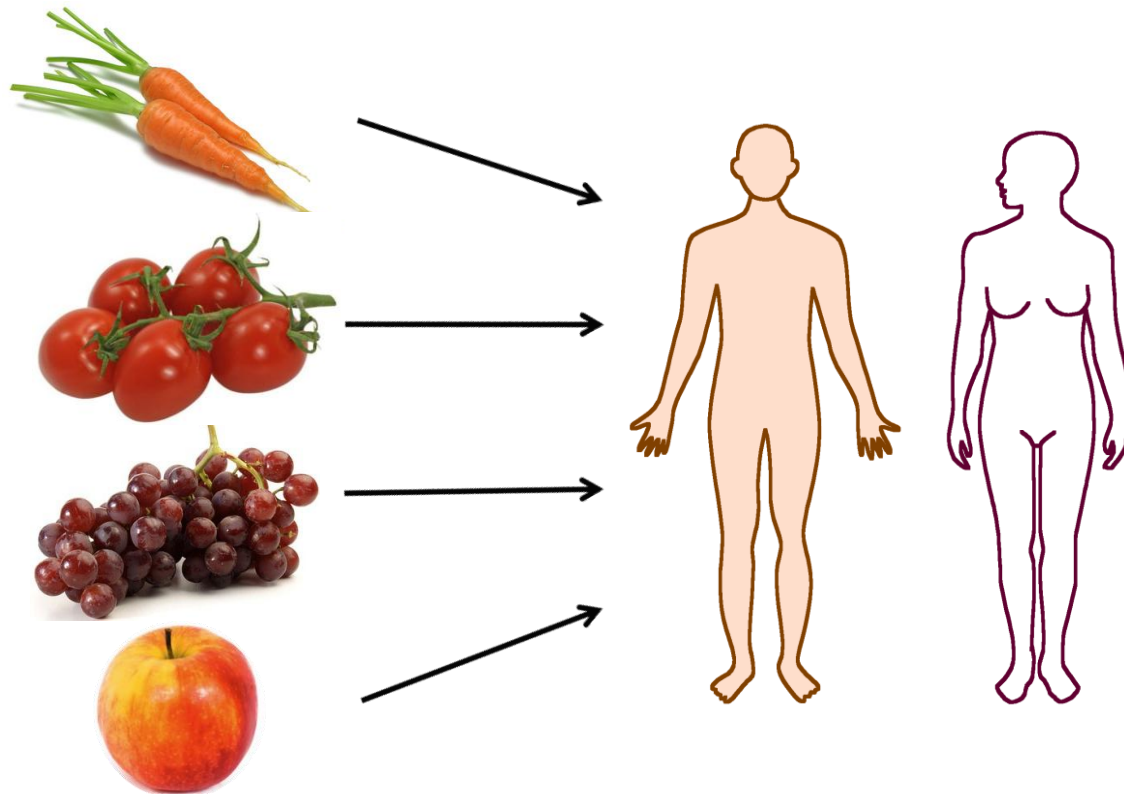


Effects of mixtures of (tri)azole fungicides in subacute feeding studies in rats as compared to those of individual substances

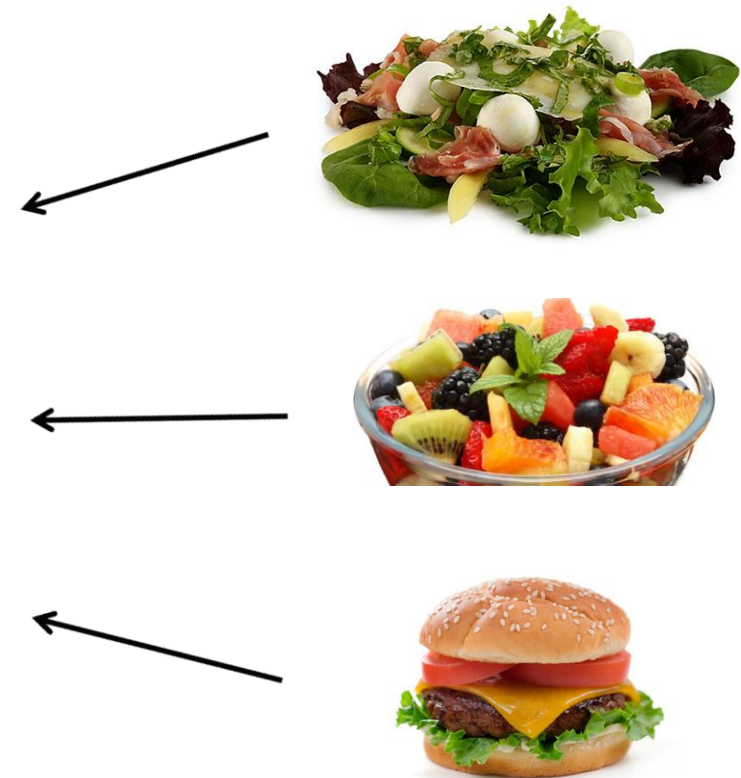
Lars Niemann

Combination effects of pesticides must be considered

Primary cocktails



Secondary cocktails



- Regulation of single substance – Consumer exposure to multiple residues **unavoidable**
- Are there **adverse combination effects** in a dose range that is **relevant for consumers**?

