Centrum pro výzkum toxických látek v prostředí

BIOMARKERS AND TOXICITY MECHANISMS

09b –Nuclear Receptors

AHR – Arylhydrocarbon Receptor

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

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

EVROPSKÁ UNIE esf

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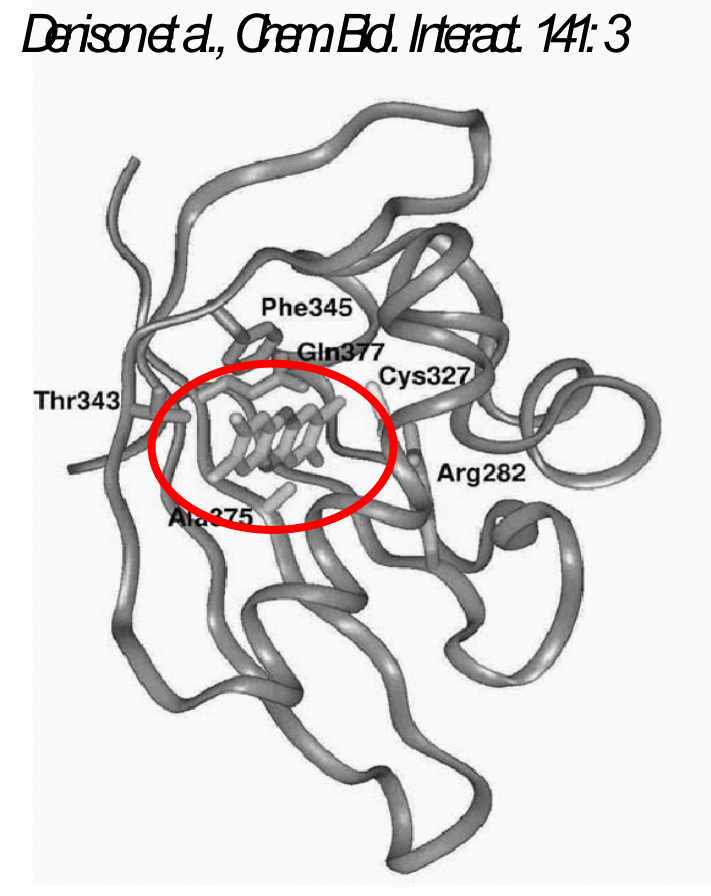
OP Vzdělávání pro konkurenceschopnost

UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

AhR (Arylhydrocarbon receptor)

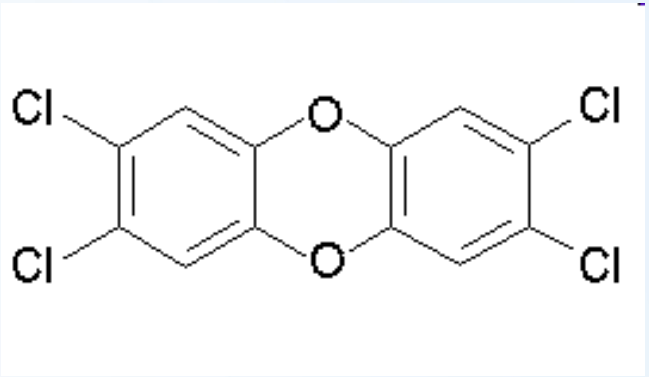
AhR structure



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

AhR

- Also known as „dioxin-receptor“ (activation/modulation results in so called „dioxin-like“ activity or toxicity)
- Similarly to all NRs AhR is a ligand-activated transcription factor
- AhR has effects on many different genes *(see next slide)*
- Important mediator of toxicity of POPs: AhR is primarily activated by **planar aromatic substances**
  → regulates xenobiotic metabolism (induction of CYP450; activation of promutagens etc)
- Crossactivation/crosstalk with other NRs
- **Known ligands of AhR**
  - **TCDD – strongest ligand:** exogenous, higly stable and very toxic compound produced during burning of materials
  - **Many other** compounds (see following slides)



AhR regulated genes

- Many different 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 exposure to toxicants**
    … also with possible toxic consequences (oxidative stress, activation of promutagens accelerated clearance of hormones)
  - **Other genes** – key roles in the regulation of cell cycle and apoptosis
    - Bax (apoptosis control), p27Kip1, Jun B (MAP-kinase), TGF-b (tumor growth factor)
      → *Various adverse toxic effects - cancer, immunotoxicity, reproduction toxicity*

