University of Basel

DEPARTMENT OF PHARMACEUTICAL SCIENCES

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Schweizerisches Zentrum für Angewandte Humantoxikologie
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In vitro assays to assess effects of EDCs on steroid hormone action

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Chemicals interfering with the endocrine system

EDCs: xenobiotics that disrupt endocrine functions and cause adverse health effects

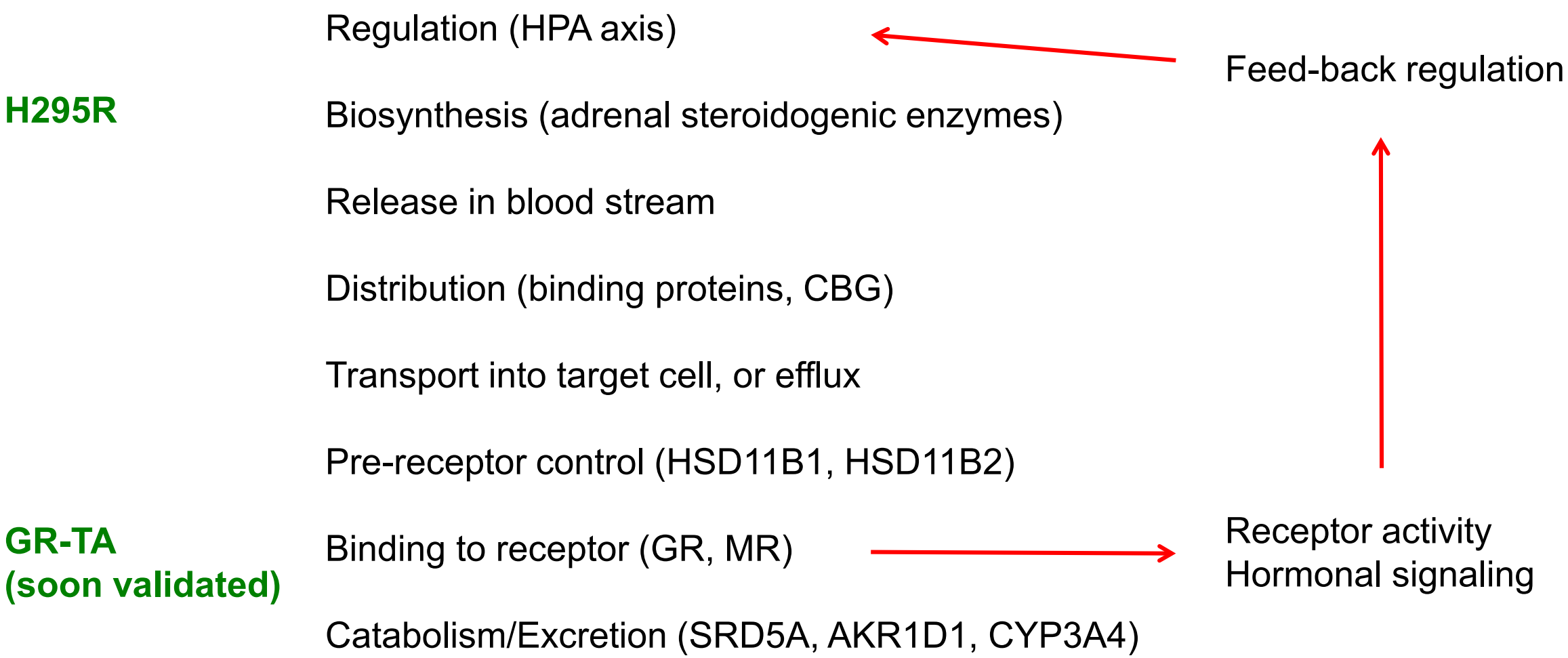
Few substances have been convincingly shown to cause adverse health effects; there is a large number of suspected EDCs

Suspected EDCs: few studies in human; most evidence from animal studies, many of them in aquatic species; descriptive studies on phenotypical changes, underlying mechanism remains unclear

Hazardous substances:
in vitro assays → identification and characterization, uncover mechanism of action
Predictivity for toxicological outcome often low
Large lab-to-lab differences in results, often insufficiently described assay protocols

Simplified levels of the regulation of the endocrine system

(example cortisol)



How can EDCs interfere with a given target?