Our experimental approach

- Investigation of mixture effects of pesticides from a chemical group with presumed additive mode of action
- Comparison of these effects to those of single substances
- Combination of *in vivo* (feeding study in rats = **focus of this presentation**), *ex vivo* (selected tissues) and *in vitro* (cell cultures) methods
- Inclusion of **low** dose levels that might reflect consumer exposure by intake of residues

Why triazoles?

- Widely used in agriculture (but also in medicine), common fungicidal mode of action, toxicity well investigated
- Target in fungi: Inhibition of ergosterole synthetase (CYP51), disturbance of cell membranes formation
- Targets in mammals: **Liver** (common); **Evidence of ED** (substance-specific effects on adrenals, ovaries, reproduction, development) - Dose additivity to be expected; EFSA CAGs for acute and chronic (liver) effects



Selected substances (technical active ingredients)

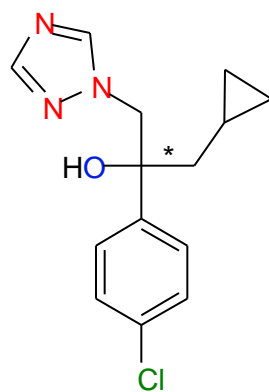
Triazoles:

- Cyproconazole
- Epoxiconazole
- Propiconazole
- Tebuconazole

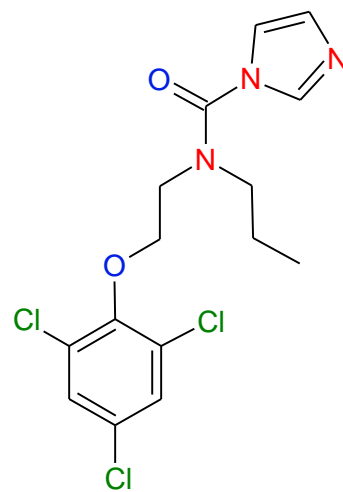
Non-triazoles:

- Prochloraz (Hepatotoxicity, ED properties)
- Phenobarbital (“positive control“ for liver toxicity, one dose of 500 ppm, not for combinations)