Physiological role of AhR

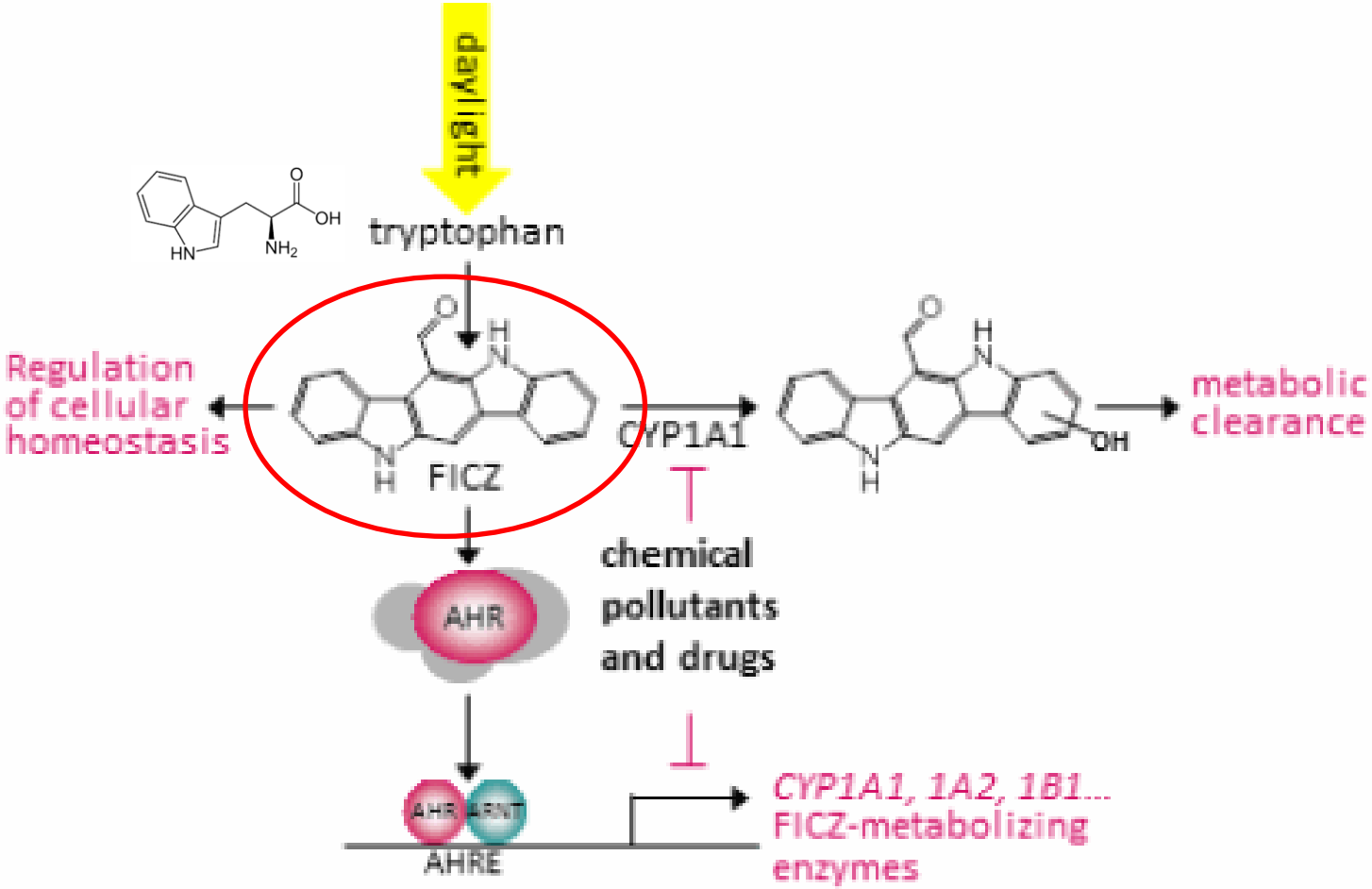
- **Physiological role of AhR still not known completely**
  - Most likely: “protection” against toxicants
    → induction of detoxification

- 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 such as TCDD can bind and activate AhR)

