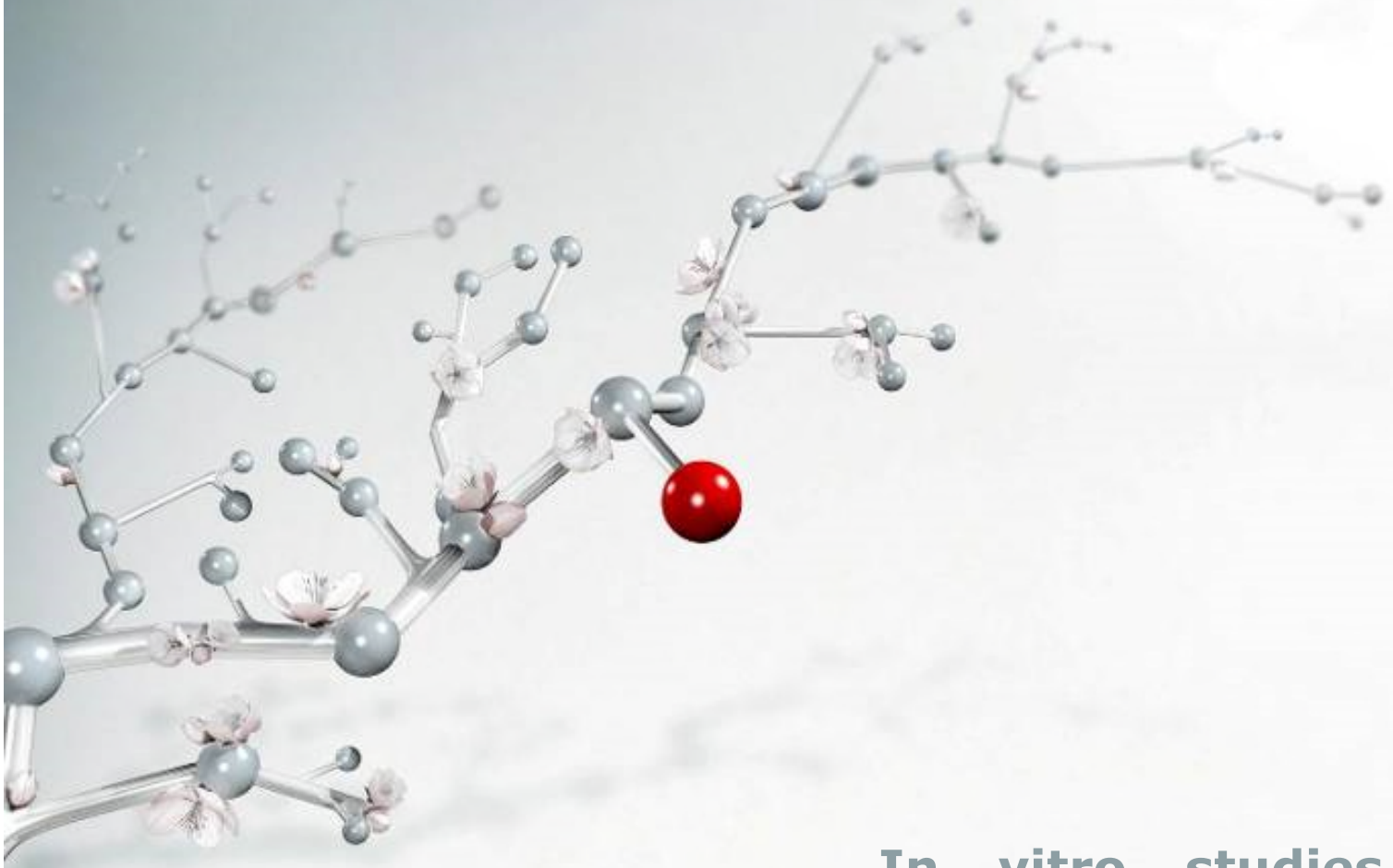


In vitro studies to assess liver enzyme induction across species.

Standards and criteria for acceptability and interpretation.

Simona Marzorati, PhD
Accelera Srl, Milan-Italy

Workshop on "In vitro comparative metabolism studies in Regulatory Pesticide Risk Assessment" Parma, EFSA, 15-16 November 2018

Outline

- Introduction: Comparison of species dependent metabolism;
- Induction study regulations and overview of models for testing;
- Protocol details:
  - ✓ Protocol for liver slices;
  - ✓ Protocol for cells lines;
  - ✓ Protocol for primary hepatocytes: change in gene expression (PCR methods);
  - ✓ Protocol for primary hepatocytes: change in enzyme activity (LC-MS/MS methods);
  - ✓ Comparison between read-out methods.
- Conclusions and future perspectives.

