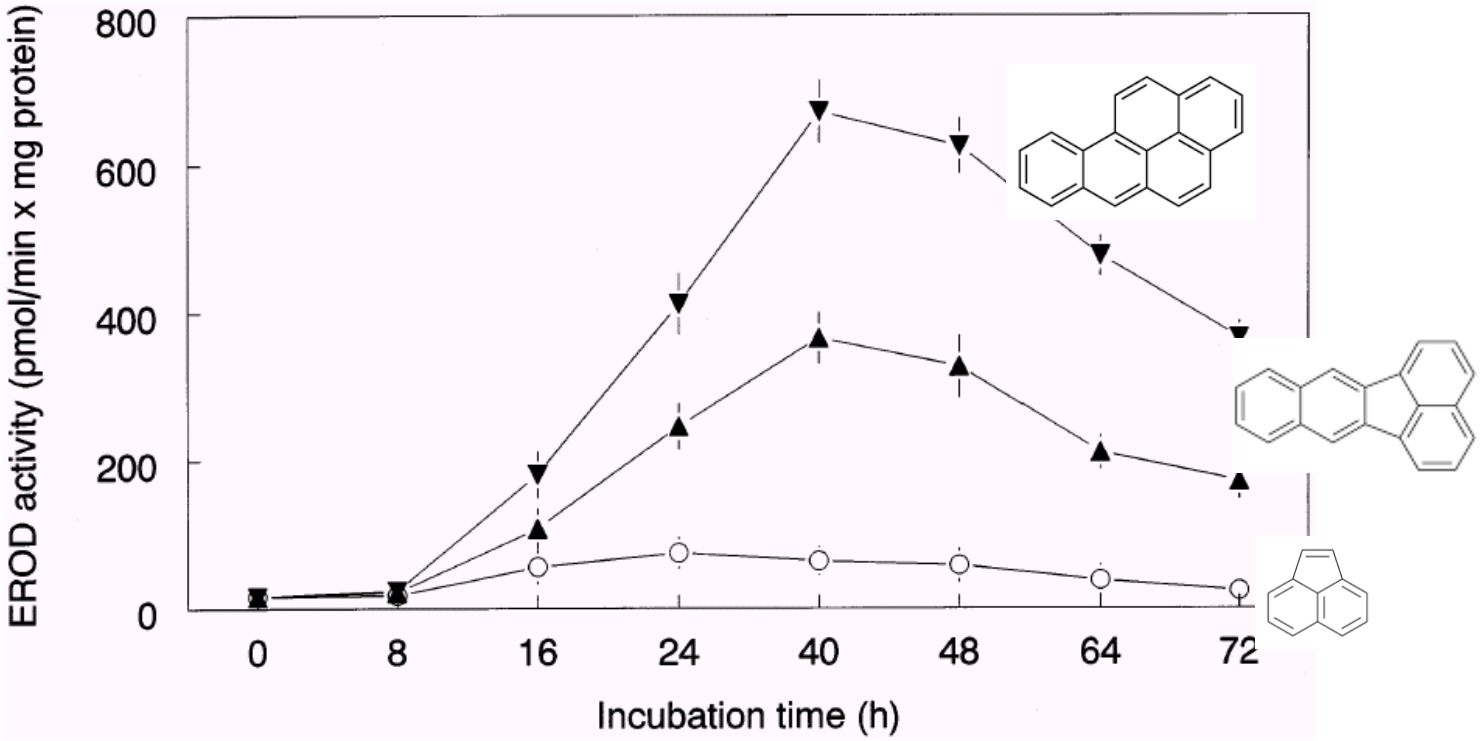