Structures of triazoles and imidazoles

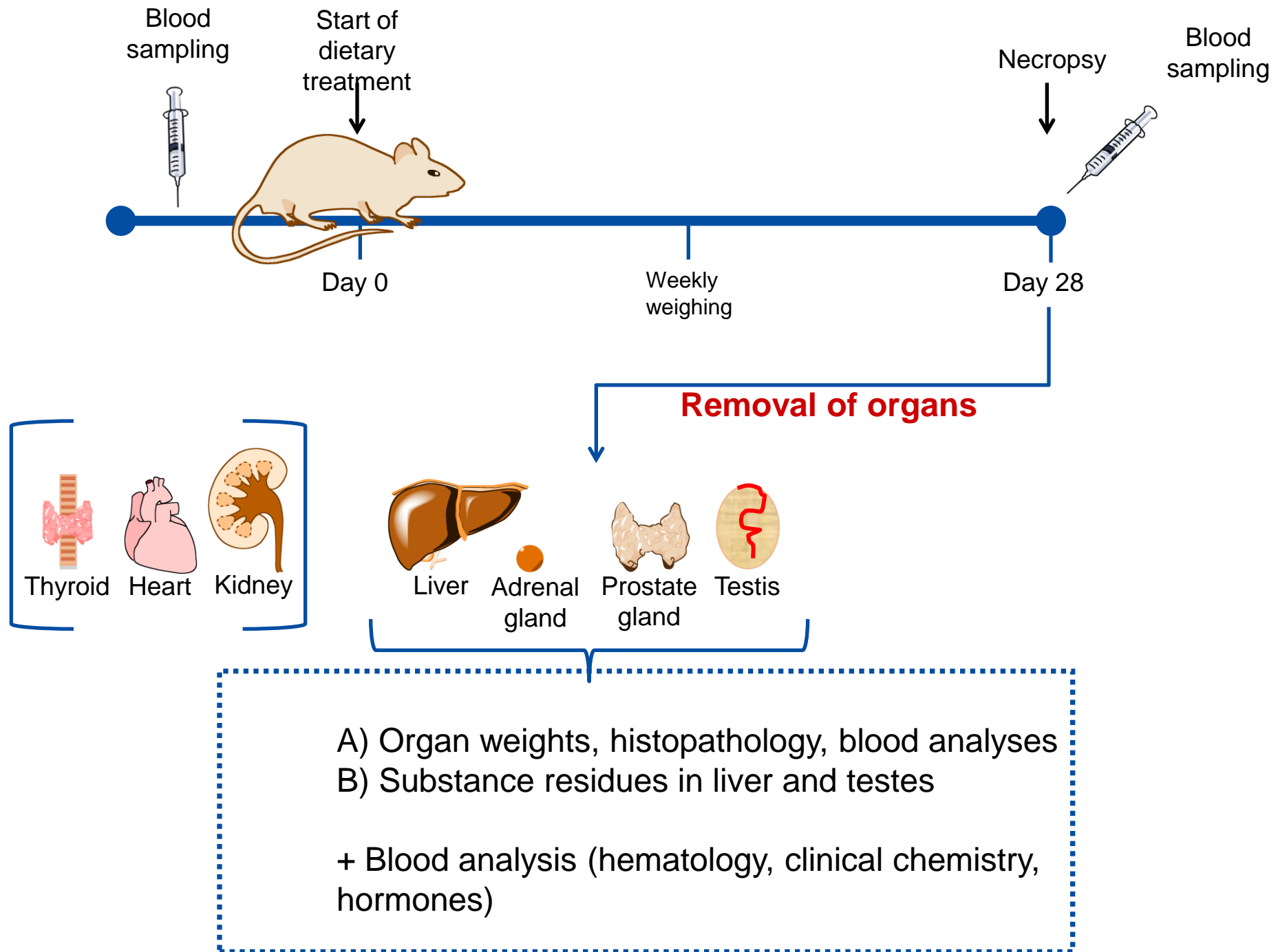


Cyproconazole



Prochloraz

The *in vivo* method: 28-day feeding study in male Wistar rats

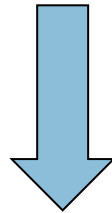


What did we want to know from these studies?

- **May we confirm the (20 – 40 years old) NOAELs/LOAELs as obtained in previous short-term studies with the individual substances provided by the manufacturers?**
- **Will the NOAELs/LOAELs go down when substances are given in combination?**
- **Are effects of mixtures different from those of single substances?**
- **Is there some interaction on toxicokinetic level?**

Dose selection and animal number (single substances)

NOAEL from previous 90-day studies in rats (taken from EU evaluations) = basis for dose selection (90 – 240 ppm for individual test compounds)



10 x NOAEL (5 males, 2nd study)
3 x NOAEL (5 males, 1st study)
NOAEL (5 males, 1st study)
NOAEL / 10 (5 males, 2nd study)
NOAEL / 100 (5 males, 2nd study)

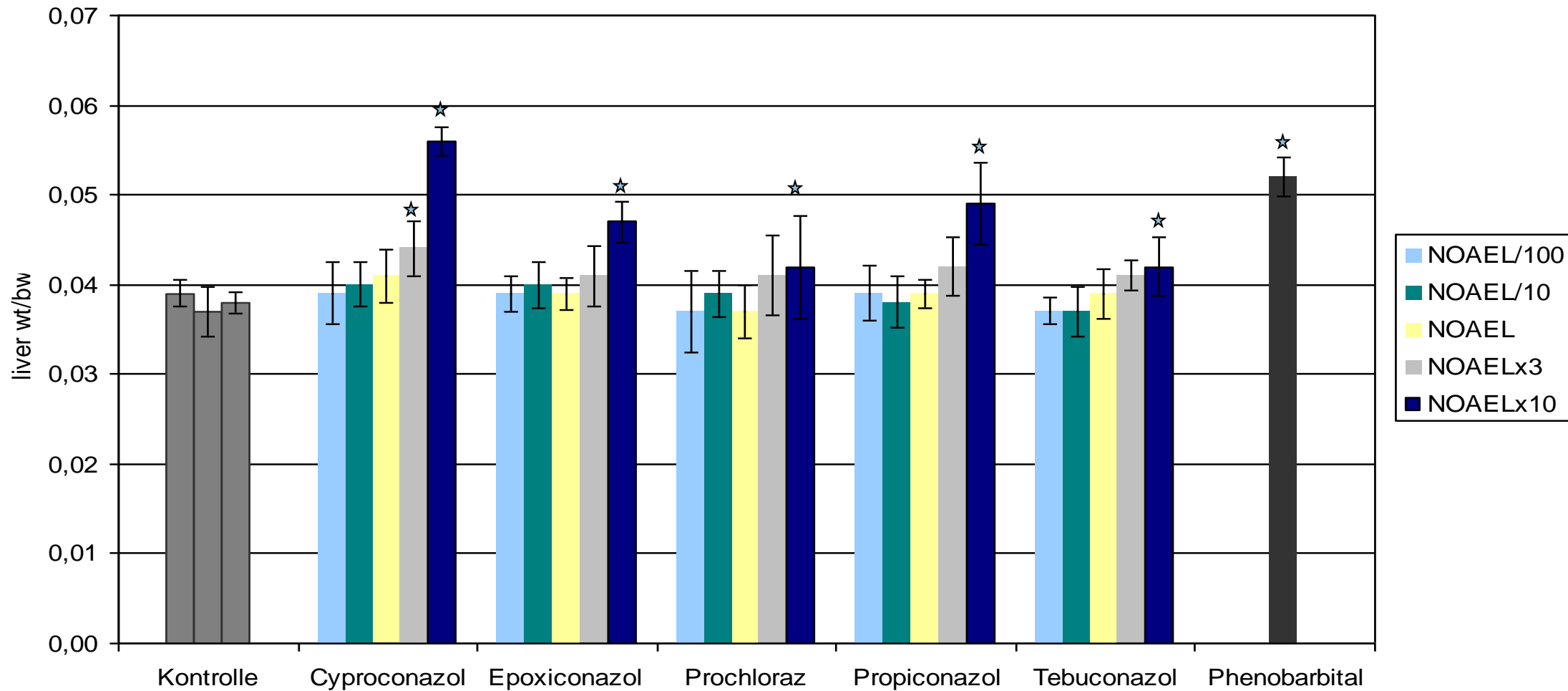
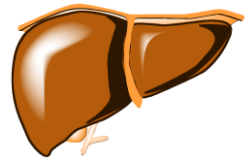
Single substances: Results and (statistical) limitations

No mortality, clinical signs, hematological, clinical chemistry or gross pathological findings up to highest dose levels.