Comparison of species dependent metabolism

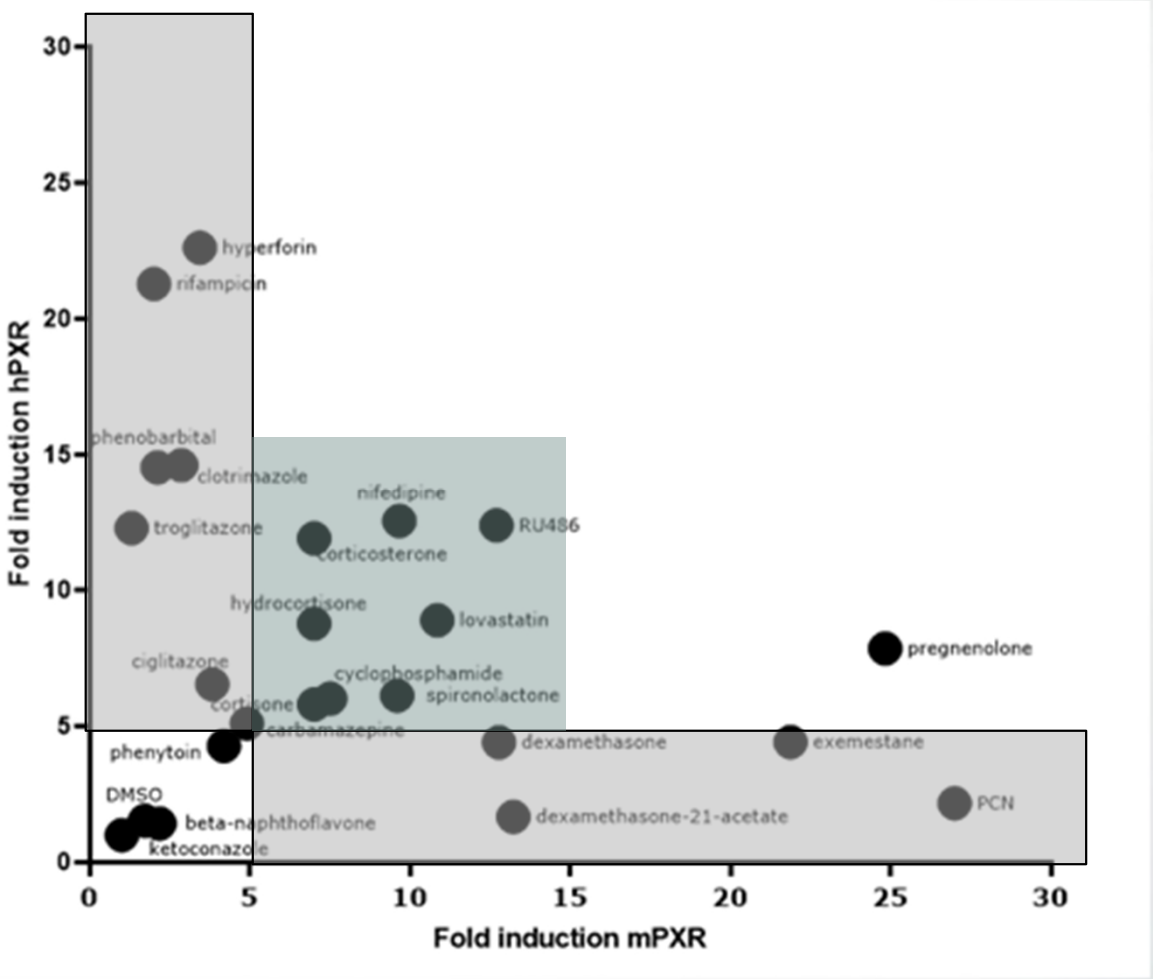
The expression of drug metabolising enzymes shows significant interspecies differences, and variability among human individuals.

Many induction studies, performed in various animal species, have proven to be beneficial.

However, species differences in the induction of some hepatic enzyme make the extrapolation from animals to humans very difficult or even impossible in some cases.

Humans differ from animals with regards to:

- Isoform composition;
- Expression and catalytic activities of drug metabolising enzymes.



REF: L.A. Vignati et al. / Toxicology 199 (2004) 23–33

Difference of metabolic enzyme cross species

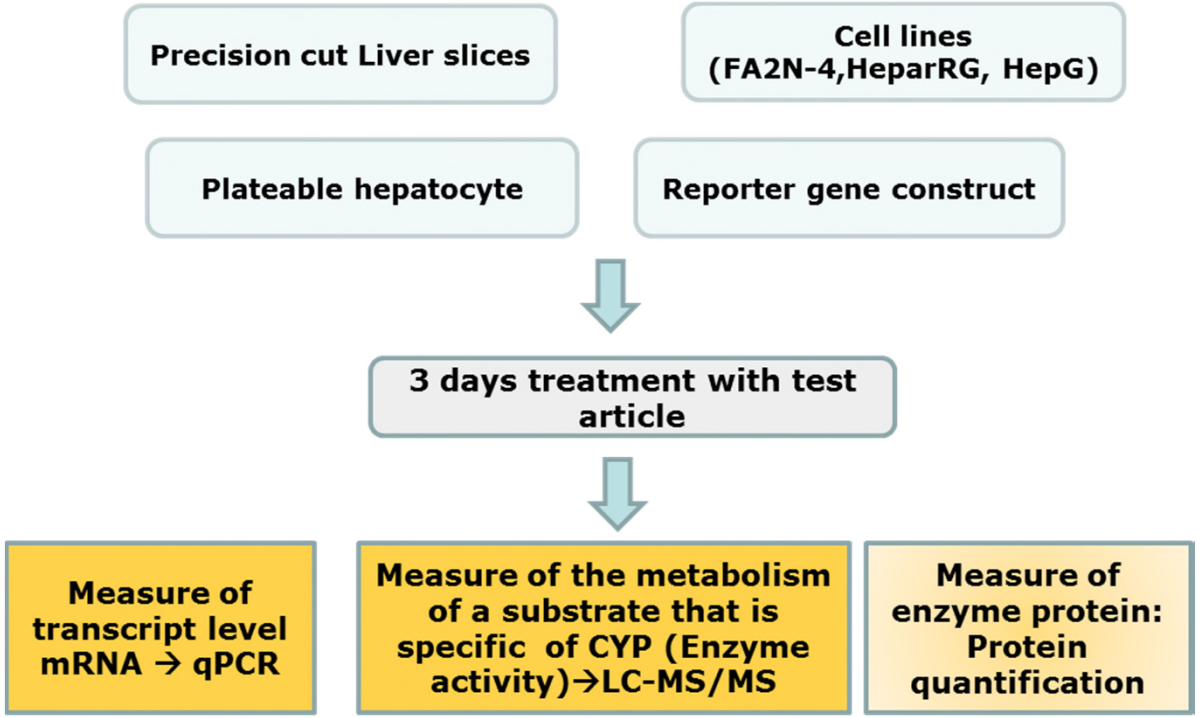
	ENZYME	DIFFERENCES
Nuclear receptor involved in CYP expression*	AhR, PXR and CAR	Mouse and rat, exhibit significant differences in specificity (in term of discrepancies in the ligand-binding domain).
Phase I enzyme *	CYP1A	Strong conservation among species; Omeprazole: example of species differences: man yes; mouse, rat no.
	CYP1B	Difficult to make species comparison; Little is known of this CYP in rodent and no-rodent.
	CYP2(A6-A7-A13)	Considerably different among species.
	CYP2B	Expressed in all species in liver; Highly expressed in intestine of mouse and rat, not in human.
	CYP2C	The largest and complicated subfamily in several species; Sex dependent in rat; Substrates specificities are largely different between human and animal; Is not expressed in dog.
	CYP3A	Extrapolation from animal to man quite hazardous since presents different isoform among species; Is inducibile but with some variables (es. rifampicin); The high inducibility is the cause of large interindividual variations.

