What do we really have versus what is really needed?

The Industry-View

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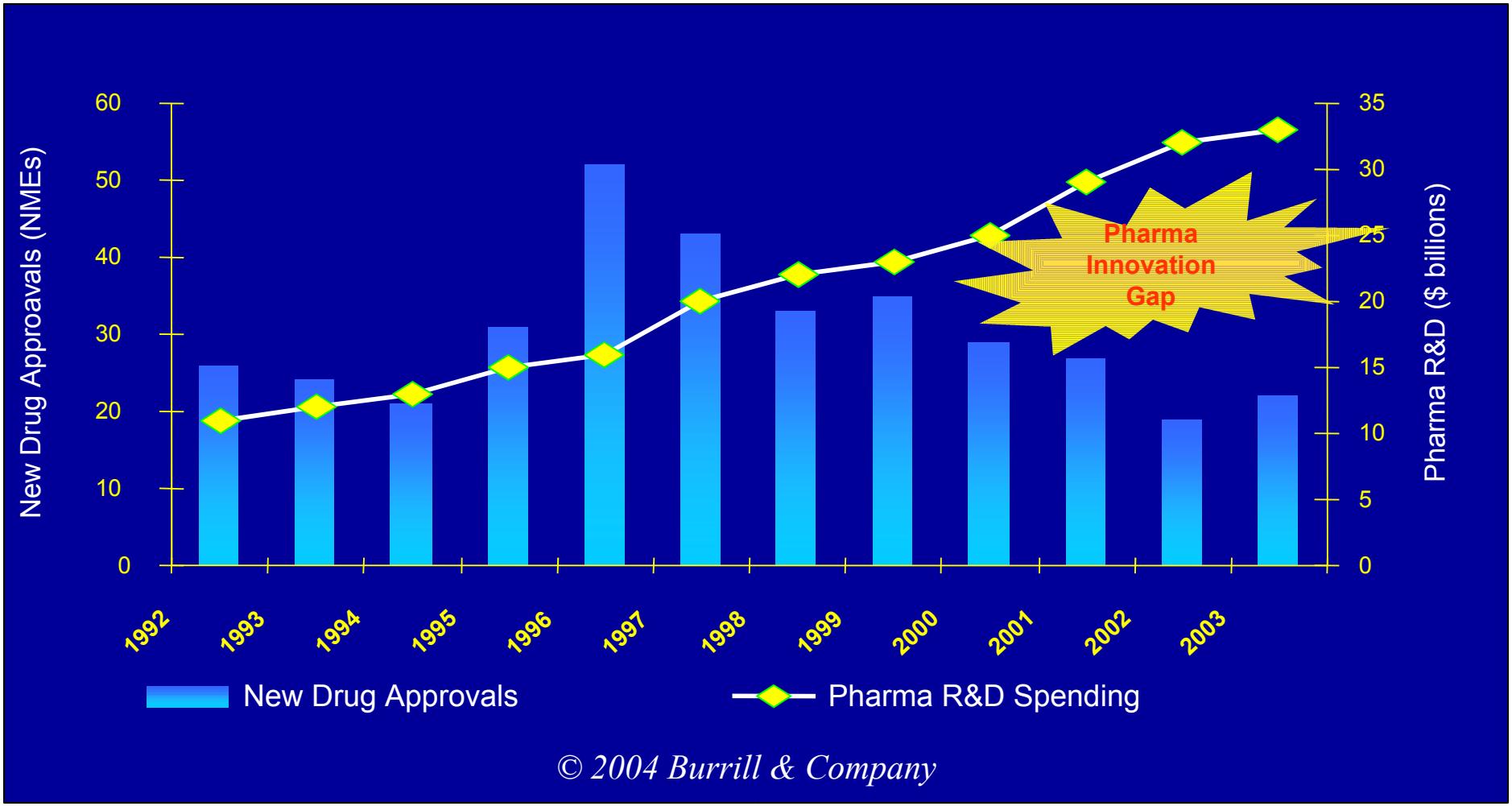
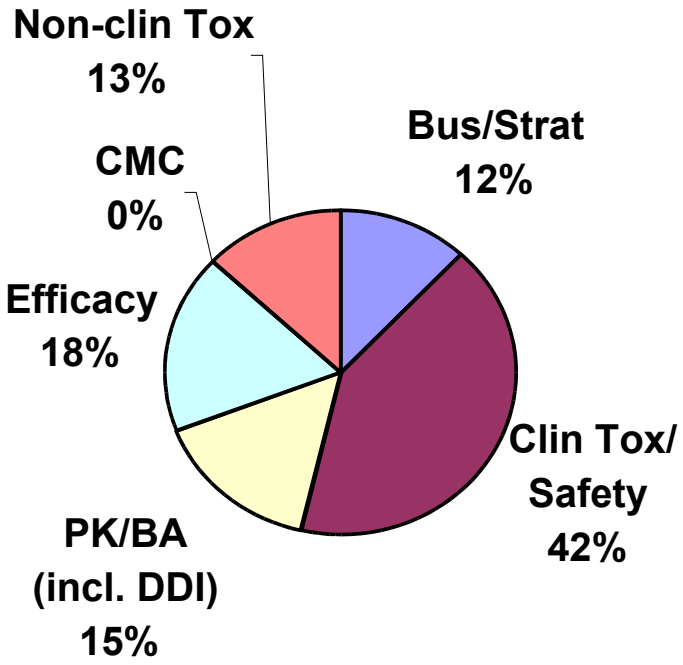
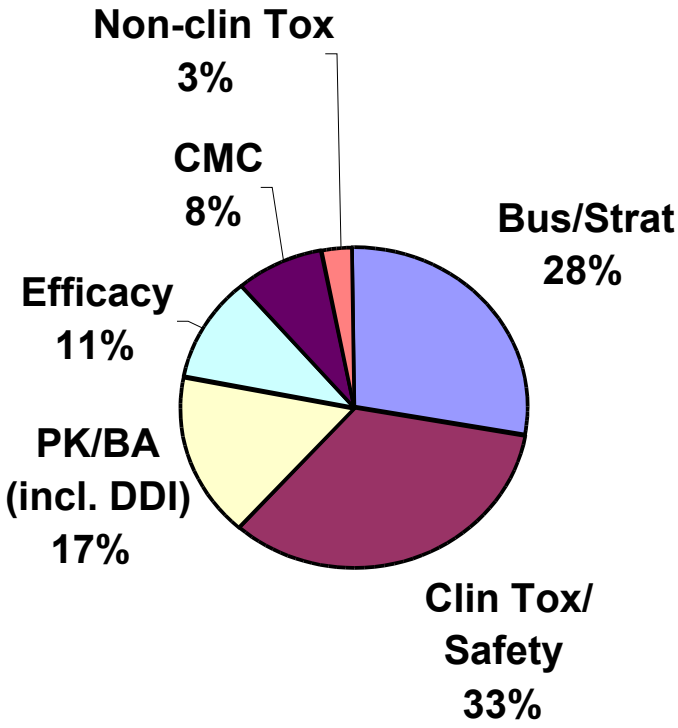