Direct modulation: reversible/irreversible inhibition, PTM

protein stability

intracellular distribution

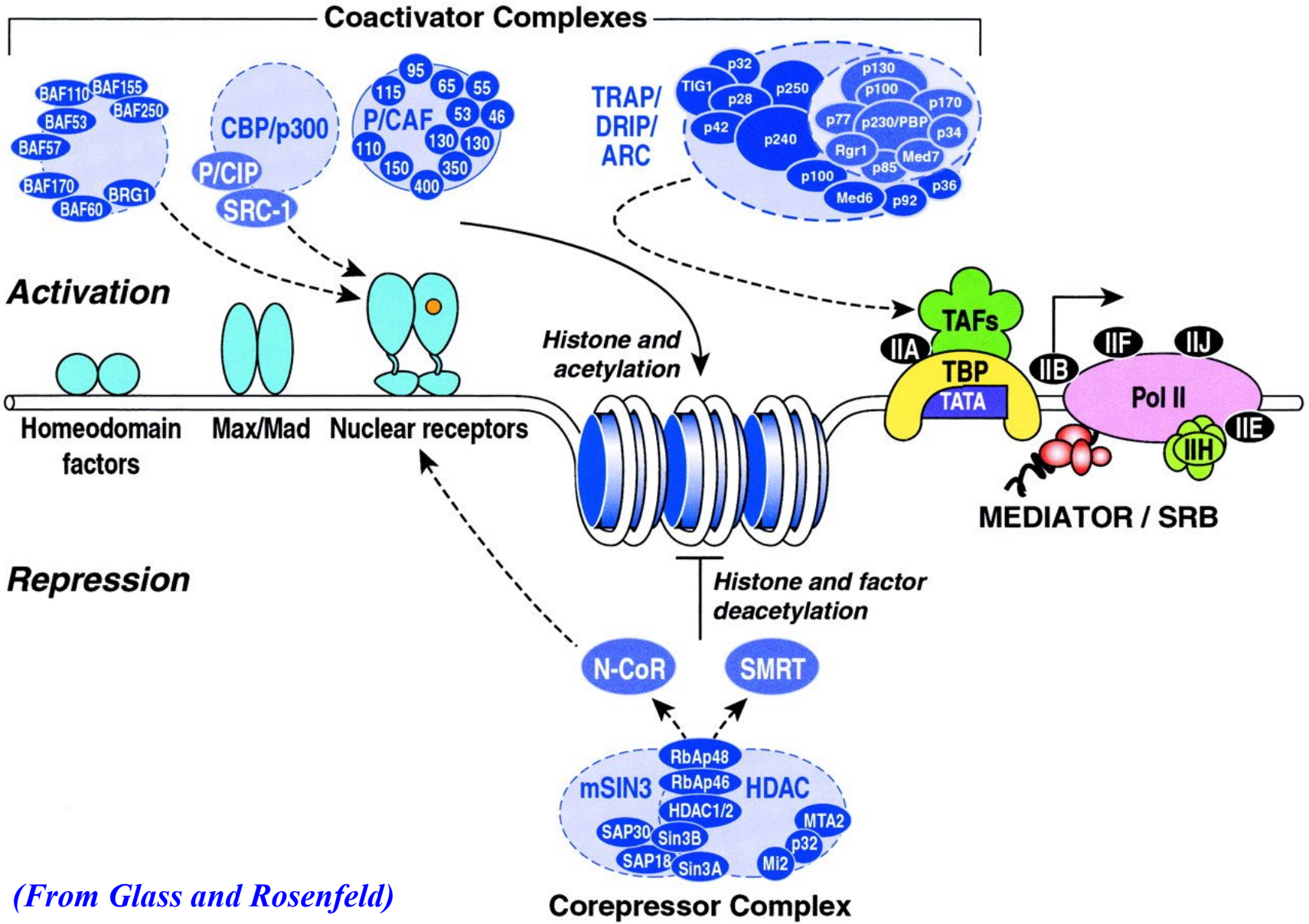
protein-protein interactions

epigenetic modifications

Limitations of steroid receptor transactivation assay protocols

- What cell line to choose?
- What promoter-reporter gene to use?
- Choose endogenous receptor expression or overexpressing recombinant receptor?
- Medium composition, w/wo serum present?

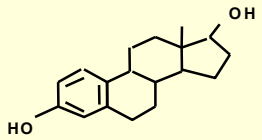
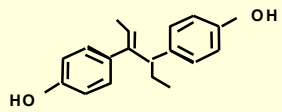
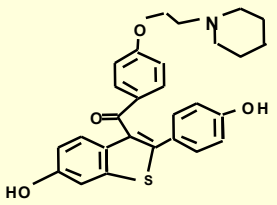
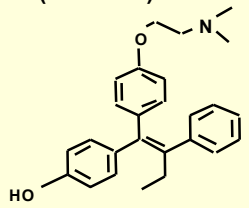
Steroid receptor activity involves large protein complexes



(From Glass and Rosenfeld)

Example Selective Estrogen Receptor Modulators (SERMs): tamoxifen

Estrogen receptor ligands elicit different tissue-specific responses

Estrogen target tissues	estradiol 	Z-pseudo diethylstilbestrol 	raloxifene 	4-hydroxytamoxifen (Z-OHT) 
Breast	agonist	agonist	antagonist	antagonist
Uterus	agonist	partial agonist	antagonist	partial agonist
Bone	agonist	agonist	agonist	partial agonist
Liver	agonist	agonist	????	partial agonist
CNS	agonist	agonist	antagonist	antagonist

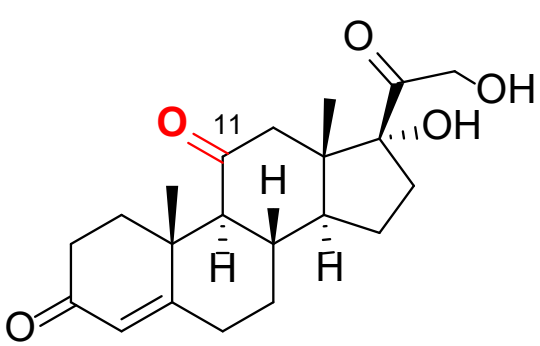
What cell line to choose for TA?