* Martignoni M., Ex opinion Drug Metab Toxicol (2006).

Induction study regulations and overview of models for induction testing

Accelera is a CRO with expertise in Pharmaceutical →GUIDELINES: EMA 2012/FDA 2017.

Initially investigated in vitro phase I enzyme*:*CYP1A2, CYP2B6, CYP3A4 (CYP2C for FDA if CYP3A is positive).



In vivo interaction studies are recommended to determine the extent of changes in the investigational drug's pharmacokinetics.

CYP induction method is based on xenobiotic-receptor binding → *in vitro* CYP induction test methods are predictive for any class of compounds that can interact with receptors (i.e. Pesticides).

For Agrochemical: GUIDELINES: introduced in the data requirements (Commission Regulation (EU) No 283/2013) for EU Regulation 1107/2009 (EU Commission, 2009; 2013a; 2013b).

Important factors to consider: test system

Test system:

- Human hepatocytes (cost 1K € vial, difficulty of standardization, human liver tissue is only sparsely available, and the number of sources of healthy tissue is limited; fresh vs cryopreserved);
- Cell lines express multiple functional Phase 1 and 2 drug metabolising enzyme activities at comparable levels to those in cultures of primary human hepatocytes: FA2N-4,HepaRG, HepG2;

"Acceptable if it can be demonstrated with positive controls that CYP3A4 and CYP1A2 are inducibile in these cell lines"

- Culture media (William's E, InVitroGRO, DMEM, hepatocyte maintenance, Chee's, HepatoSTIM) and media supplements (serum or serum free);
- Primary cultures of animal hepatocytes "is not recommended" for induction studies for predicting human response.

Important factors to consider: experimental design and analytical method.

Assay Variables:

- Effect of the duration between plating and start of treatment (24-48 hrs);
- Addition of drug time of treatment (24 hrs vs 72 hrs);
- Number of testing concentrations:
  - ✓ based on the expected human plasma drug;
  - ✓ 3 concentrations spanning the therapeutic range;
  - ✓ if information is not available, concentrations ranging over at least 2 orders of magnitude.
- Methods for assessing cytotoxicity (i.e. cell morphology, enzyme leakage, ATP content, or MTT);
- Vehicle of test system (DMSO may have induction potential on CYP3A4).

End-point and analytical method:

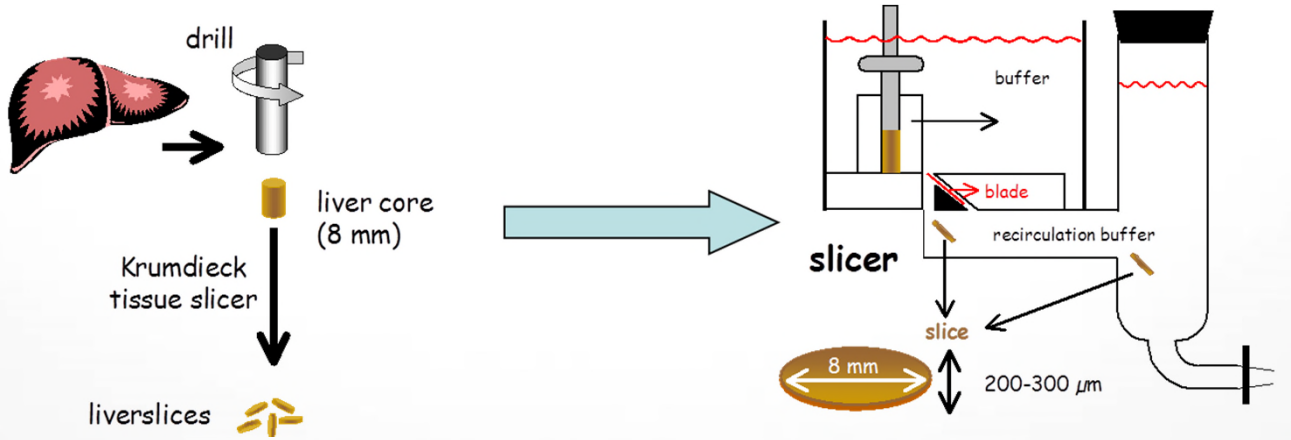
- mRNA expression, housekeeping genes (GAPDH, 18S, or actin);
- mRNA read out: fold-induction vs vehicle control or induction response vs pos control;
- Evaluation of enzymatic activity by LC-MS/MS analysis to assess the amount of metabolites formed: fold induction of treated samples over vehicle control and percentage of induction in treated samples versus positive controls;
- Other methods: protein quantification (immunoblotting or LC-MS/MS).