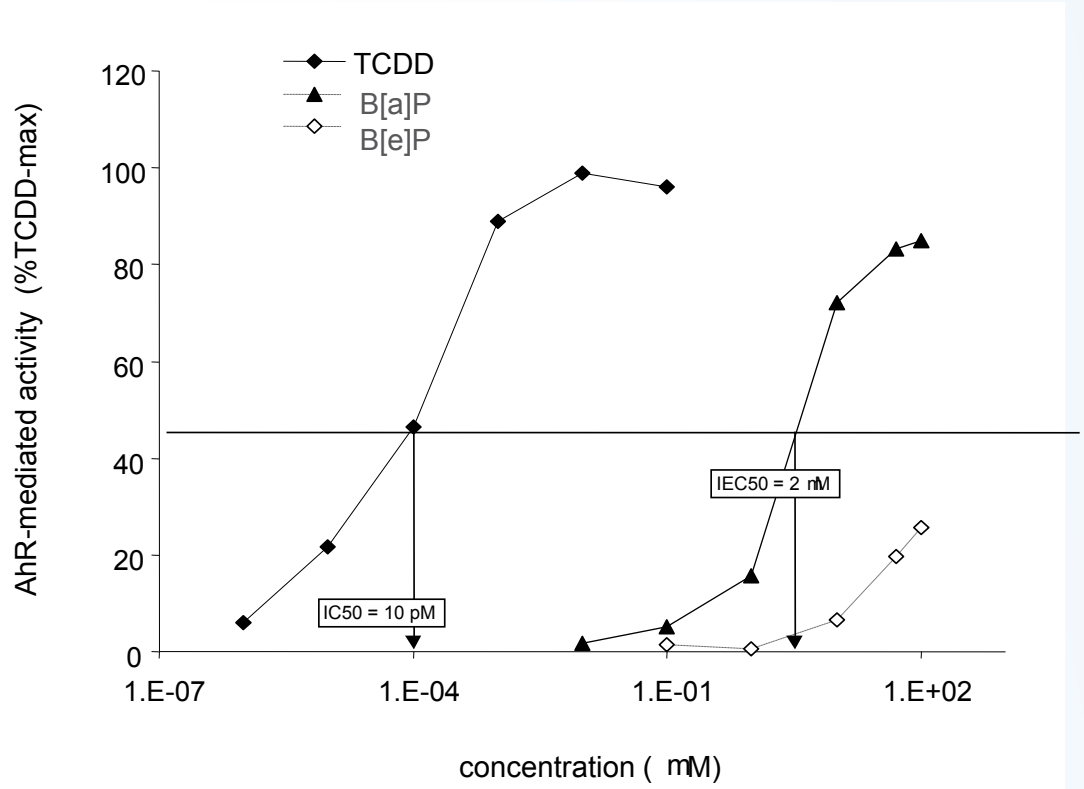