New test models and lower attrition rate of drug candidates can help to address the "Pharma Innovation Gap"

Primary reasons for discontinuation in Phase I



PBF* Benchmark 2000-04
n=195 (incl. JNJ, excl. reasons <10%)



JNJ Pharma 2001-05
n=18

*Pharmaceutical Benchmarking Forum: Abbott Labs, Amgen, AstraZeneca, BMS, Lilly, GSK, J&J, Merck, Novartis, Pfizer, Roche, Schering-Plough

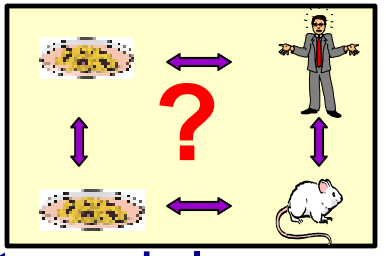
Safety findings J&J Pharma: ~33% of Phase 1 terminations

Reason for discontinuation after starting clinical studies	Frequency	
	Preclinical findings	Clinical findings (Phase I)
Cardiotoxicity	1	3
Hepatotoxicity	0	3
Nephrotoxicity	0	1
IV irritation	0	1
Skin rash	0	1

- Hepatotoxicity accounts for 27% of drugs withdrawn from the market in the period 1960-2002.

Need for better in vitro and in vivo liver toxicity test models

- Define new and better testing strategies



- Make better use of existing regulatory test models

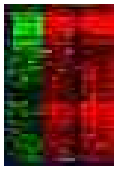
Try to get more with less animals

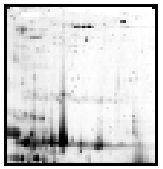
- Develop new in vitro and in vivo test models

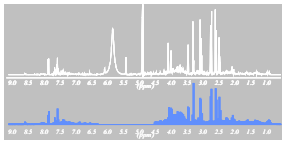
- Implement HTS models for compound selection

Know more earlier

- Integrate new techniques to define the mechanism of action







"Think out of the box"

"Discovery consists in seeing what everyone else has seen and thinking what no one else has thought"
Albert Szent-Gyorgi (1893-1986)

New in vitro models: cytotoxicity testing

Pfizer study [O`Brien et al. Arch. Toxicol, 2006; 80 (9): 580-604]

- 611 compounds tested in vitro on HepG2 cells (48h incubation; 7 parameters)
  - 42 severely human hepatotoxic compounds
  - 283 moderately human hepatotoxic compounds
  - 286 non-toxic drugs

		Sensitivity	Specificity
1	DNA synthesis	10	92
2	Protein synthesis	4	97
3	Gluthathione depletion	19	85
4	Superoxide induction	1	97
5	Caspase-3 induction	5	5
6	Membrane integrity	2	99
7	Cell viability	10	92
	Combination of above tests 1,3, 7	25	83

Late indicators of cell stress

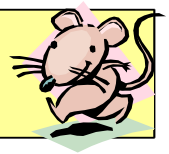
LDH, NR

ATP

(J&JPRD)

High need for improved cytotoxicity assays

	Regulatory animal toxicity studies	52	-

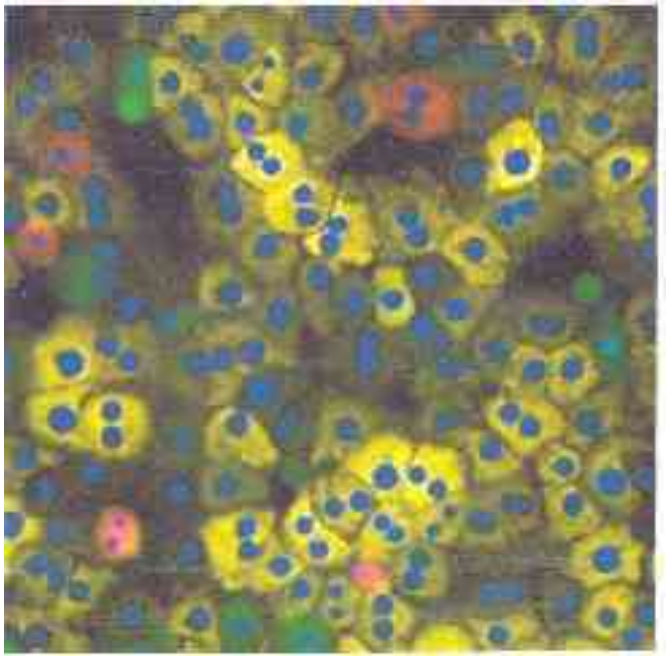


Pfizer study [O`Brien et al. Arch. Toxicol, 2006; 80 (9): 580-604]