Some available protocols

Test system	Rat liver slices	***Ref:*** Accelera SOP and M. Martignoni et al. Chemico-Biological Interactions (2004)
	Cells lines	***Ref:*** Accelera SOP and L.A. Vignati et al. Toxicology (2004)
	Cryopreserved human hepatocytes	***Ref:*** Accelera SOP
End-point	Reporter assay	***Ref:*** Accelera SOP and L.A. Vignati et al. Toxicology (2004)
	mRNA measurement	***Ref:*** Accelera SOP
	Enzyme activity measurement	***Ref:*** Accelera SOP

Rat liver slices as tool to identify CYP induction-Method-

	vitro	*vivo*
Test system	Rat Liver Slices	Rats
Test articles	β–Naftoflavone (50 μM) Dexametasone (100 μM) Phenobarbital (1mM) 6MW culture plates in Williams'Medium E	β–Naftoflavone (80 mgkg-1day-1) Dexametasone (100 mgkg-1day-1 Phenobarbital (80 mgkg-1day-1)
Duration of treatment	48 hr + 6 hr EROD (5 mM) and testosterone hydroxylase (250 mM) activities investigation	3 days treatment
Endpoints	MTT RT PCR (CYP1A1, CYP1A2, CYP2B1, CYP3A1)	RT-PCR (CYP1A1, CYP1A2,CYP2B1,CYP3A1)



REF: M. Martignoni et al. / Chemico-Biological Interactions 151 (2004) 1–11

Rat liver slices as tool to identify CYP induction-Results-

Comparison of induction (qualitative and quantitative) observed in vivo and in vitro in rat liver after exposure to βNF, PB and DEX.

	CYP1A1		CYP1A2		CYP2B1		CYP3A1	
	vivo	vitro	vivo	vitro	vivo	vitro	vivo	vitro
β-Naftoflavone	+	+++	+	+	-	++	-	-
Phenobarbiltal	-	+	-	-	+++	+++	+	+
Dexametasone	-	-	-	+	+	+++	++	++

Legend: + < 25 fold induction; ++ 25-100 induction;+++> 100 fold induction

PRO	CONS
• The induction profiles (qualitative and quantitative) observed in vivo and in vitro are quite similar; • Liver slices are a useful and predictive tool to study CYP induction.	Various shortcomings related to fresh liver availability (human) and difficulties in maintaining enzymatic activities for prolonged periods.

REF: M. Martignoni et al. / Chemico-Biological Interactions 151 (2004) 1–11

Human and mouse reporter gene assay-Method-

An alternative way to measure CYP induction is to detect the activation of nuclear receptors transcription factors.

Day 1

- Transfection of HepG2 cells
  - -Lipofectamine Plus
  - -0.5 ug/ml plasmid 4xGal4re-LUC
  - -0.05 ug/ml plasmid Gal4-hPXR LBD
- Seeding of transfected cells
  - -96 well plate
  - -100000 cells/well