Breast cancer cell

Endometrial cell

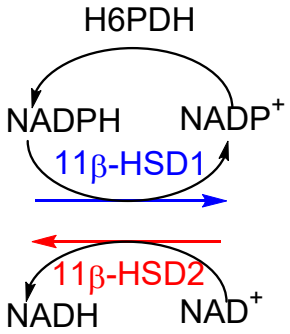
How to test substance X?

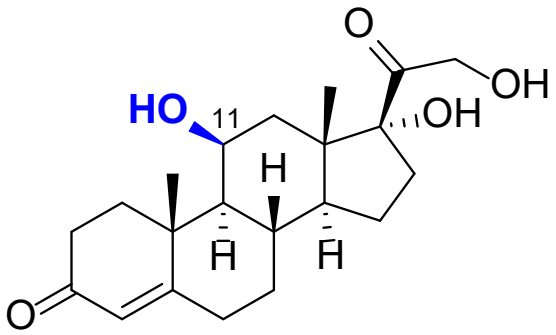
Intracrinology: pre-receptor enzymes of GR and MR: 11β-HSD1 and 11β-HSD2



cortisone

(11-dehydrocorticosterone, prednisone)

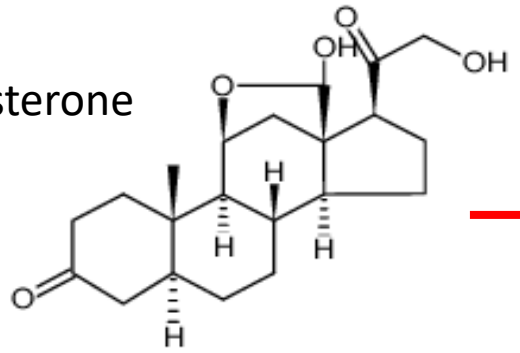




cortisol

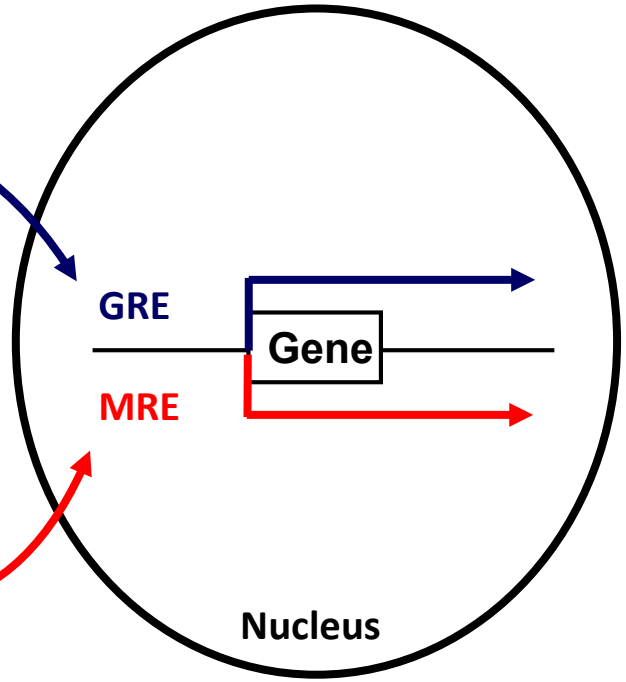
(corticosterone, prednisolone)

aldosterone



GR

MR



Cell type-specific glucocorticoid regulation:

None	dependent on extracellular cortisol
11β-HSD1 (H6PD)	own production of cortisol from circulating cortisone
11β-HSD2	inactivation of cortisol, relatively «resistant»

GR and MR respond differently to corticosteroids in different cell types:

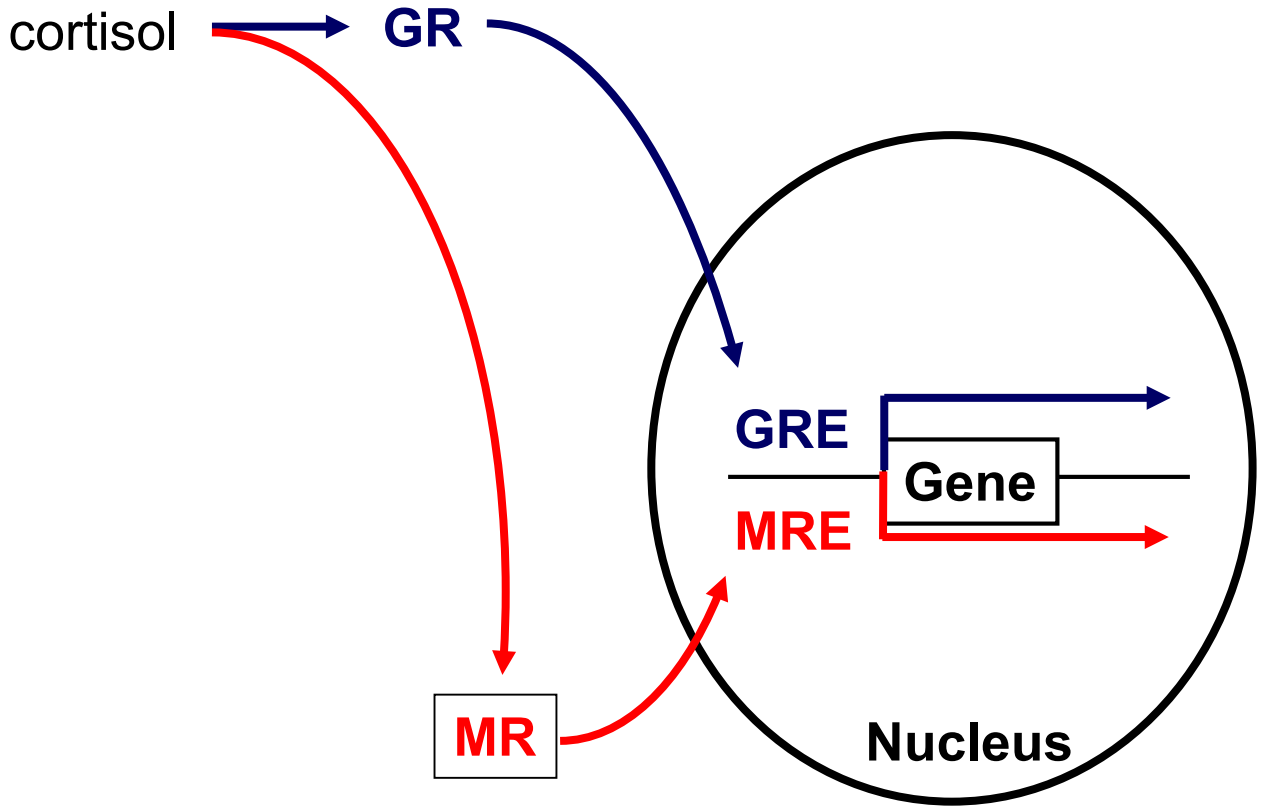
Renal CCD:	GR, MR, 11β-HSD2	11β-HSD2 renders specificity of MR for Aldo
Cardiomyocyte:	GR, MR	Cortisol acts as antagonist for MR
Macrophage:	GR, MR, 11β-HSD1	Balance of MR, GR (opposite effects)

What cell background to choose for GR and MR transactivation assays?