Some effects on food consumption, food efficiency, body weight (all ↓), liver weight (↑), liver histology (hypertrophy, vacuolisation) **confined to 3x / 10x NOAEL**; adrenal weight ↓ and cortical atrophy with epoxiconazole (**10x NOAEL**)

Statistical analysis difficult because of low animal number, comparison to only one or two control groups and inclusion of many parameters: “multiple testing” problem, p – level should be lowered to 0.016

Relative liver weight following exposure to single substances



★ p<0.05

→ Significant increase confined to high dose levels (3x or 10x NOAEL)

Combination experiments: Substance and dose selection

Substances:

■ Cyproconazole ■ Epoxiconazole ■ Prochloraz ■ ~~Propiconazole~~ ■ ~~Tebuconazole~~

Selected combinations and dose levels

(10 animals per group, two separate control groups):

■ Cyproconazole +
Epoxiconazole

■ Cyproconazole +
Epoxiconazole +
Prochloraz

NOAEL/100

NOAEL

NOAELx10

Dose levels for 1st combination, compared to ADI and ARfD

Compound	Dose level (ppm)	Mean daily intake (mg/kg bw)	ADI (mg/kg bw)	ARfD (mg/kg bw)
Cypro-conazole	10x NOAEL (1000)	57.7	0.02	0.02
	NOAEL (100)	6.4		
	NOAEL/100 (1)	0.06		
Epoxi-conazole	10x NOAEL (900)	59.6	0.008	0.023
	NOAEL (90)	5.9		
	NOAEL/100 (0.9)	0.06		

Combination results

No mortality, clinical signs, hematological or gross pathological findings up to highest dose level (10x NOAEL).

Some effects on food consumption, food efficiency, body weight (all ↓), clinical chemistry, liver weight (↑), liver (hypertrophy, vacuolisation) **confined to 10x NOAEL**. Liver confirmed as main target. Adrenal effects (weight↓, histology) less severe than with epoxiconazole alone.

→ **Combination effects only observed at concentrations at which the individual substances proved also toxic!**

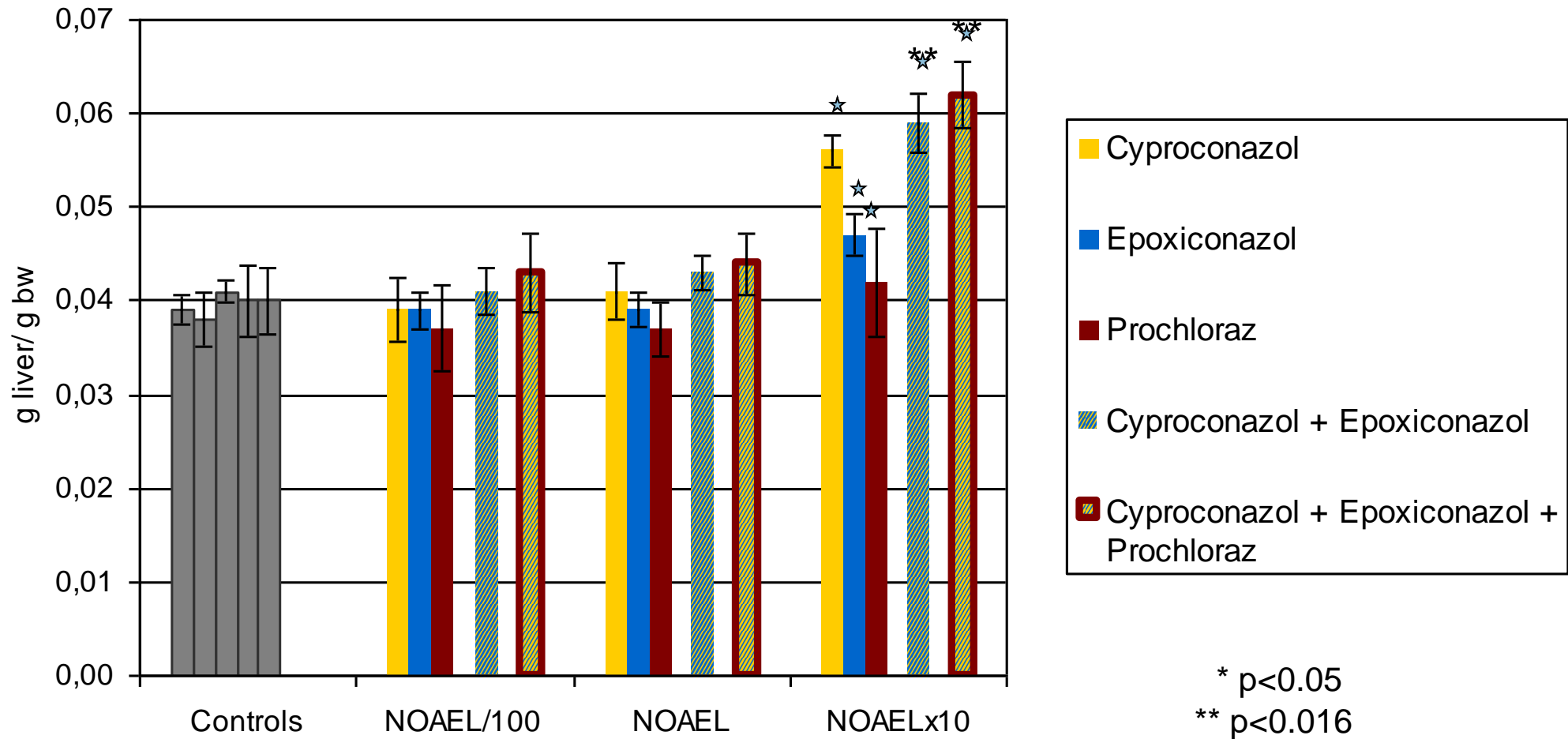
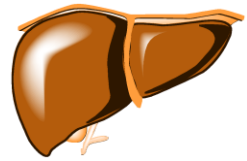
Evidence of increased effects with combinations (I)