overnight

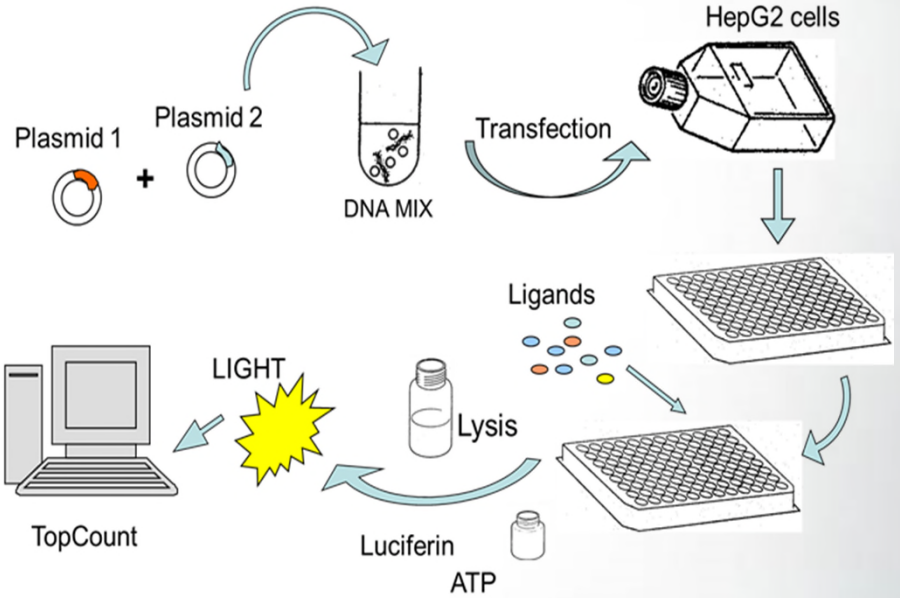
Day 2

- Treatment with test compounds

24h

Day 3

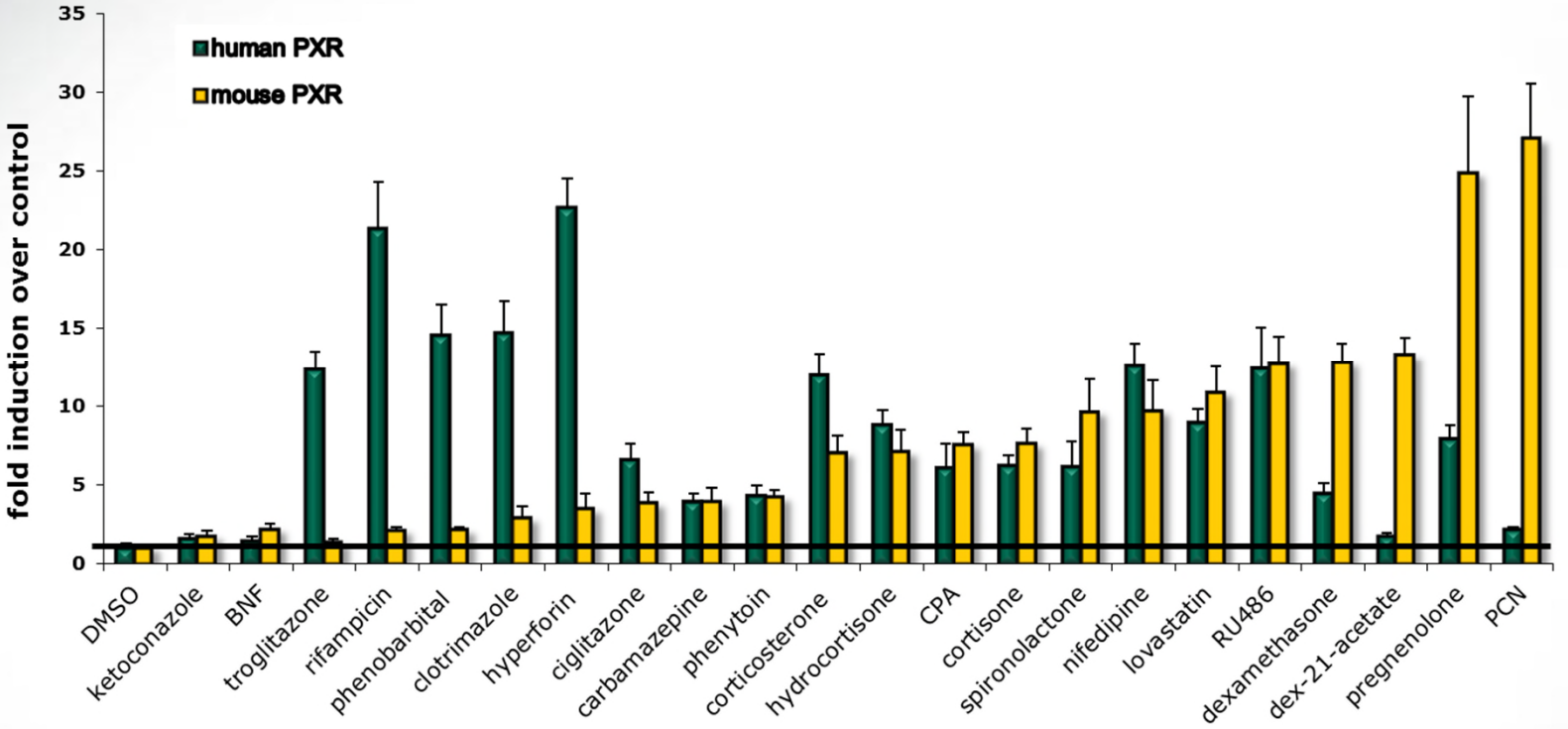
- Luciferase assay and MTT/Alamar Blue test



- 8 concentrations (0-100 µM)
- 6 replicates
- Control: DMSO 0.1%

REF: L.A. Vignati et al. / Toxicology 199 (2004) 23–33

Human and mouse reporter gene assay-Result-



PRO	CONS
Rapid and easy read-out for drug screening; Reporter gene assay is a useful and predictive tool to study CYP induction; High sensibility and reproducibility.	Cannot assess the ability for induction via other mechanism (i.e, via other nuclear receptor); It does not give information about post-transcriptional induction.

REF: L.A. Vignati et al. / Toxicology 199 (2004) 23–33

Pharmaceutical CYP induction protocol details

The use of human hepatocytes in testing for induction remains the gold standard test system for EMA/FDA for making in vitro assessments of induction.

Especially for informing clinical drug interaction study strategies and inclusion of data in drug registration dossiers and product labels.