Other cytotoxicity markers than the classical ones were tested in a high content screen

Tested 4 fluorophores in HepG2 for 3 days

- **Hoechst33342: nuclear size and cell number: nuclear shrinkage is hallmark of apoptosis. (late stage cytotox parameter).**
- **Fluo-4 AM: intracellular free calcium: early indicator of cell stress.**
- **TMRE: mitochondrial membrane potential: indicator of respiratory capacity of the cell (very early cytotox marker)**
- **TOTO3: plasma membrane permeability: late stage tox indicator (post mortem)**



243 drugs

- **Sensitivity for human toxicity increased to 93%**
- **Specificity for human toxicity increased to 98%**

Results of a follow-up study by J&JPRD at CEREP

- The cytotoxicity assays with new parameters in HepG2 cells seem to be superior to classic cytotoxicity assays (LDH, ATP, neutral red, MTT, AlamarBlue, …)
- In most of calculated IC_{50s}, the mitochondrial membrane potential was the most sensitive parameter
- Lowest IC_{50s} with the new parameters are always lower than the in house IC_{50s} values with LDH, NR and ATP
- Some evidence that the Lowest Toxic Concentration (LTC) is more predictive than the IC_{50}
- When the compounds are ranked according to their Therapeutic Index [TI = LTC/Cmax], a better prediction to hepatotoxicity is obtained.

Results (2)

How predictive is this assay?

- **Currently database of 186 compounds (internal data, Cerep data, published Cerep data).**

	LTC<10 µM	LTC<30 µM	LTC<100 µM	TI<10 LTC	TI<30 LTC	TI<100 LTC
Severely hepatotoxic (42)	14+ 28- =33%+	24+ 18- =57%+	29+ 13- =69%+	31+ 11- = 74%+	36+ 6- = 86%+	42+ 0- =100%+
Moderately hepatotoxic (60)	26+ 34- =43%+	35+ 25- =58%+	48+ 12- =80%+	37+ 23- = 62%+	49+ 11- = 82%+	53+ 7- = 88%+
Toxic to other organs (48)	22+ 26- =46%+	29+ 19- =60%+	39+ 9- =81%+	27+ 21- = 56%+	37+ 11- = 77%+	42+ 6- = 88%+
Non-toxic drugs (36)	3+ 33- =8% false+	4+ 32- =11% false+	14+ 22- =39% false+	1+ 35- =3% false+	1+ 35- =3% false+	4+ 32- =11%false+

Results (3)

How predictive is this assay?

Internal classifier LTC

(LTC<30 µM = cytotoxic)

		Liver toxic in vivo	
		yes (*)	no
Cytotoxic in vitro	yes	59	4 FP
	no	43 FN	32

(*): Significant/Moderate Human Hepatotoxic

	%
Sensitivity	58
Specificity	89
Concordance	66

Internal classifier TI

(TI<30 = cytotoxic)