Inhibition of renal 11β-HSD2

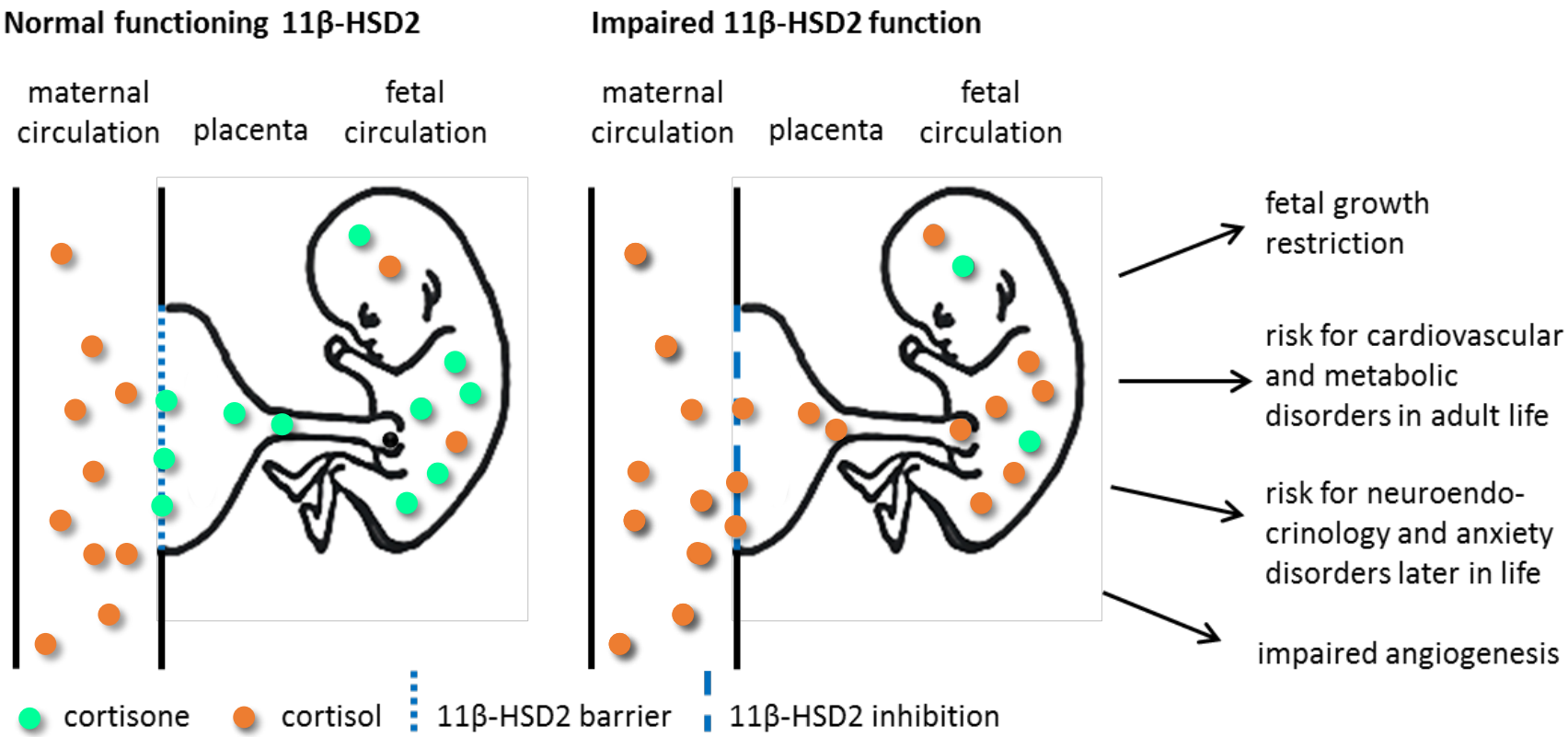
Excessive licorice consumption: apparent mineralocorticoid excess (AME)

- Low renin, low aldosterone hypertension
- Hypokalemia, hypernatremia



Inhibition of placental 11β-HSD2

Cortisol mother to cortisol fetus = 10 to 1, due to placental 11β-HSD2 activity



→ avoid non-intentional inhibition or downregulation of 11β-HSD2

Transactivation assay in cells overexpressing MR ± 11β-HSD2

Transactivation activity: 50 nM cortisol, 10 µM inhibitor or vehicle

Cell type	MR	MR + 11β-HSD2	MR + 11β-HSD2 inhibitor	inhibitor
HEK293	+++	-	-	fluoxymesterone
COS1	+++	-	-	fluoxymesterone
V79	+++	-	**+++**	fluoxymesterone
HEK293	+++	-	-	posaconazole
COS1	+++	-	-	posaconazole
V79	+++	-	**+++**	posaconazole

What cell background to choose for testing substance X?

11β-HSD2 enzyme activity assay: inhibition by fluoxymesterone

11β-HSD2 overexpressed in HEK293: IC50 >>10µM (50 nM cortisol as substrate)