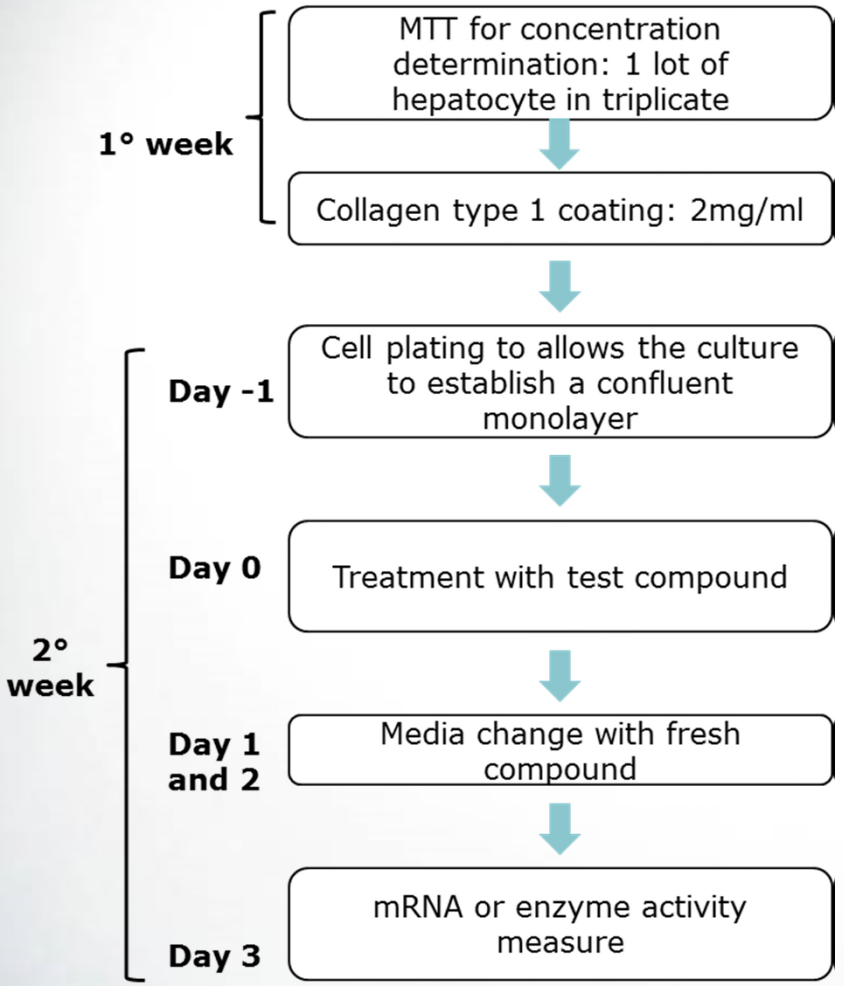
Focus on:

- Protocols;
- Data interpretation.

Hepatocytes culture and treatment protocol

Test System

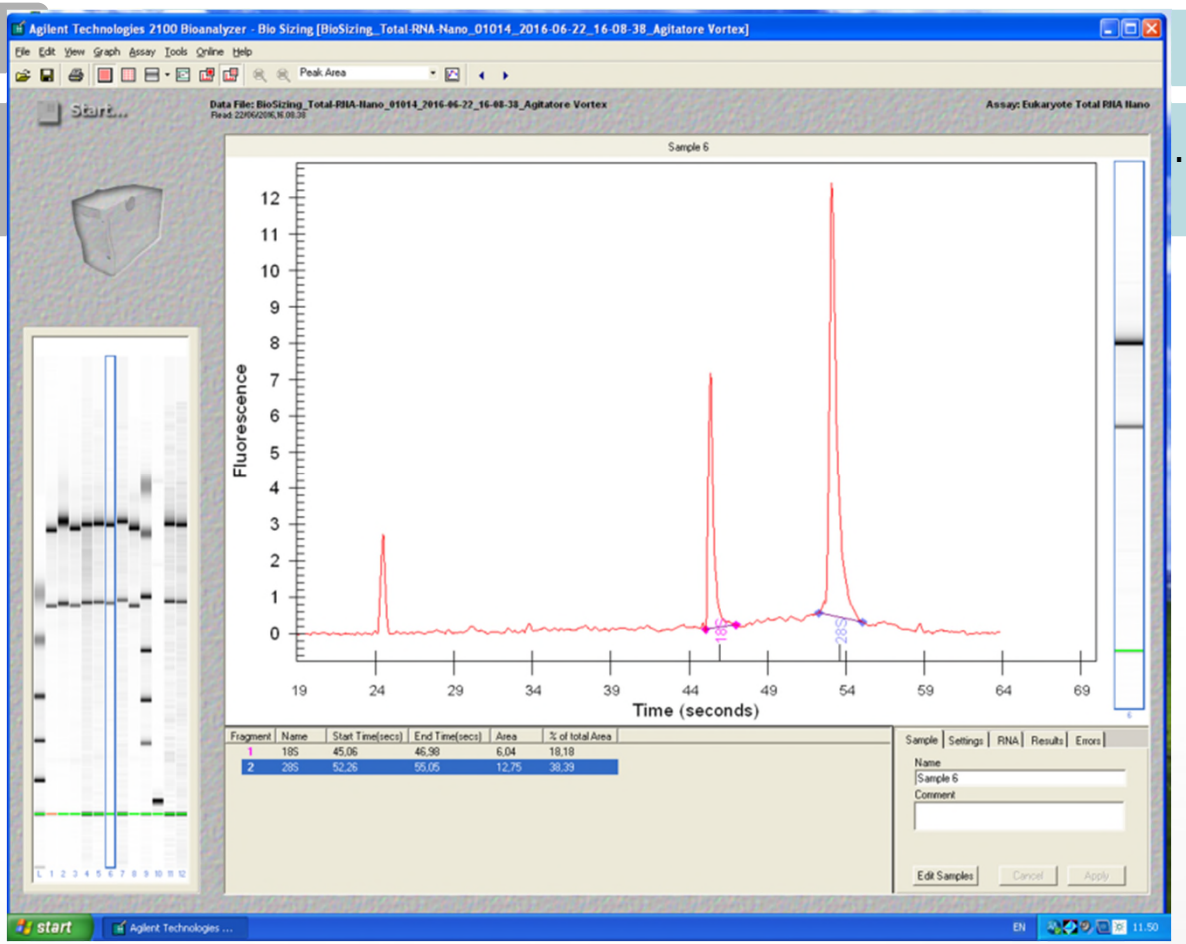
Plateable human cryopreserved hepatocytes from 3 donors (Donor characteristics that should be avoided:a high BMI or fatty livers; liver disease, such as viral infections; age < 6 mo or >60 yr of age. 48MW collagen coated plate (700000 cells/ml) in William's E media at 37°C, 5% CO_2.
3 testing concentrations in triplicate.