		Liver toxic in vivo	
		yes (*)	no
Cytotoxic in vitro	yes	85	1 FP
	no	17 FN	35

(*): Significant/Moderate Human Hepatotoxic

	%
Sensitivity	83
Specificity	97
Concordance	87

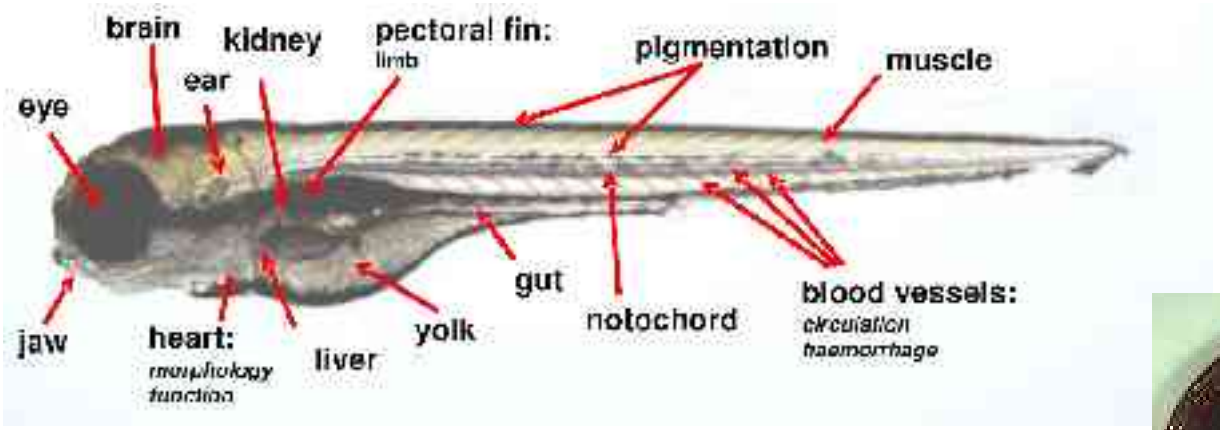
Pfizer study: sensitivity with conventional parameters was only 25%

Conclusion on cytotoxicity testing

Sensitivity improved by using other parameters
and
by looking to the data in a different way.

New in vivo test models: the zebrafish (1)

- physiology and development parallels that of mammals
- optical transparency of the larvae makes real time observations of its internal organs simple



- are small and inexpensive to maintain

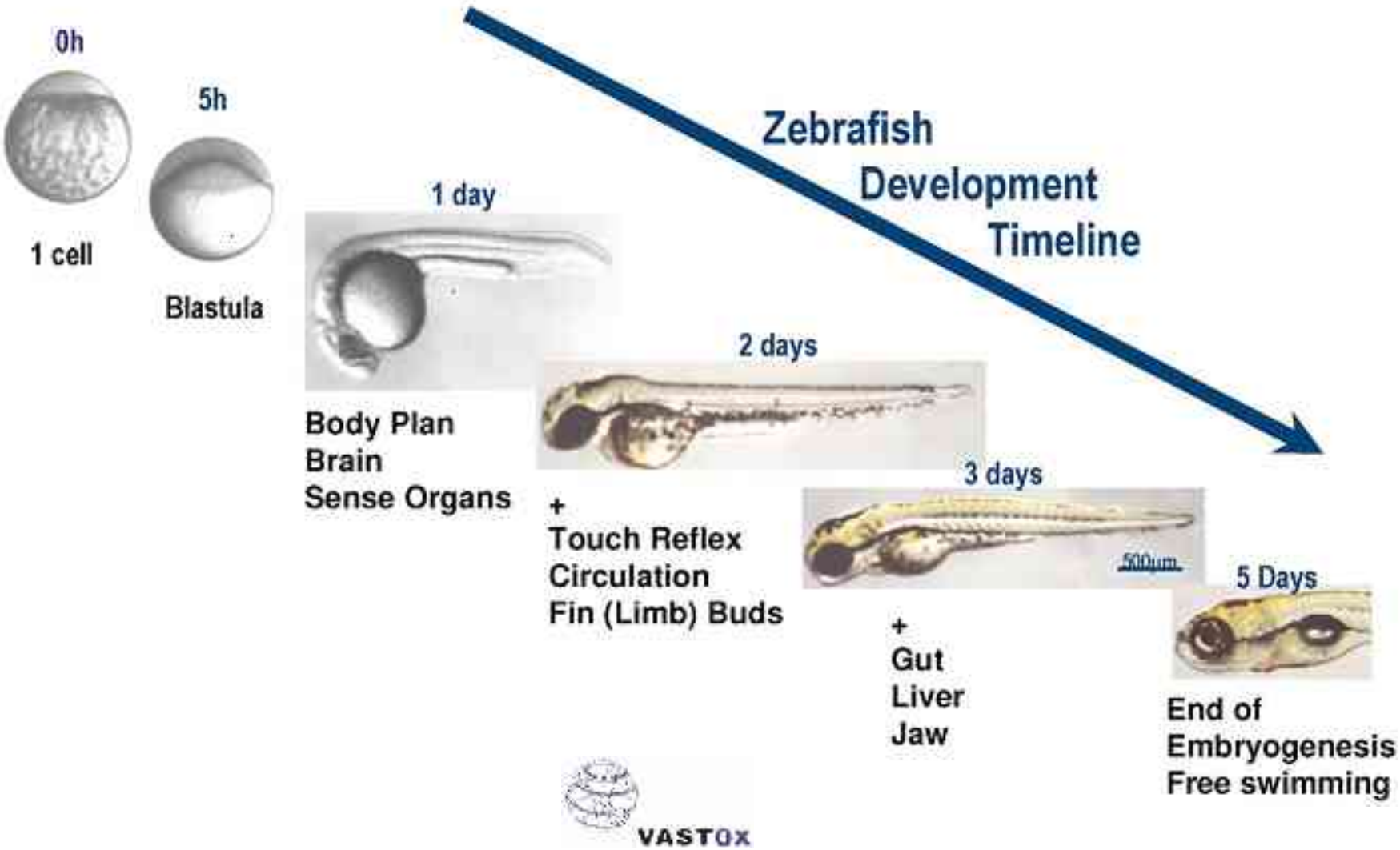
The zebrafish as test model (2)