11β-HSD2 endogenously expressed in SW620: IC50 160 nM

11β-HSD2 endogenously expressed in MCF7: IC50 530 nM

11β-HSD2 overexpressed in HEK293, lysates: IC50 80 nM

→ **Differences most likely due to efflux transporters**

Impact of medium composition: 11β-HSD1 enzyme activity assay

Our research group/pharma company in CH

Pharma company in US

Lysates of HEK293 expressing 11β-HSD1

Potent inhibition by glycyrrhetinic acid

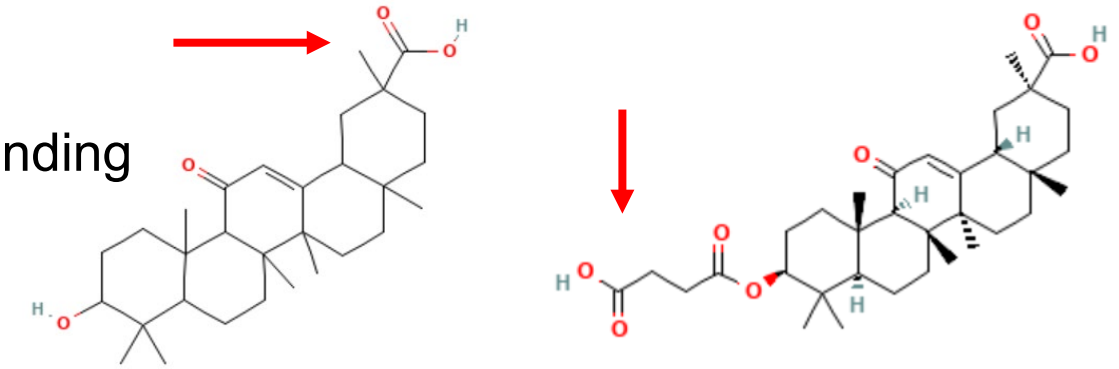
Potent inhibition by carbenoxolone

No inhibition by carbenoxolone

no serum albumin added to lysate

serum albumin added to lysate

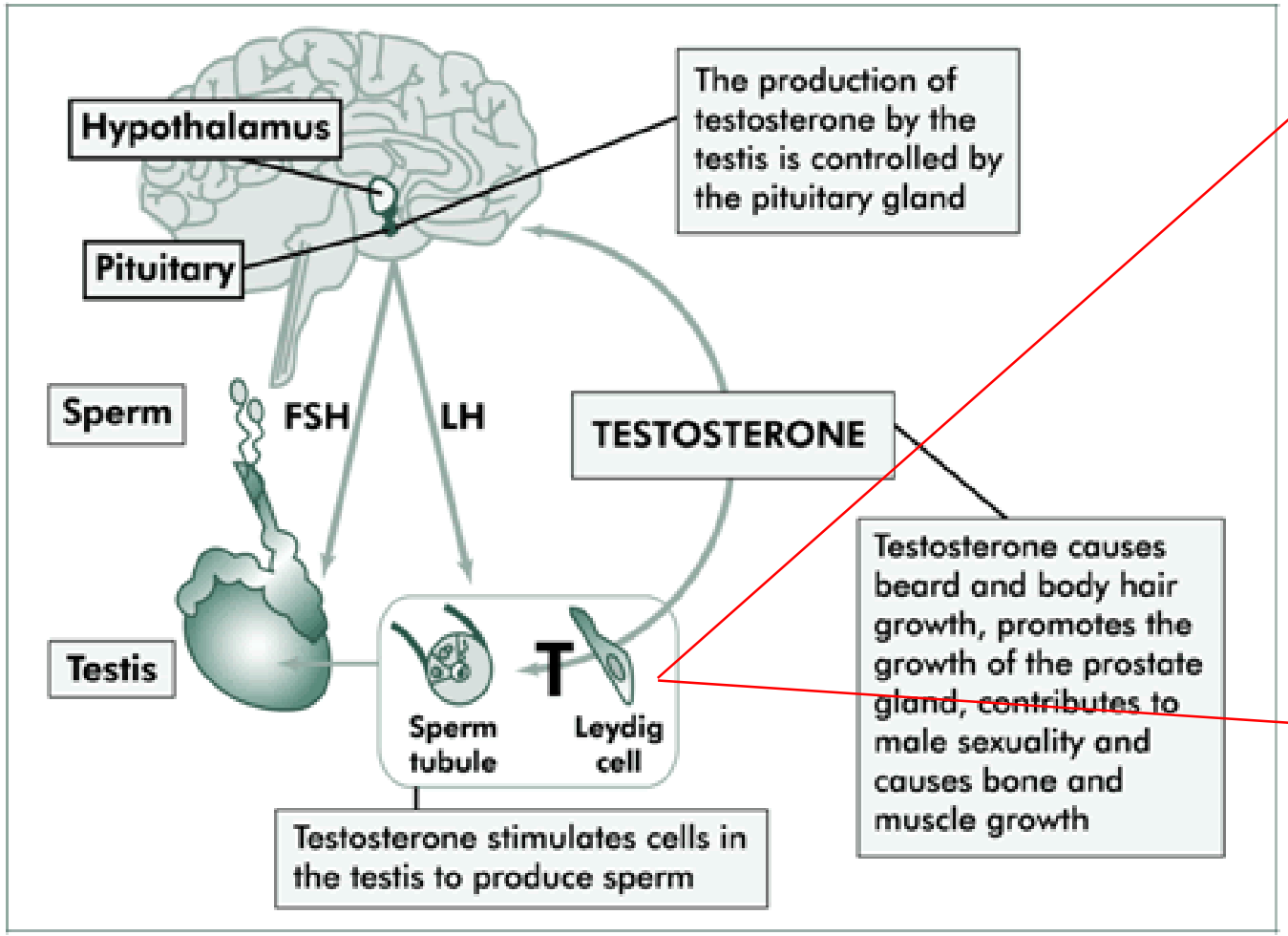
Glycyrrhetinic acid binding to serum albumin: ~ 50%



Carbenoxolone binding to serum albumin: > 95%

→ Assay protocol: medium composition must be well-defined

Appropriate use of models: example male reproductive toxicity cell models



Leydig Cell

Pregnenolone

↓ CYP17A1

17α-Hydroxypregnenolone

↓ CYP17A1

DHEA —3β-HSD2→ **Androstenedione**

↓ 17β-HSD3 ↓ 17β-HSD3

Androstenediol —3β-HSD2→ **Testosterone**

Appropriate use of models: example male reproductive toxicity cell models

No human Leydig cell system available

Alternative: mouse Leydig cell lines MA-10, TM3, BLTK1
>40 scientific publications showed testosterone production using antibody-based quantification

Mass-spectrometry-based methods detect very low or no testosterone!