Pos control	**CYP1A**	Omeprazole (50μM)	Fold induction>10
	CYP2B e (CYP3A4)	Phenobarbital (1 mM)	Fold induction>5
	CYP3A e CYP2C	Rifampicin (25μM)	Fold induction>2
Negative control		DMSO 0.2%	

mRNA expression protocol -I-

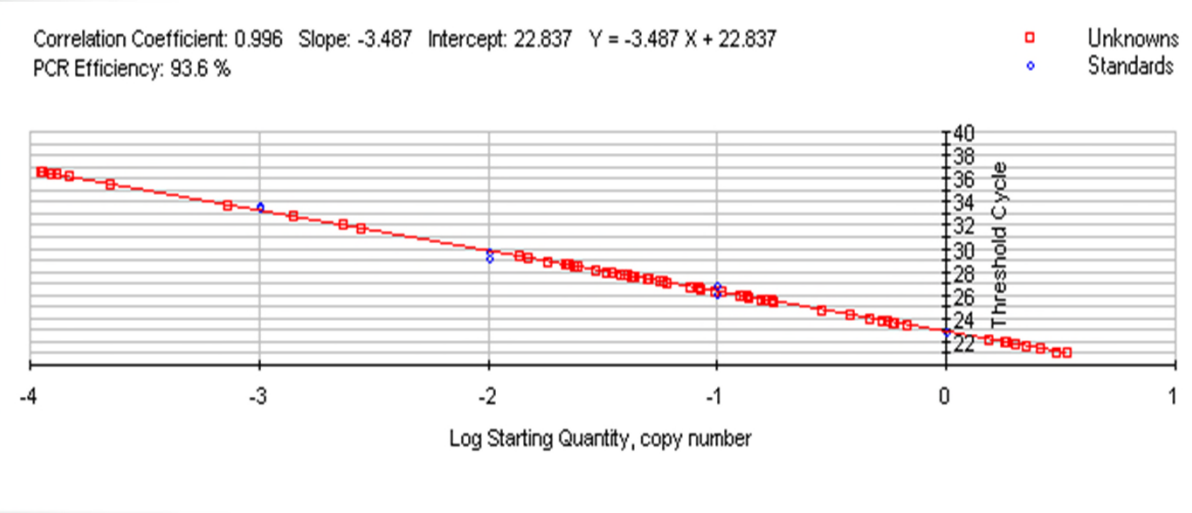
Test System	Plateable human cryopreserved hepatocytes from 3 donors in 48MW collagen coated plate (700000 cells/ml) see previous slide.
RNA isolation	
Quality/quantitative evaluation	.7



Retrotranscription parameters	INSTRUMENT: iCycler BioRAD. Enzyme: Superscript II, Invitrogen. Protocol: DENATURATION protocol: 65°C, 10'manufacture protocol.

mRNA expression protocol-II-

PCR parameter	PROBE: Taqman (FAM); PRIMER: commercial.
Standard curve	hcDNA (at least 5 dilution 1:10); starting quantity is extrapolated via standard curve.



Criteria acceptability standard curve:

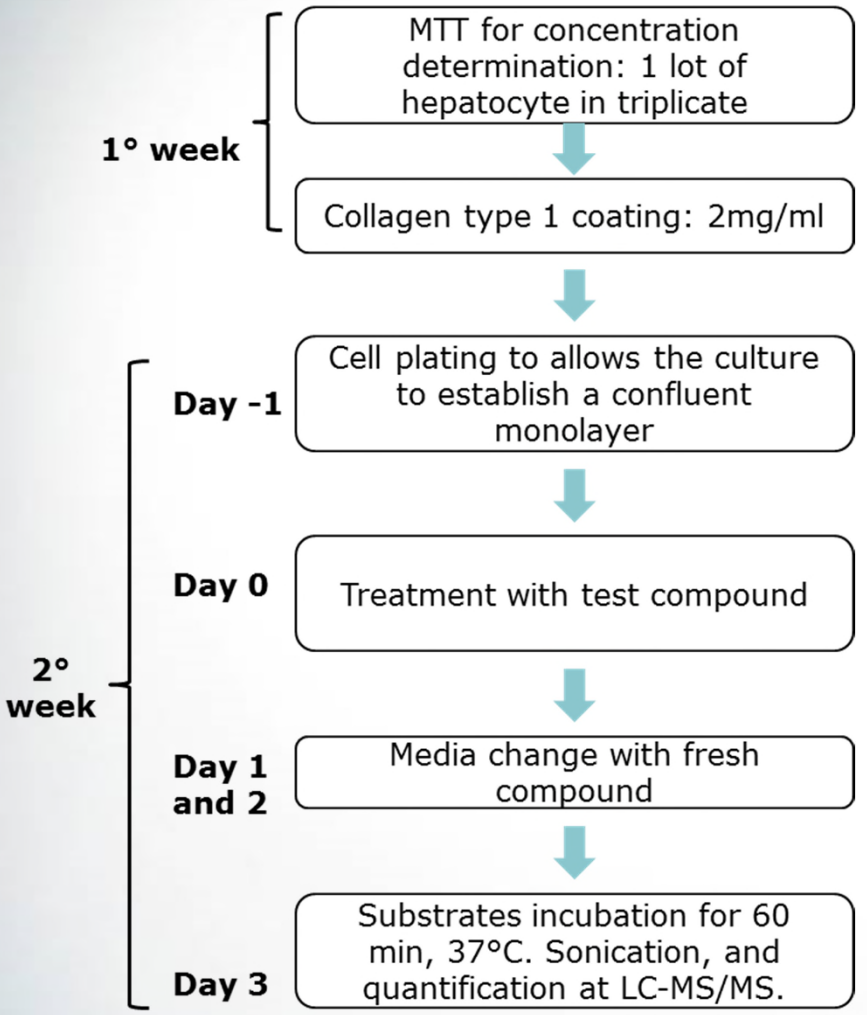
- Efficiency: 80-120%
- Slope: 3-3.8

PCR acceptability criteria	Based on results of reference substances and controls.
Fold change calculation	SOFTWARE: iCycler IQ/CFX Maestro BioRad. Threshold: manual: where the increase of fluorescent signal gets out of the background noise and is exponential based on standard Ct. Normalization: Housekeeping: β-Actin (Hs99999903_m1 TaqMan®Gene Expression Assay).

Enzyme assay activity protocol (LC-MS/MS)-I-

Test System

Plateable human cryopreserved hepatocytes from 3 donors in 48MW collagen coated plate (700000 cells/ml) see previous slide.



Pos control	**CYP1A**	Omeprazole (50μM)
	CYP2B e (CYP3A4)	Phenobarbital (1 mM)
	CYP3A e CYP2C	Rifampicin (25μM)
Negative control		DMSO 0.2%

	Substrate
CYP1A	7-ethoxyresorufin (100μM)
CYP2B	Bupropion hydrochloride (200μM)
CYP3A	Testosterone (250μM)

Enzyme assay activity protocol (LC-MS/MS)-II-

HPLC-MS INSTRUMENT AND CONDITIONS	
LC-system	HP 1100 binary pump or similar.
Injector	Waters 2777 Autosampler 100 µl sample loop- 100 µl syringe or similar.
Mass spectrometer	Triple quadropole API 4000 or similar.
Scan Mode	MRM.
Total run time	2.5 min
Software	Analyst (AB-Sciex).

	Substrate	**Metabolite**	**Standard curve (µM)**
CYP1A	7-ethoxyresorufin (100µM)	Resorufin	0.005-1 µM
CYP2B	Bupropion hydrochloride (200µM)	(2S,3S) Hydroxybupropion	0.0005-1 µM
CYP3A	Testosterone (250µM)	6 β-hydroxytestosterone	0.005-1 µM

mRNA expression vs enzymatic activity measure

Methods	PRO	CONS
mRNA (qPCR)	• Detect inducers that are enzyme inhibitors. • Reliable and predictive to detect induction at transcriptional and post transcriptional mechanism.	In cases where CYP induction is mainly regulated by a post-translational mechanism the mRNA levels has not a predictive value.
Enzymatic activity (LC-MS/MS)	• The sensitivity and selectivity (superior to other analytical techniques). • Detect effective enzymatic activity.	May miss inducers that are also enzyme inhibitors: some drugs can cause an increase in transcriptions of CYP3A4 yet also be mechanism based inactivators.

Conclusions

- The best option is understanding the pros and cons of each approach to choose the best tool for a given situation. To measure induction of drug-metabolizing enzymes in vitro, several endpoints can be chosen;
- Hepatocytes contain the full complement of enzymes, so this test system has an advantage for several studies over the other test systems;
- Careful selection of human hepatocyte lots is important for the evaluation of induction; at least three donor hepatocytes, with experiment meeting the acceptance criteria for each donor;
- An inducer is a compound that shows ≥ 2-fold increase in mRNA/ enzyme activity and a response ≥ 20% of the response of the positive control;
- A positive result in at least one of the three donor hepatocytes is considered an indication of induction.

Future perspectives

- More complex cell-based assay may provide an improvement in predictive power of mechanistic models;

- CYP induction *in vitro* test method evaluates the potential of a test item to induce CYP mediated via PXR/CAR (CYP3A4, CYP2B6) and the Ah receptor (CYP1A2). Phase II enzymes have historically attracted less attention most likely because of the lack of consolidated tools (UGT validation).

Contact informations

www.accelera.org

email: simona.marzorati@accelera.org