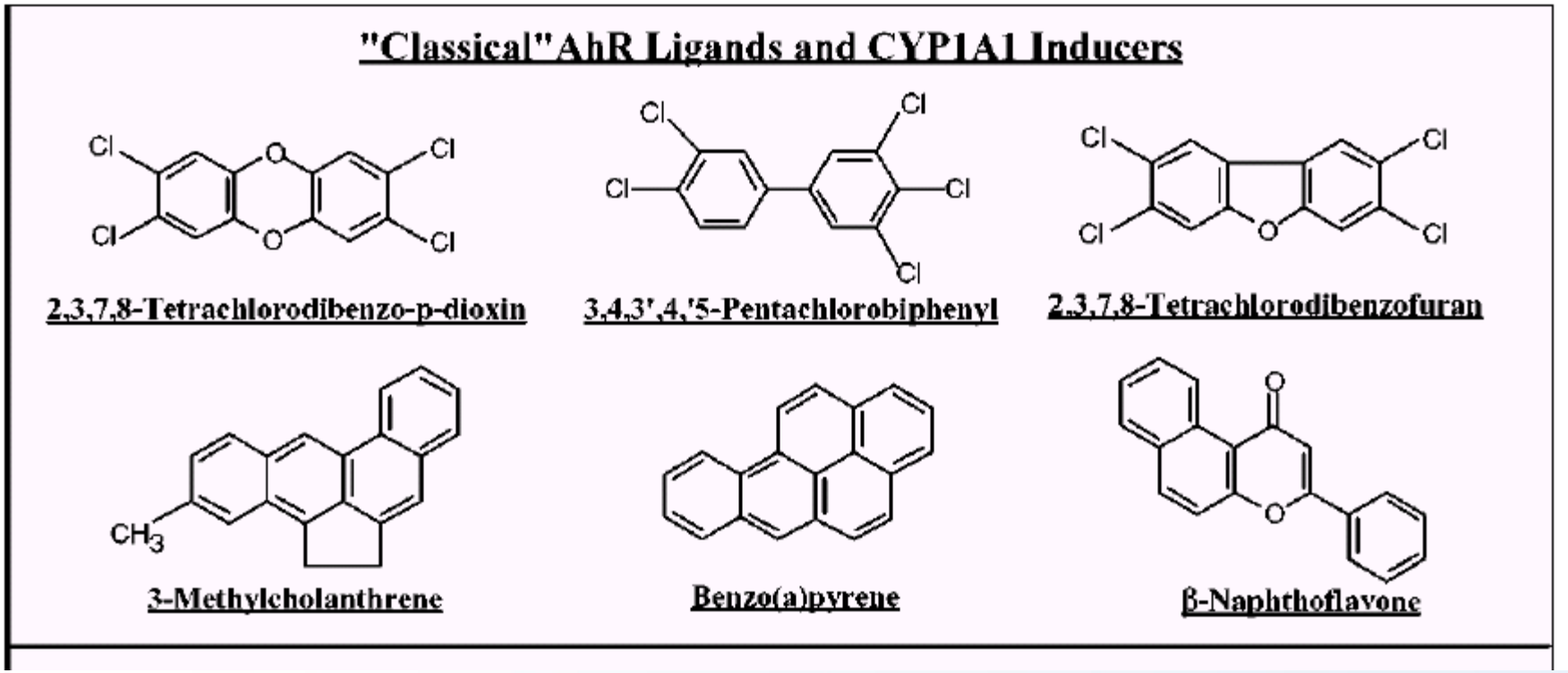
What are the natural (endogenous) physiological ligands of AhR ?

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



AhR ligands

Classical = planar structures (strong binding and activation of AhR)

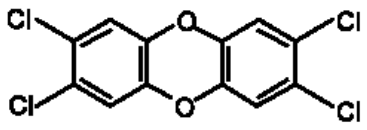


Denison & Nagy, Annu. Rev. Pharmacol. Toxicol. 43:309

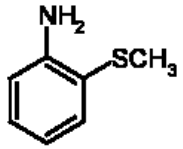
„Non-classical“ AhR ligands – various structures, also activate of AhR