Initial reduction of food consumption:

- Clear effect with cyproconazole, weak effect with prochloraz, no effect with epoxiconazole
- More pronounced with both combinations than with any substance alone

Reduced body weight gain due to cyproconazole and prochloraz alone (not with epoxiconazole) but **body weight losses with combinations**, due to lower food consumption and impaired food efficiency

Relative liver weight following exposure to combinations



→ Significant increase only above NOAEL and only slightly enhanced by combinations

☆

Evidence of increased effects with combinations (II)

Substance related increase in liver weight slightly more pronounced with combinations as compared to individual substances.

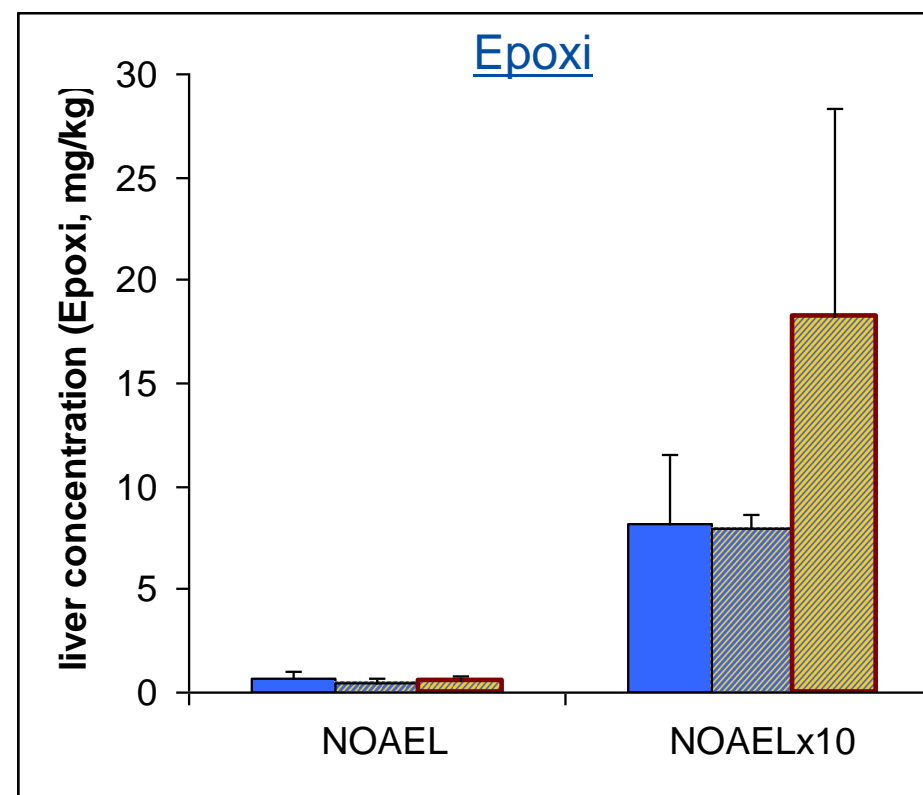
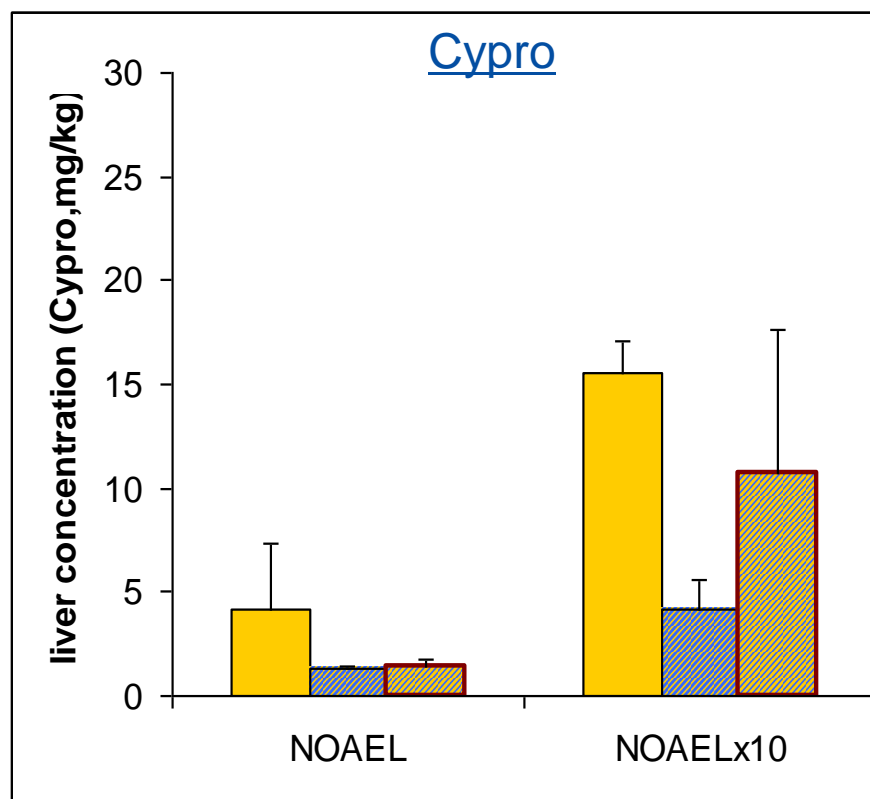
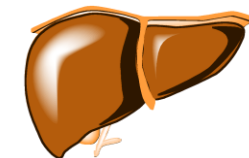
In contrast:

Histological changes observed with individual substances in the liver and, with epoxiconazole, in the adrenals were confirmed but not enhanced when combinations were given.

Not seen with any single substance: γ -GT increase ($\mu\text{kat/L}$)

All controls (n=35)	All single compounds, all doses (n=130)	Both combinations, low and mid doses (n=40)	Cypro, 1000 ppm + Epoxi, 900 ppm (n=10)	Cypro, 1000 ppm + Epoxi, 900 ppm + Prochlo, 1000 ppm (n=10)
- (< 0.02, LOQ)	- (< 0.02, LOQ)	- (< 0.02, LOQ)	9/10 < 0.02 0.03	5/10 < 0.02 0,12 0,07 0,05 0,05 0,05

Evidence of toxicokinetic interaction: Residues of cyproconazole and epoxiconazole in the liver



➤ Parallel administration of substance B or B and C may have an impact on liver residues of substance A resulting in concentrations that are quite different from those measured in experiments with single substances

➤ Pattern of absorption, distribution, metabolism and excretion might be altered by combined exposure – **Is that of toxicological relevance?**

- Cyproconazol
- Epoxiconazol
- Cyproconazol+ Epoxiconazol
- Cyproconazol + Epoxiconazol + Prochloraz

What did we learn from our *in vivo* studies?

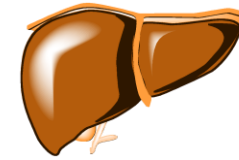
- May we confirm the (20 – 40 years old) NOAELs/LOAELs as obtained in previous short-term studies with the individual substances provided by the manufacturers?
YES !
- Will the NOAELs/LOAELs go down when substances are given in combination?
NO !
- Are effects of mixtures different from those of single substances?
YES, partly ! (but most are confirmed and may be a bit stronger)
- Is there some interaction on toxicokinetic level?
YES !

Ex vivo data: Use of recent molecular and biochemical methods

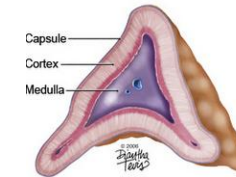
- Measurement of enzyme activities (EROD, PROD, BROD, MROD)