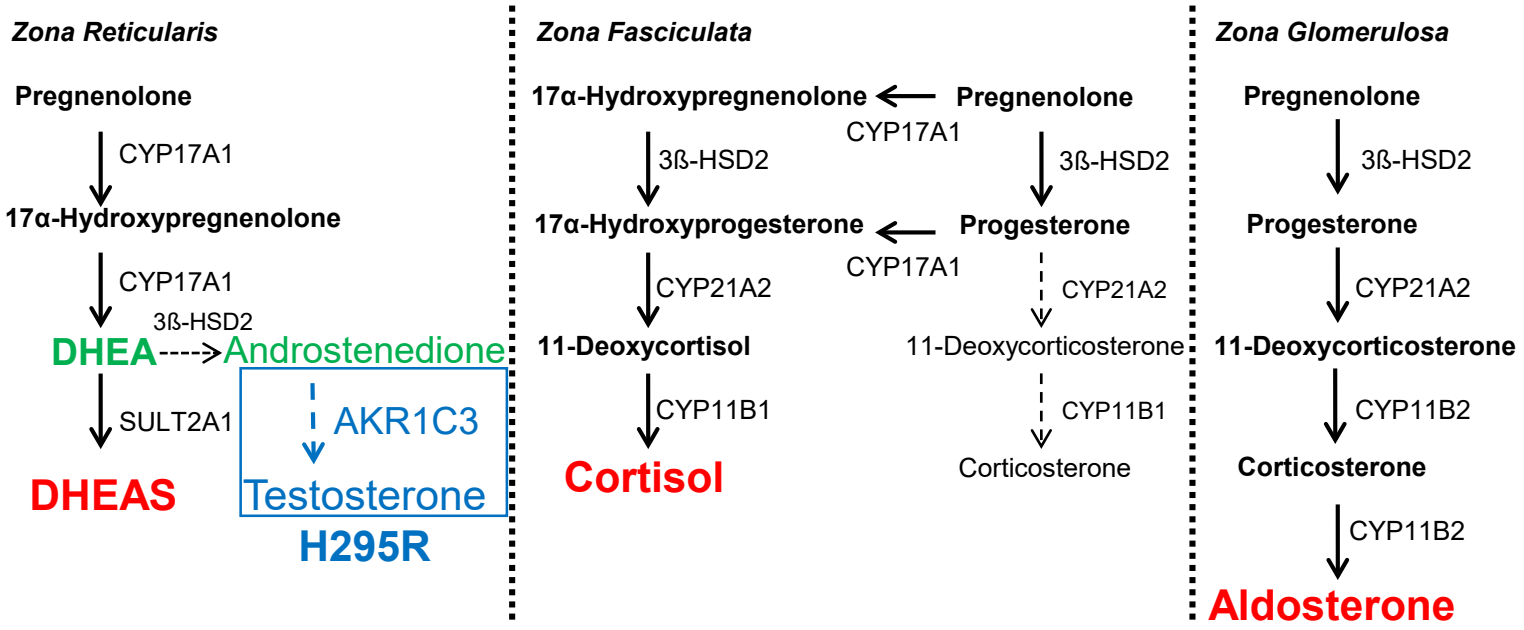
Steroid production down to progesterone quantifyable using mass-spec methods

	Testis	MA-10	BLTK	TM3
→	*Hsd17b3*	22.4 ± 0.9	31.2 ± 0.2 **	31.36	n.d
→	*Akr1c14*	23.6 ± 0.7	17.3 ± 0.2 **	14.21	21.54
	Srd5a1	21.8 ± 0.3	20.7 ± 0.5	19.77	25.05
	Hsd3b1	17.6 ± 1.2	19.0 ± 0.5	12.35	23.31
→	*Cyp17a1*	17.4 ± 0.9	23.3 ± 1.0 *	25.98	28.55

→ Use appropriate analytics!

Alternative: OECD TG 456 human H295R cells with T as readout

Adrenal steroid synthesis



H295R: derived from a female patient suffering from an adrenal tumor

Adrenal androgens **Glucocorticoids** **Mineralocorticoids**

H295R does not express 17β-HSD3 but AKR1C3

	Complete medium	
	Ct value DMSO 0.1%	Fold change GOI/GAPDH Forskolin 10 μM
GAPDH	13.7 ± 0.5	1.0 ± 0.4
StAR	15.9 ± 0.5	2.2 ± 0.4*
CYP11A1	17.5 ± 0.5	2.4 ± 0.8*
3β-HSD2	24.6 ± 0.6	27.9 ± 5.2*
CYP17A1	19.5 ± 0.4	3.4 ± 0.6*
CYP21A2	20.0 ± 0.7	8.0 ± 1.9*
CYP11B1	29.1 ± 0.7	2.9 ± 1.0*
CYP11B2	25.9 ± 0.6	9.1 ± 2.1*
CYP19A1	23.4 ± 0.5	7.7 ± 1.6*
AKR1C3	21.6 ± 0.6	0.5 ± 0.1*
17β-HSD3	> 32	> 32

Substance-induced alteration of T:
either upstream steroidogenic enzymes, or AKR1C3 (wrong terminal enzyme!)