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

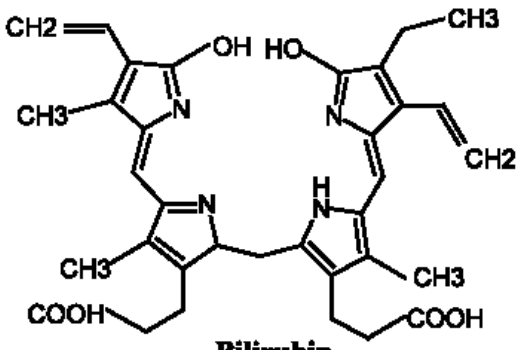
“Classical” ligand



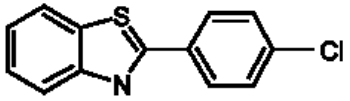
2,3,7,8-Tetrachlorodibenzo-p-dioxin



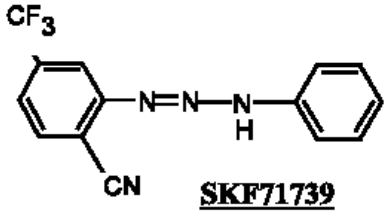
2-(Methylmercapto)aniline



Bilirubin



2-(4'-Chlorophenyl)benzothiazole



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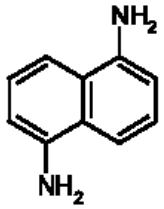
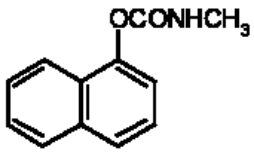
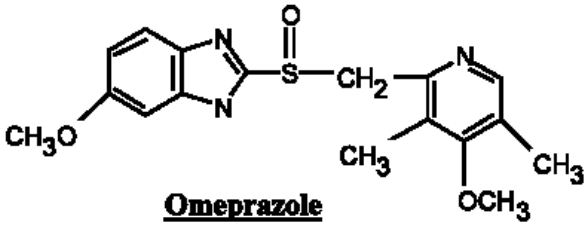