Fig. 2. Time course of induction of CYP1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity in primary cultures of rat hepatocytes, after addition of 1.7×10^{-5} M benzo[*a*]pyrene (-▼-), 1.9×10^{-6} M benzo[*k*]fluoranthene (-▲-) or 9.4×10^{-5} M acenaphthylene (-○-). EROD activity was determined in cell homogenates. The data represent means ± S.D. from four independent experiments.

Comparing toxicity of compounds → Application in Risk Assessment

- Quantification of effects (EC_{50})
- Comparison with the effect of reference toxicant (2,3,7,8-TCDD)
  - → relative potencies (REPs) to TCDD
    (= in vitro "Toxic Equivalency Factors" ~ TEFs)



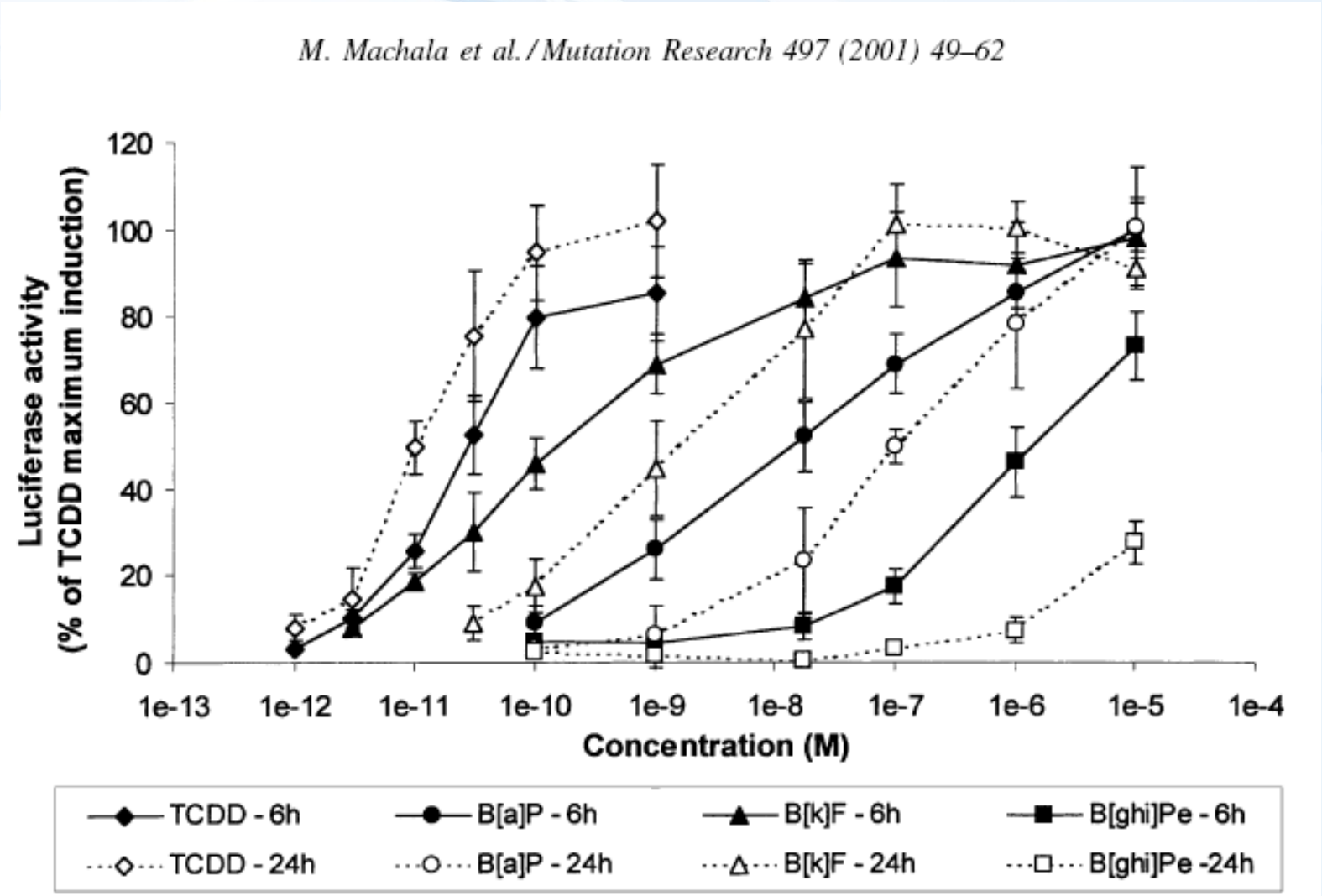
TCDD: IC_{50}
PAH: IEC_{50}

Relative Potency (REP)
= Induction Equivalency Factor

$$IEF = IC_{50} / IEC_{50}$$

REP interpretation: How many times is the compound "weaker" inducer than TCDD ?

Example - relative potencies of PAHs (two exposure periods) „CALUX“ assay



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Longer period: lower induction due to (partial) metabolization of PAHs (CYPs, oxidation) to products less potent to AhR

Table 2
IEFs of PAHs relative to TCDD or B[a]P derived from EC50 or EC25 values in 24 and 6 h exposure assays