- larvae can live several days in a single well of standard 24, 96 or 384 well plates surviving on nutrients stored in their yolk sac

Zebrafish larvae at 6 days post fertilization in a 96-well plate

- easily bred in large numbers (a single pair of adults can routinely lay hundreds of fertilized eggs in a single morning)
- larvae absorb compounds in the surrounding water (Danieau's solution) through their skin and gills
- the liver constitutes 9% of the biomass
- compounds are solved in fish water or DMSO (tolerate up to 1.5% DMSO)

Zebrafish as test model (3)

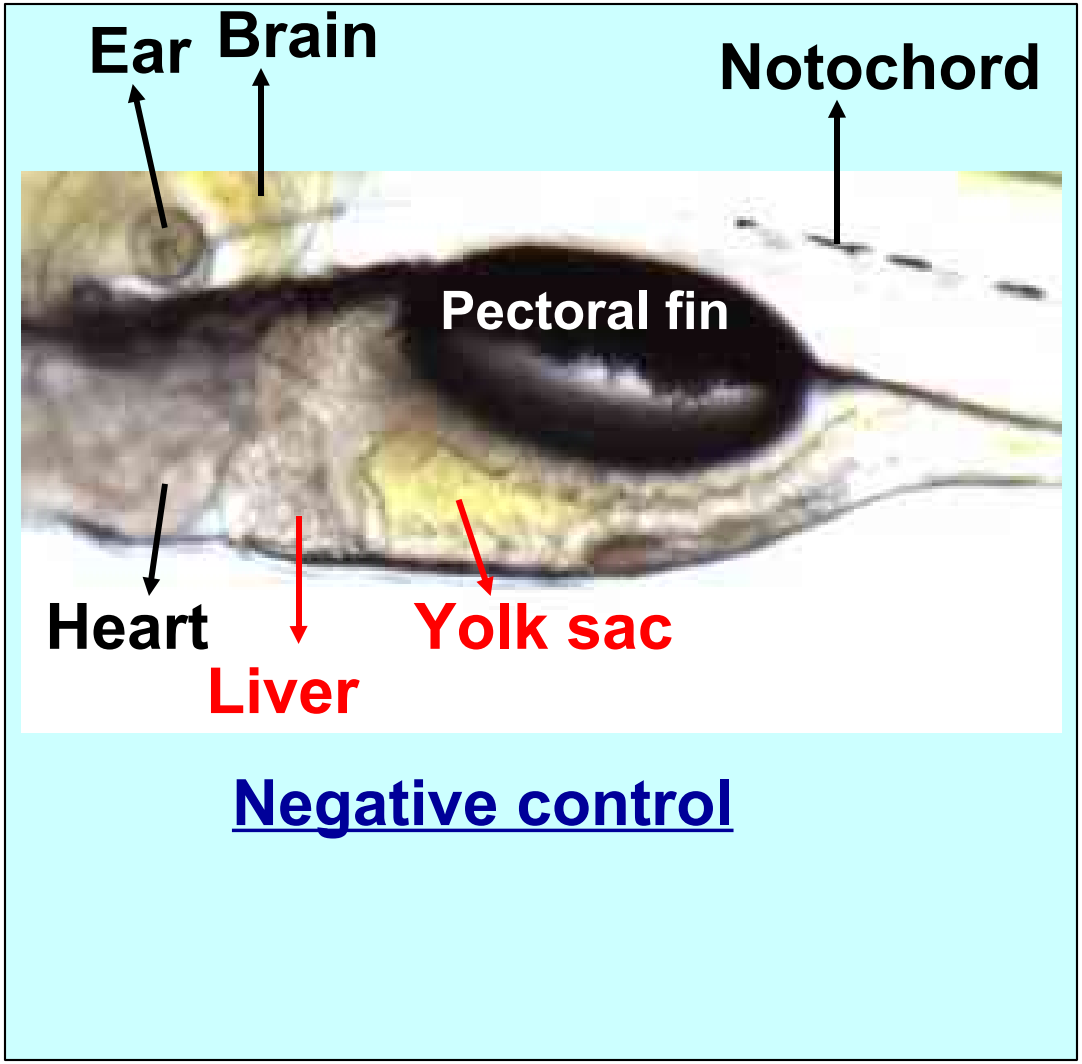


Test design: phenotypic screen for hepatotoxicity

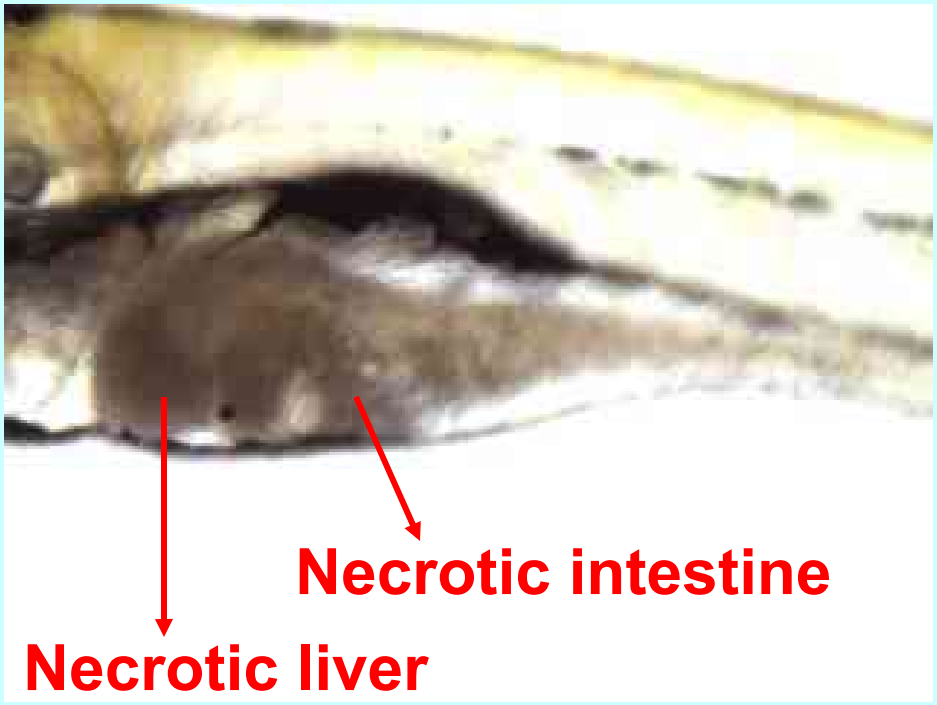
- Concentrations: 0.5, 5, 10, 50, 100 and 500 µM
- 14 larvae per concentration
- Dosing takes place at 96 hpf (day 4) at which time the liver is fully developed
- Assessment for liver toxicity at 144 hpf
- Embryos are screened using a stereo dissecting microscope for the following endpoints:

  - Liver necrosis
  - Changes in size and shape of the liver
  - Yolk abnormality (yolk sac oedema)
  - Lethality

Test design (2)



Negative control



Liver toxicity
(500 µM Acaftadine)

Results (1)

Name	Pharmacological activity	Hepatotoxic in mammalians	Hepatptotoxic in zebrafish
Lusaperidone	Antidepressant	Monkey and human hepatotoxic	
Clofibrate	PPAR-alfa	Rat and a few cases in humans	ATN
Oxyphenisatin	Laxative	Human hepatotoxic	
Ketoconazole	Antifungal	Rat hepatotoxic	(*)
Itraconazole	Antifungal	Rat hepatotoxic	
Ridogrel	Thromboxanesynthetase inh.	Rat hepatotoxic; not human hepatotoxic	
Acaftadine	Anti-histaminic	Rat hepatotoxic	
NCE 1	V1a-antagonist	Rat hepatotoxic	ATN
NCE 2	CRF-1 antagonist	Monkey & human hepatotoxic; neg. in rat & dog	
Amiodarone	Antiarrhythmic	Severely human hepatotoxic	
Danazol	Steroid hormone	Severely human hepatotoxic after metabolisation	
Valproate	Anticonvulsant	Severely human hepatotoxic after metabolisation	(*)
Furazolidone	Antibiotic	Moderately human hepatotoxic	
Tamoxifen	Estrogen receptor modulator	Mod. human hepatotoxic after metabolisation	
Troglitazone	PPAR-gamma activator	Human hepatotoxic	
HP-Beta-CD	-		
NCE 3	Antiviral		
Sucrose	-		
Gentamycin	Antibiotic	Not hepatotoxic but nephrotoxic and ototoxic	
Praziquantel	Anthelmintic		
Biotine	Vitamin H		

ATN: Additional Testing Needed

(*): Bioanalysis needed to prove bioavailability

Negative

Equivocal

Positive

Results (2)

Phenotypic screen only
(19 compounds)

		Liver toxic in rodents and/or humans	
		yes	no
Liver toxic in zebrafish	yes	10	3 FP
	no	3 FN	3

	%
Sensitivity	77
Specificity	50
Concordance	68

Results (3)

Compound	Concentration (µM)														Conclusion
	0.5	5	7.5	10	20	25	30	50	75	100	200	300	500	1000	
Amiodarone										P			P		
Danazol															
Troglitazone															
Lusaperidone															
NCE2															
Tamoxifen															
Itraconazole									L	L					
Furazolidone										L			L		
Gentamycin															
Praziquantel															
Ridogrel											L				
Acaftadine													L		
													L		
NCE3															
Ketoconazole															
Oxyphenisatin															
HP-beta-CD															
Sucrose															
Valproate															
Biotine															
NCE1															Additional testing
Clofibrate															Additional testing

No effect	Liver necrosis	P: Precipitation	Not tested	Lethal
	Weak liver toxic			L: Lethal

Conclusion on the zebrafish

- The zebrafish model is a suitable and promising model to screen for liver toxins. An acceptable sensitivity index was obtained.
- Human specific liver toxins were detected to be hepatotoxic to the zebrafish at low concentration levels.
- False positives were only obtained at high concentration levels.
- Phenotypic screen may be used as a first filter for liver toxicity.

Case by case, compounds are tested in the zebrafish for hepatotoxicity.

Acknowledgement

Mechanistic Toxicology

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DMPK

Snoeys Jan

Thanks

Questions?