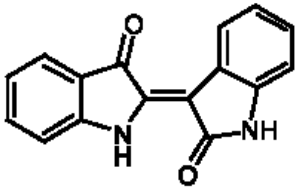
1,5-Diaminonaphthalene



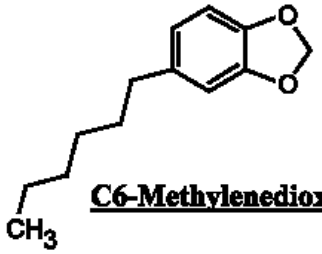
Carbaryl



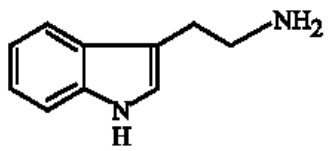
Omeprazole



Indirubin

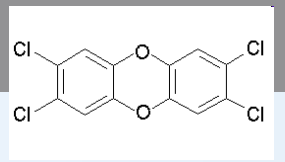


C6-Methylenedioxybenzene



Tryptamine

Biological responses to TCDD & AhR ligands



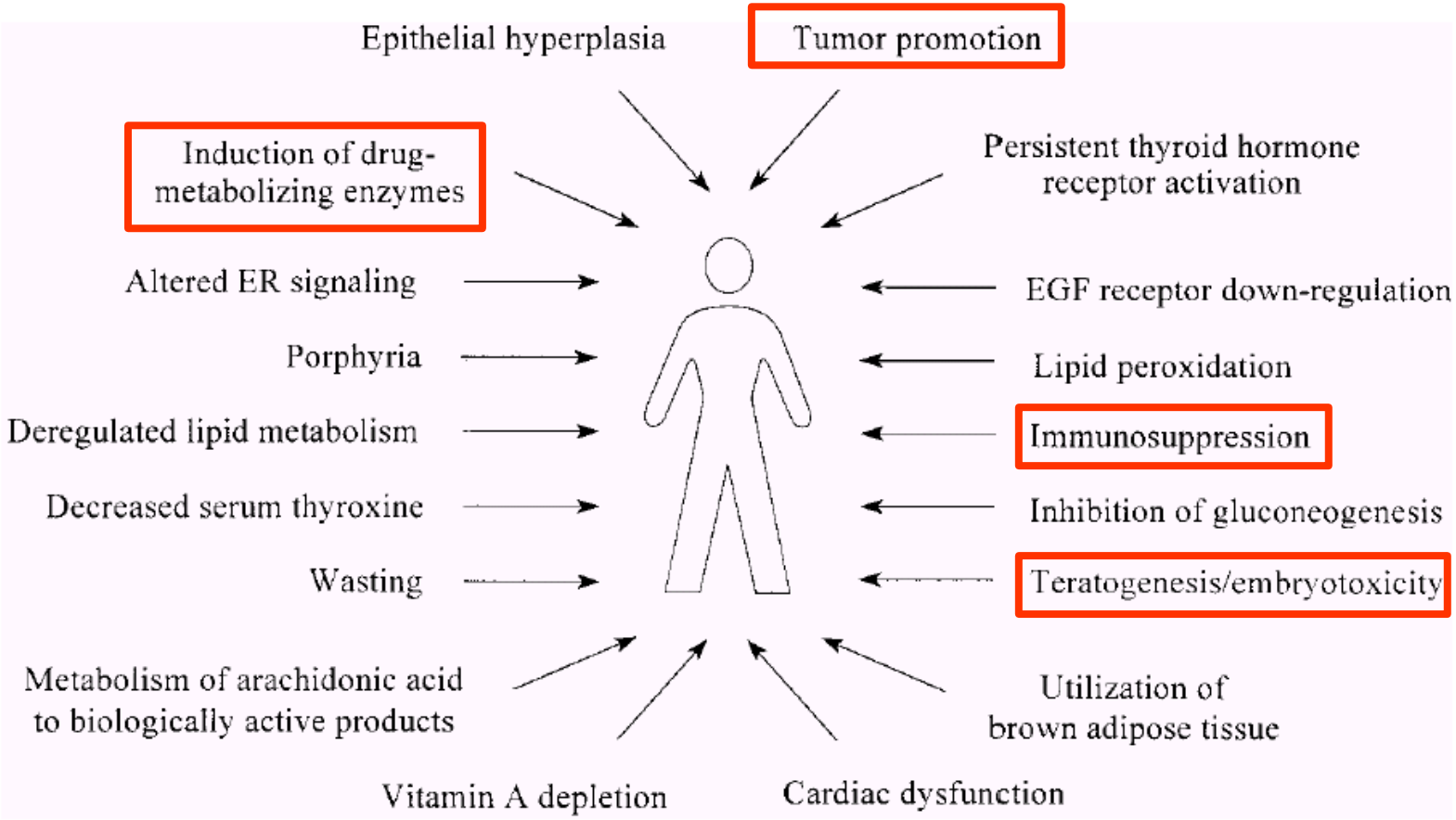


Figure 1 Biological responses to TCDD. A wide variety of cellular processes have been shown to be affected by TCDD.

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

Toxic equivalency factors (TEF)/TEQ concept

- Toxicity of compounds with similar toxicological as TCDD (i.e. compounds activating AhR) are evaluated through TEF/TEQ concept
  - TEF = Toxic Equivalency Factor (potency of a chemical to activate AhR)
  - TEQ = Toxic Equivalent (sum of TEFs of individudal compounds x their concentrations)

- **TEFs are consensus values** based on REPs (relative potencies of individual compounds)
  - TEFs have been established from number of endpoints (from in vitro effects: modulation of AhR to chronic in vivo toxicity)

- **TEQs provide a simple and single number** that is indicative of **overall toxicity of a sample** containing complex mixture of dioxin-like acting compounds**.**

- The total potency of a mixture of many compounds is expressed as one TEQ – i.e. concentration corresponding to the effect that would be induced by TCDD (the strongest ligand)

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

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

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

How to derive Relative Potencies (REPs)? Example PAHs – B[a]P and B[e]P

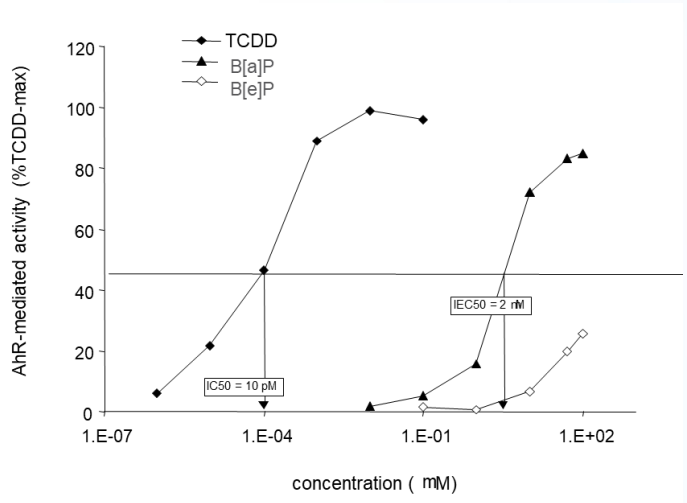
- **In vitro assay (see also next slide):** genetically modified human liver cells, transfected with luciferase gene (originally from firefly). Luciferase gene has DRE in the promoter, i.e. expression of luciferase gene (and protein) is under the control of AhR.
  - stronger the activation of AhR in the cells after exposure to dioxin-like acting compound, more luciferase is synthesized (and more luminescence is detected)

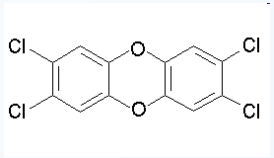
- Quantification of effects (EC_{50}) of studied compounds (BaP and BeP)

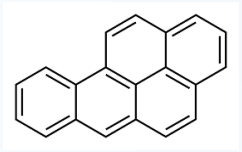
- Comparison with the effect of reference toxicant – i.e. 2,3,7,8-TCDD.