Strajhar P et al Toxicology 381 (2017) 51–63

Conclusions

- Predictivity of available *in vitro* assays is low, limiting use to obtain relevant data for regulators
- Large lab-to-lab differences
- Detailed description of assay protocols, including analytical methods
- *In vitro* assays cover only few levels of steroid homeostasis
- Cell- and tissue-specific differences need to be considered
- Correct interpretation of results requires detailed understanding of steroid pathways/physiology

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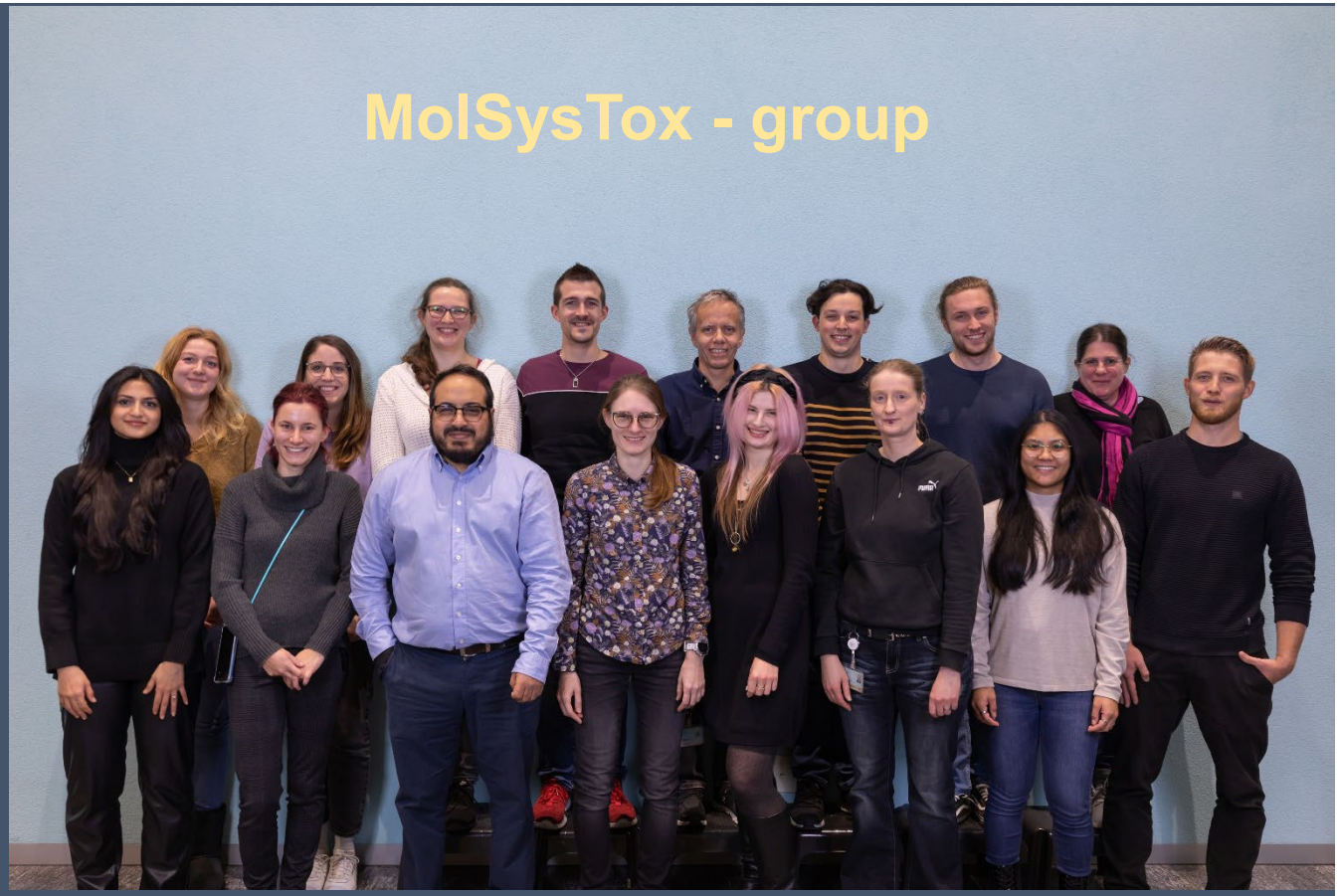

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NEWS ITEM: ESTROGEN-IMITATING CHEMICALS IN THE ENVIRONMENT SUSPECTED OF WIDE-RANGING BIOLOGICAL ANOMALIES
(INCLUDING HERMAPHRODISM IN ANIMALS AND LOWER SPERM COUNTS IN HUMAN BEINGS)
We in the business community prefer a cautious 'wait-and-see' approach over needless media scare-mongering...