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Use of human hepatocytes for the *in vitro* determination of cytochromes P450 induction

Hepatocyte Expert Programme™
June 6-8 2012, Paris

Hanan Osman-Ponchet , Alexandre Gaborit, Karine Sevin, Magali Kouidhi, Pierre Comby.

DMPK Unit

hanan.osman-ponchet@galderma.com

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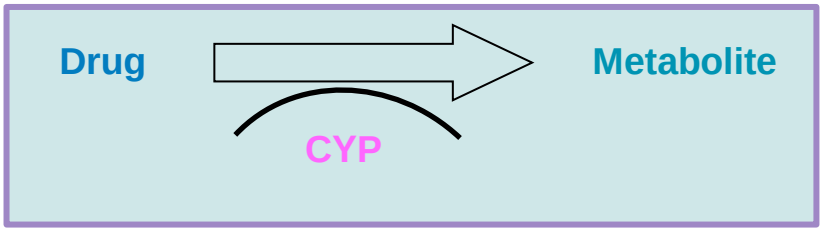
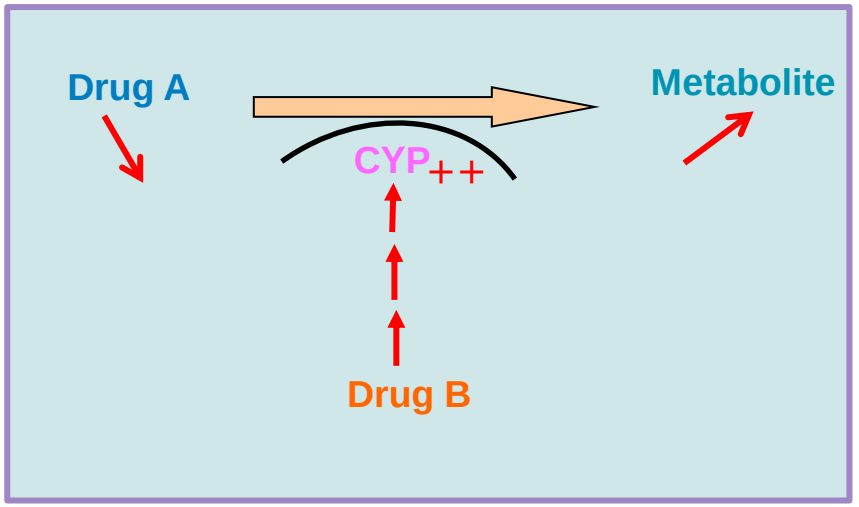
Outline of the presentation

- Introduction: Enzyme induction
- Consequences of enzyme induction
- Regulatory guidances
- Time and dose effect
- Molecular mechanisms
- Species differences
- *In vitro* models to assess enzyme induction
  - Human hepatocyte model
- General procedure: *in vitro* induction study
- Example of results
- Conclusion

INTRODUCTION

- Enzyme induction studies assess the potential for a drug candidate to induce (up-regulate) the expression of drug-metabolizing enzymes.





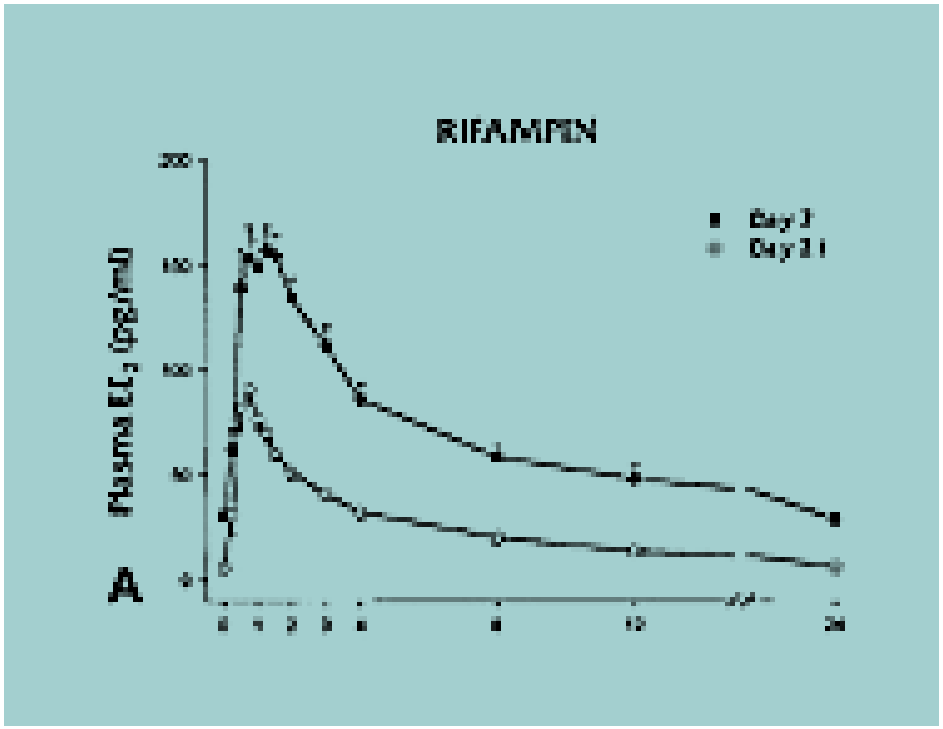
INTRODUCTION

- A drug that induces a specific drug metabolizing enzyme (e.g. a specific P450 isoform) would have the potential to enhance the metabolism of a co-administered drug that is a substrate of the induced pathway.
- Enzyme induction is a major mechanism of pharmacokinetic drug-drug interactions.

Consequences of enzyme induction

- Increase of drug's elimination,
  - Lower drug concentration
  - Decrease the drug's efficacy.