Derived from	$IEF_{TCDD(24h)}$		$IEF_{TCDD(6h)}$		$IEF_{B[a]P(6h)}$	
	EC50	EC25	EC50	EC25	EC50	EC25
Flu	ni[a]	ni	ni	ni	ni	ni
Ant	ni	ni	ni	ni	ni	ni
Fla	2.27E−8	9.31E−7	9.84E−5	1.11E−4	1.05E−2	5.59E−3
Py	1.78E−6	3.38E−6	2.59E−5	4.45E−5	7.57E−3	6.21E−3
B[a]A	7.04E−6	9.60E−6	7.64E−7	2.40E−6	0.39	0.50
Chry	1.01E−4	1.07E−4	1.41E−2	3.26E−2	3.25	2.04
B[b]F	3.35E−5	4.82E−5	4.90E−2	2.32E−1	8.83	12.81
B[k]F	1.64E−3	2.94E−3	0.28	0.57	67.76	36.33
B[a]P	9.01E−5	1.99E−4	1.11E−2	2.02E−2	1.0	1.0
DB[ah]A	1.17E−3	1.52E−3	0.06	0.20	11.46	11.72
I[123-cd]P	2.96E−4	5.01E−4	0.86	1.24	44.20	29.70
B[ghi]Pe	ni	ni	2.27E−5	4.68E−5	5.47E−3	2.99E−3
DB[al]P	4.90E−6	1.13E−6	2.52E−5	3.26E−5	2.36E−2	1.88E−2
NPyr	2.05E−4	3.83E−4	5.80E−3	1.31E−2	1.10	0.88
CPP	2.48E−7	6.53E−7	6.20E−6	1.72E−5	4.23E−3	3.38E−3
B[a]Pe	6.19E−6	6.28E−6	2.27E−4	3.05E−4	3.37E−2	1.68E−2
DB[ae]F	9.30E−6	1.18E−5	2.75E−5	1.33E−4	1.74E−3	6.74E−3
DB[ai]P	1.65E−4	4.41E−4	4.29E−2	3.82E−2	2.59	1.75
DB[ae]P	1.80E−5	3.90E−5	1.08E−3	3.90E−3	0.49	0.13
DB[ah]P	7.14E−5	3.70E−4	2.65E−2	5.43E−2	2.80	2.68
DB[ak]F	1.23E−3	1.37E−3	1.55E−2	2.02E−2	2.69	1.65
5-MeChry	9.48E−5	1.59E−4	4.05E−2	5.08E−2	3.07	2.46
DB[aj]A	3.70E−4	5.21E−4	3.07E−2	4.04E−2	2.16	2.16
B[j]F	3.68E−4	7.40E−4	4.05E−2	6.33E−2	2.25	2.51
B[c]Phe	4.49E−7	1.07E−6	6.21E−5	7.51E−5	4.64E−3	3.76E−3
B[e]P	5.15E−7	6.30E−7	3.71E−5	8.17E−5	2.27E−3	2.86E−3
DMBA	5.41E−6	1.30E−5	4.71E−2	3.98E−2	0.46	0.9
1-MePyr	2.07E−6	2.82E−6	4.80E−5	7.20E−5	8.54E−3	6.33E−3
DB[ac]A	1.92E−4	4.23E−4	3.53E−2	7.80E−2	1.75	2.78
Pic	4.11E−5	5.54E−5	1.90E−3	5.20E−3	0.12	0.25

[a] ni, no induction observed.

Summary – Nuclear receptors

- Important physiological functions,
- Important roles in pathologies and chemical toxicity (ENDOCRINE DISRUPTION)

- NRs with well studied roles in toxicity: **ER and AhR**
  - Other NRs (AR, RAR/RXR, ThR) – important but less explored

- All NRs share similar structure and mechanisms of action
  - Act as direct transcription factors on DNA
- Natural ligands of NRs are small lipophilic hormones
  - steroids, thyroids, retinoids
  - Various regulatory functions
  - Role in toxicity: NR interact with **structurally similar xenobiotics**
- Various mechanisms beyond the toxicity
  - Adverse are both STIMULATIONS and INHIBITIONS directly at the receptor site (e.g. "anti-androgenicity)
  - Additional mechanisms –in blood (Thyroids), metabolism (Thyroids) clearance (Retinoids), heterodimerization and transport of hormones, "crosstalk" of different NRs

- **Other key information to remember**
  - REPORTER GENE ASSAYS (principle, use, what is CALUX?)
  - Characterization of chemical "toxic potentials"
    - General concept of "REPs" (valid for activation of all NRs)
    - Specifically for AhR - concept of TEFs / TEQs