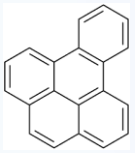
  → relative potencies (REPs) to TCDD

  (= in vitro "Toxic Equivalency Factors" are then used to derive final TEF)









TCDD: IC_{50} = 0.01 nM

BaP: IEC_{50} = 2 nM

REP: = 0.01 / 2 = 0.005

Relative Potency (REP)

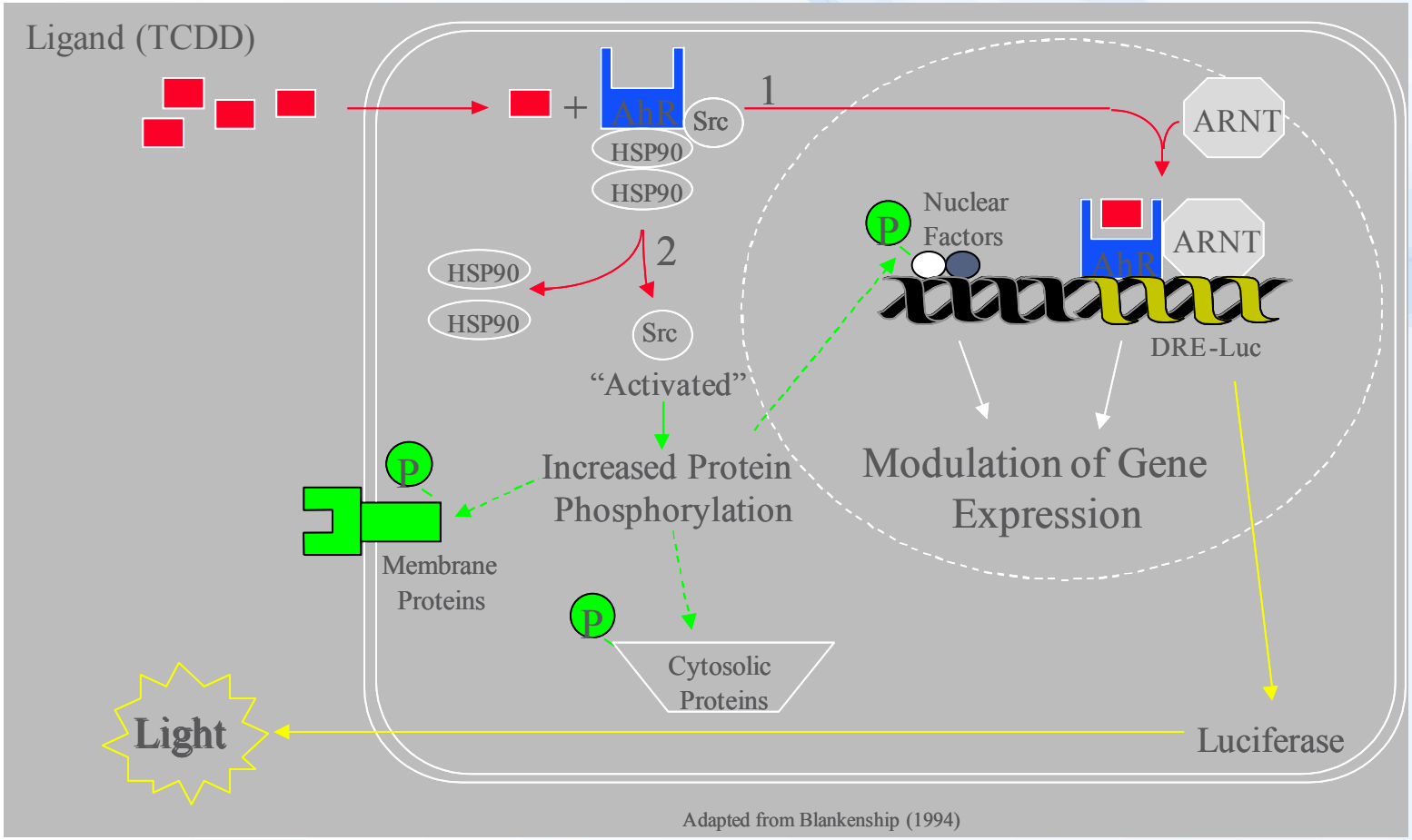
= Induction Equivalency Factor

REP (IEF) = IC_{50} / IEC_{50}

REP interpretation: How many times is the compound "weaker" inducer of AhR in comparison with TCDD ?