- Gene expression analysis

- Quantitative real-time PCR
- Low density arrays



Liver

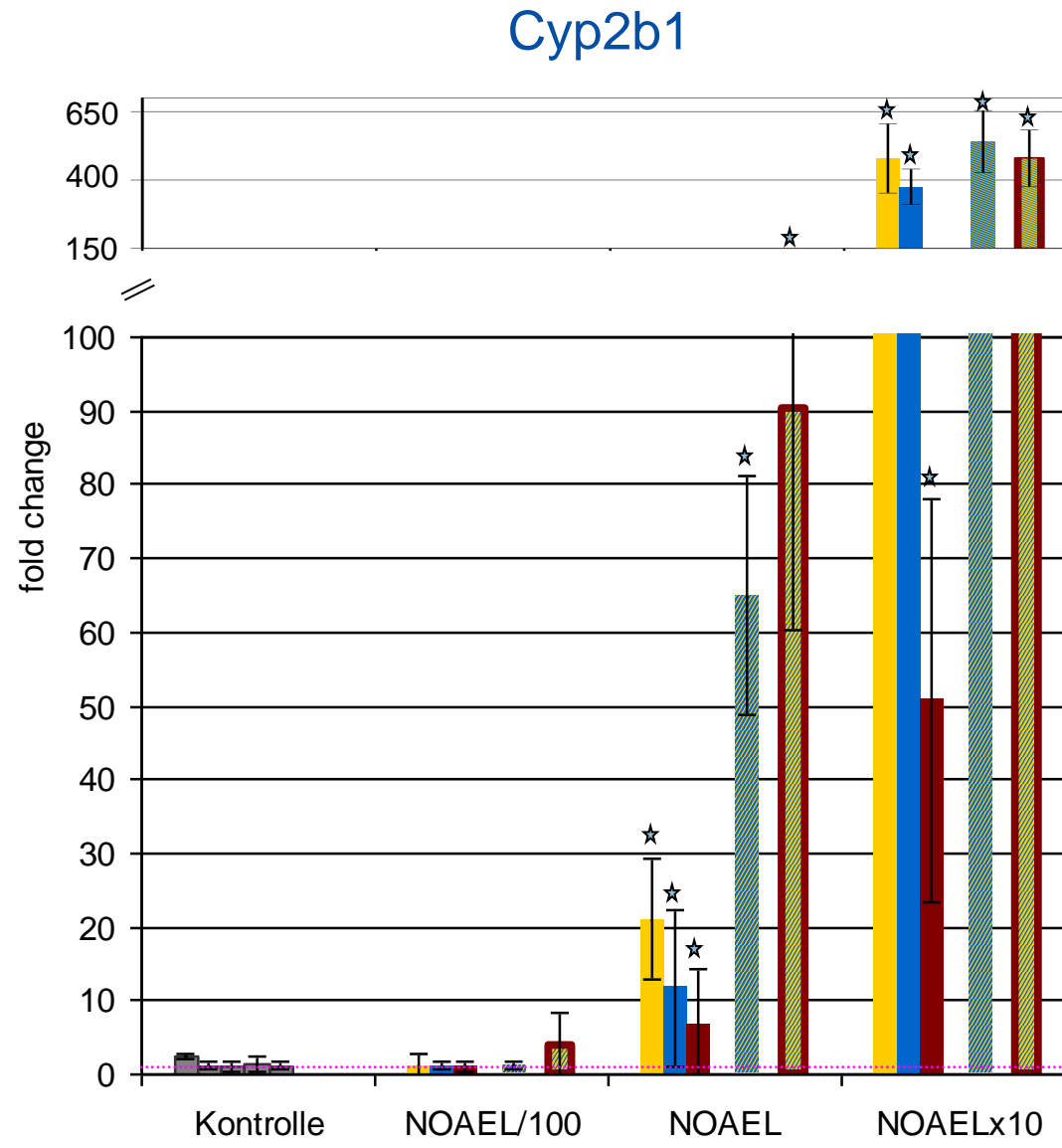
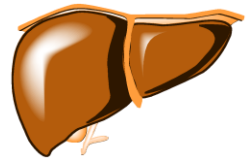


Adrenals

What are the “molecular“ NOELs/LOELs?

What is the relevance of these methods for regulation?

An example: Increase of Cyp 2b1 activity in the liver



■ Cyproconazole

■ Epoxiconazole

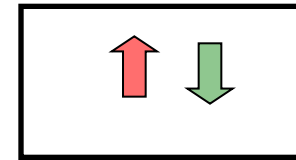
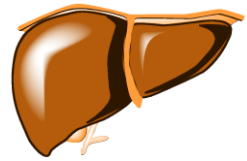
■ Prochloraz

■ Cyproconazole+
Epoxiconazole

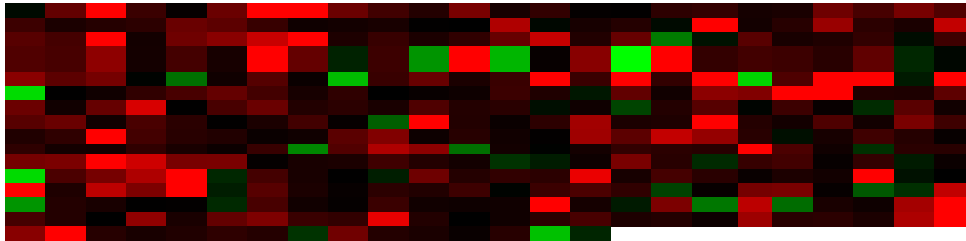
■ Cyproconazole +
Epoxiconazole +
Prochloraz

★ p<0.05

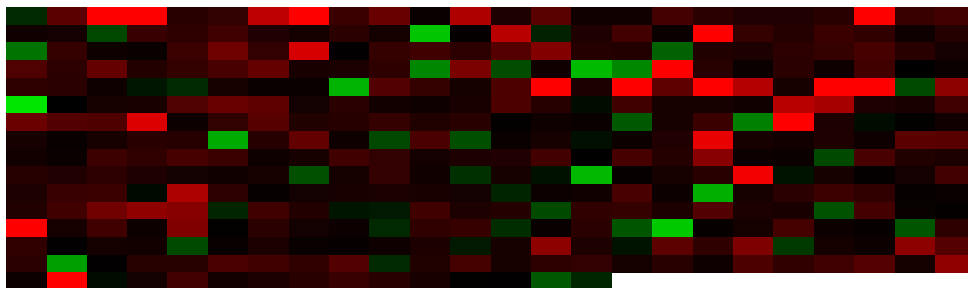
Changes in gene expression in the liver – low density array (Rat Molecular Toxicology PathwayFinder)