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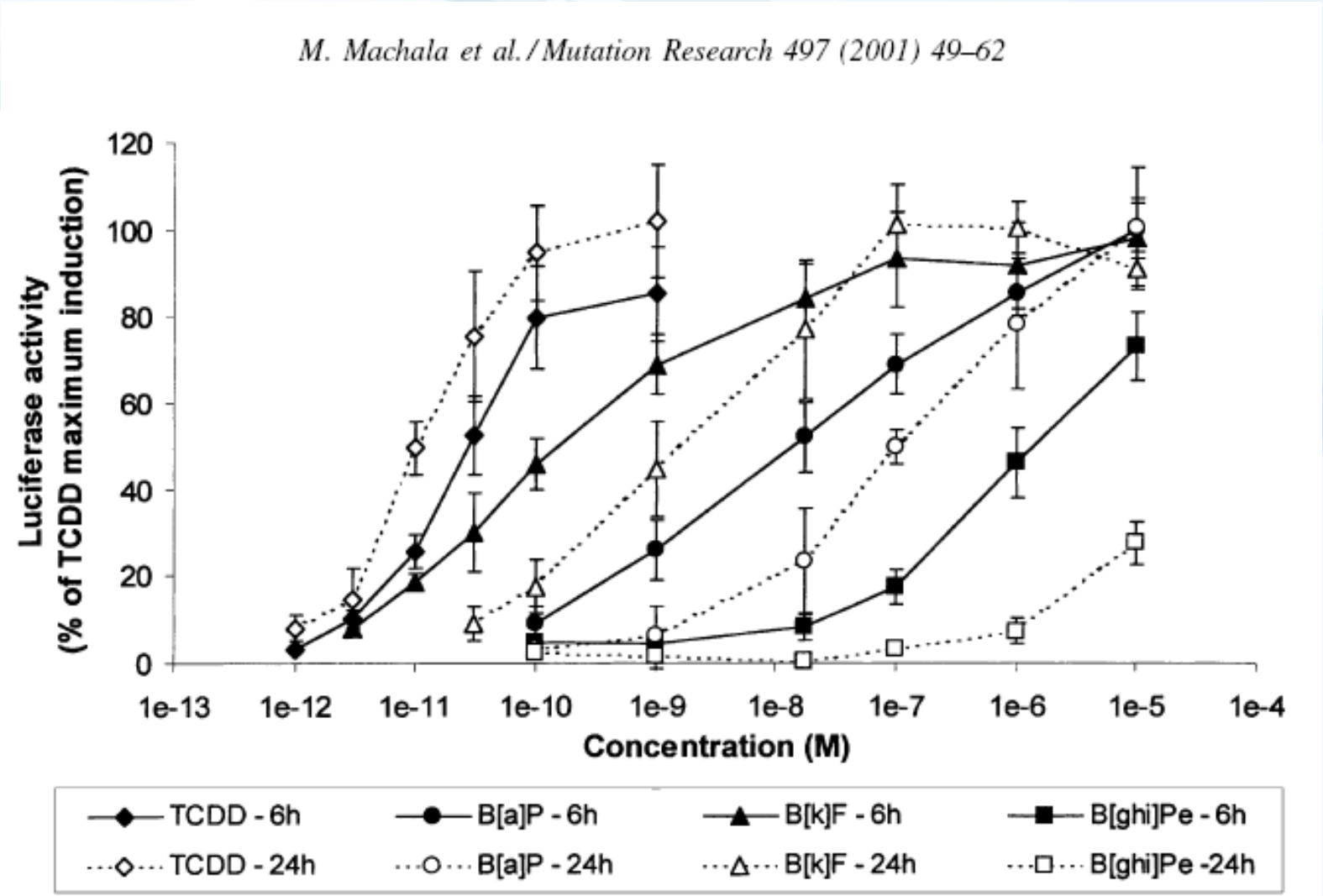
Example - relative potencies of PAHs (two exposure periods) in „CALUX“ assay



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Longer exposure period: lower induction due to (partial) metabolization of PAHs in the cells (CYPs, oxidation)

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)

DETECTION of EROD activity - example

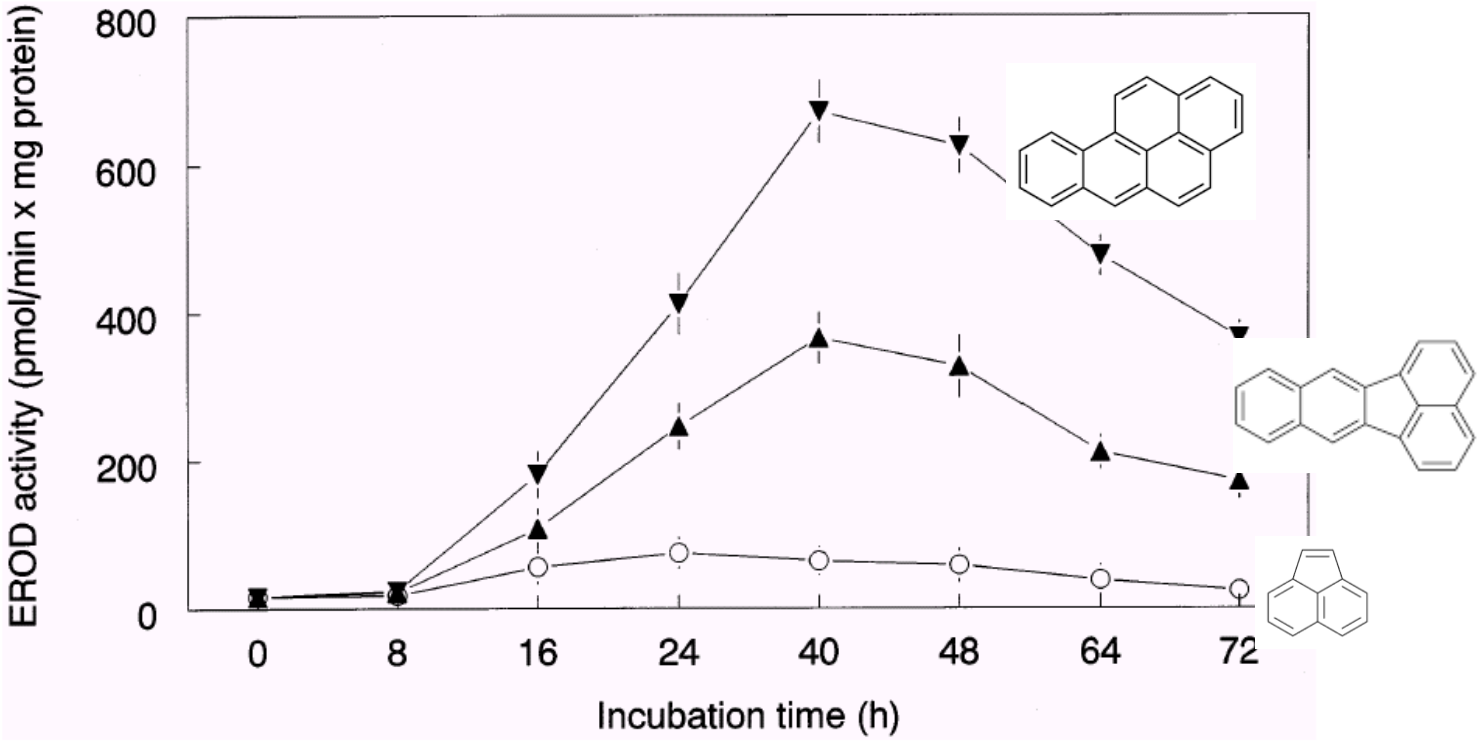


Fig. 2. Time course of induction of CYP1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity in primary cultures of rat hepatocytes, after addition of 1.7×10^{-5} M benzo[*a*]pyrene (-▼-), 1.9×10^{-6} M benzo[*k*]fluoranthene (-▲-) or 9.4×10^{-5} M acenaphthylene (-○-). EROD activity was determined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

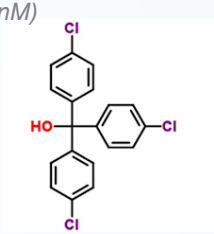
Concept of REPs is used to assess toxicity mediated by other NRs

Estrogenicity (ER):

REP (Relative Potencies) of selected compounds compared to natural ligand (reference compound E2 - REP=1)

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}

- **tris-(4-chlorophenyl)-methanol**
  - Ubiquitous contaminant of uncertain origin (probable metabolite in DDT mixtures)
  - Levels in human blood serum around 50nM *(similar to antiAR effective EC50 200nM and close to the effective dose of DH-testosteron 100nM)*



Androgenicity (AR):

Effective concentrations (EC50) of various compounds in the in vitro assay in comparison with natural androgen DHT

Compound	EC_{50} (µM)
DHT - Dihydrotestosteron	**0.1**
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**

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**

- 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
  - Important for toxicity: affected by xenobiotics sharing structural similarity with natural ligands

- Various mechanisms of toxicity
  - Directly at the receptor site (e.g. “anti-androgenicity), adverse effects have both STIMULATIONS and INHIBITIONS
  - Additional mechanisms – transport in blood (thyroids/goitrogens), metabolism and clearance (thyroids, retinoids), heterodimerization “crosstalk” of different NRs (e.g. AhR/ER)

- Other key information
  - Characterization of “toxic potentials” of individual chemicals
    - General concept of “REPs” (valid for all NRs)
    - Very important role in the risk assessment of dioxin compounds (AhR) - TEFs / TEQs
  - Reporter Gene Assays (e.g. CALUX) and its use to derive REP