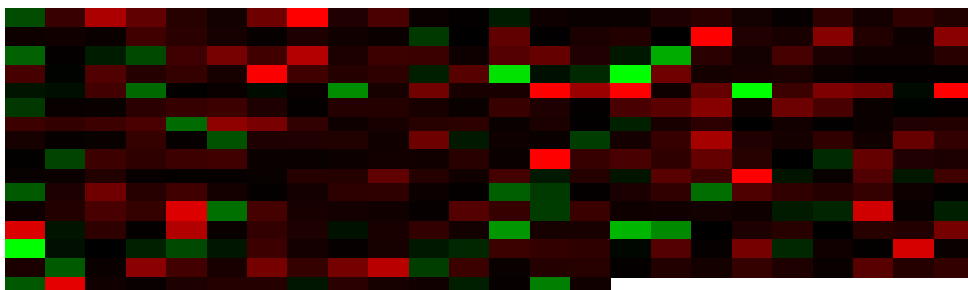
Cyproconazol NOAELx10



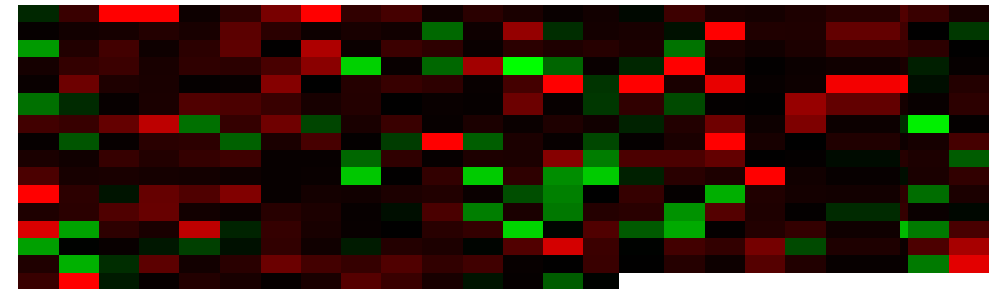
Epoxiconazol NOAELx10



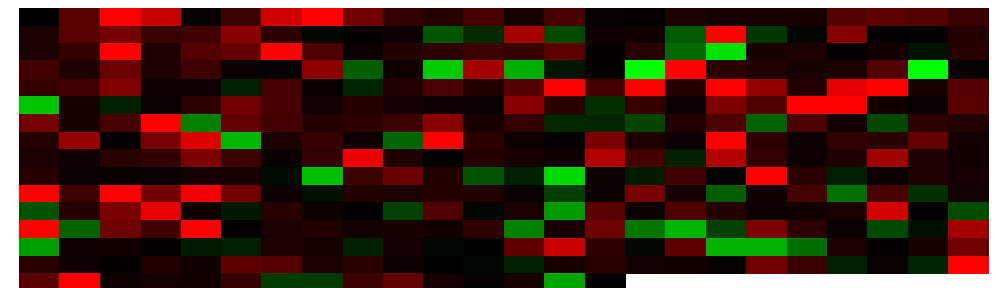
Prochloraz NOAELx10



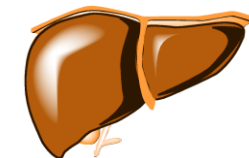
Cypro & Epoxi NOAELx10



Cypro, Epoxi, Prochloraz NOAELx10



Changes in gene expression following exposure to single substances over a wide dose range: What to do with that data?



↑ ↓	Cyproconazole			Epoxiconazole			Prochloraz			Pheno- barbital
	NOAEL /100	NOAEL	NOAEL x10	NOAEL /100	NOAEL	NOAEL x10	NOAEL /100	NOAEL	NOAEL x10	
Abcb1b	1,5	1,4	2,7*	2,0*	0,9	9,4*	1,6	1,7	1,7	0,9
Aldh1a1	2,7	2,4*	43,6*	4,6	2,0	23,6*	2,0	1,4	8,3*	18,9*
Ahr	2,1*	1,8	2,8*	2,2	1,5	2,6*	2,2*	2,1*	2,4	2,9*
Cdkn1a	0,5	1,2	0,6	0,2*	0,9	0,3*	0,8	0,7	0,1*	
Hsd17b2	5,5*	0,7	2,4*	16,0*	3,0*	12,5*	1,6	4,9*	22,4*	0,7

X-fold change as compared to controls, * $p < 0,05$

Abcb1b - ATP-binding cassette, subfamily B, member 1B – Drug transporter

Aldh1a1 - Aldehyde dehydrogenase 1 family, member A1 – Phase II metabolizing enzyme, Phospholipidosis

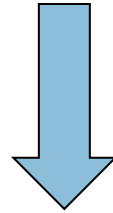
Ahr - Aryl hydrocarbon receptor – Transcription factor, drug metabolism

Cdkn1a - Cyclin dependent kinase inhibitor 1A – Cell cycle control, DNA damage response

Hsd17b2 - Hydroxysteroid (17 beta) dehydrogenase 2 - Phase II metabolizing enzyme, steroid metabolism

Challenges for the future

The multitude of combinations precludes testing all of them



- Experimental proof of theoretical concepts (based, e.g., on CAGs) urgently needed
- Regulatory relevance of *ex vivo* findings to be agreed on

A possible next step: Targeted discussion on a planned workshop with invited experts in April, 2014, at BfR in Berlin

- Development of *in vitro* methods for combination toxicity as alternatives to animal testing, perhaps using toxicokinetic data as indication of realistic doses

Our next contribution: COMBIOMICS project with University of Bielefeld and University of Veterinary Medicine in Hannover

Thanks to



Risiken erkennen – Gesundheit schützen

- All colleagues in BfR who contributed to the animal studies and *ex vivo* investigations!
- Our external partners who performed clinical and histopathology and residue analytics!
- Tanja Heise, Philip Marx-Stölting, Conny Knebel and Alex Dabrowski from our group for providing slides!

Thank you for your attention!

Lars Niemann