Contraceptive drug



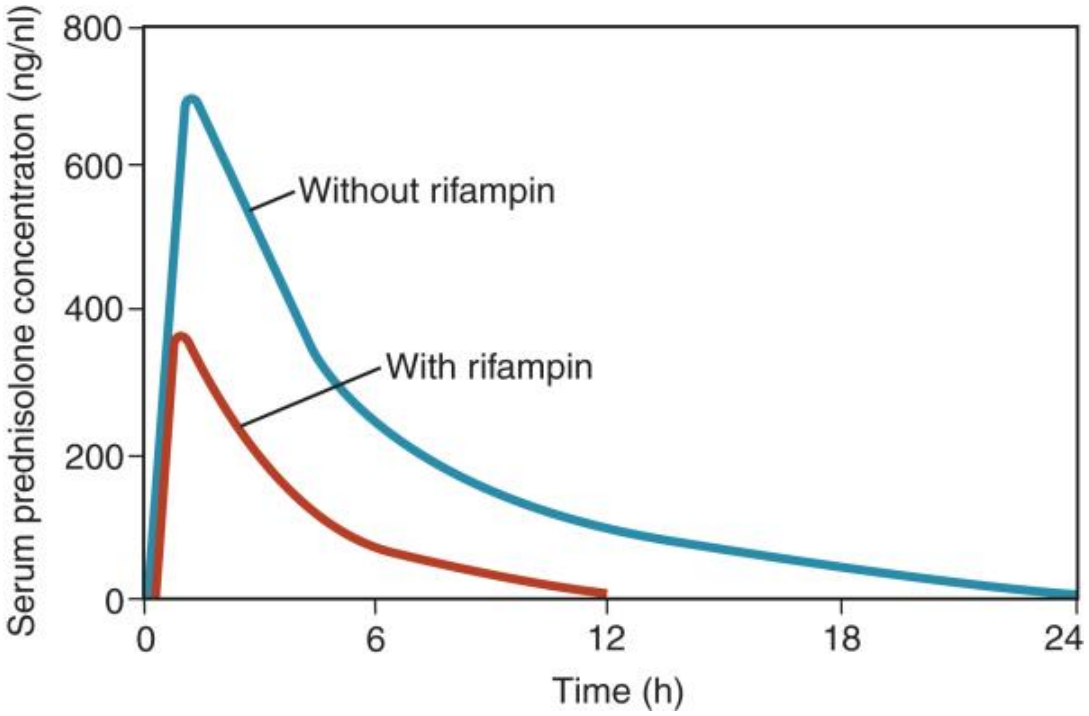
Induction of CYP3A4 by rifampin

Barditch-Crovo et al (1999).
Clin. Pharmacol. Ther

Consequences of enzyme induction

- Increase of drug's elimination,
  - Lower drug concentration
  - Decrease the drug's efficacy.

Anti-inflammatory drug



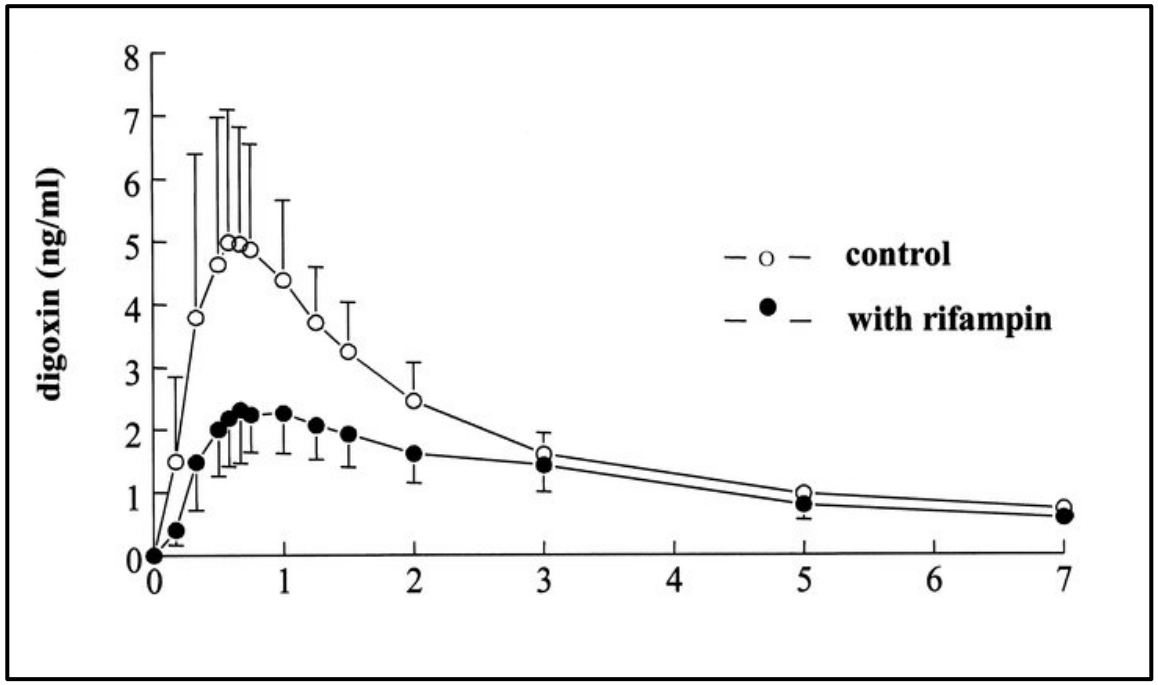
Induction of CYP3A4 by rifampin

Jacobs & Bijlsma (2008).
Kelley's Textbook of Rheumatology, 8th ed.

Consequences of enzyme induction

- Increase of drug's elimination,
  - Lower drug concentration
  - Decrease the drug's efficacy.

Digoxin



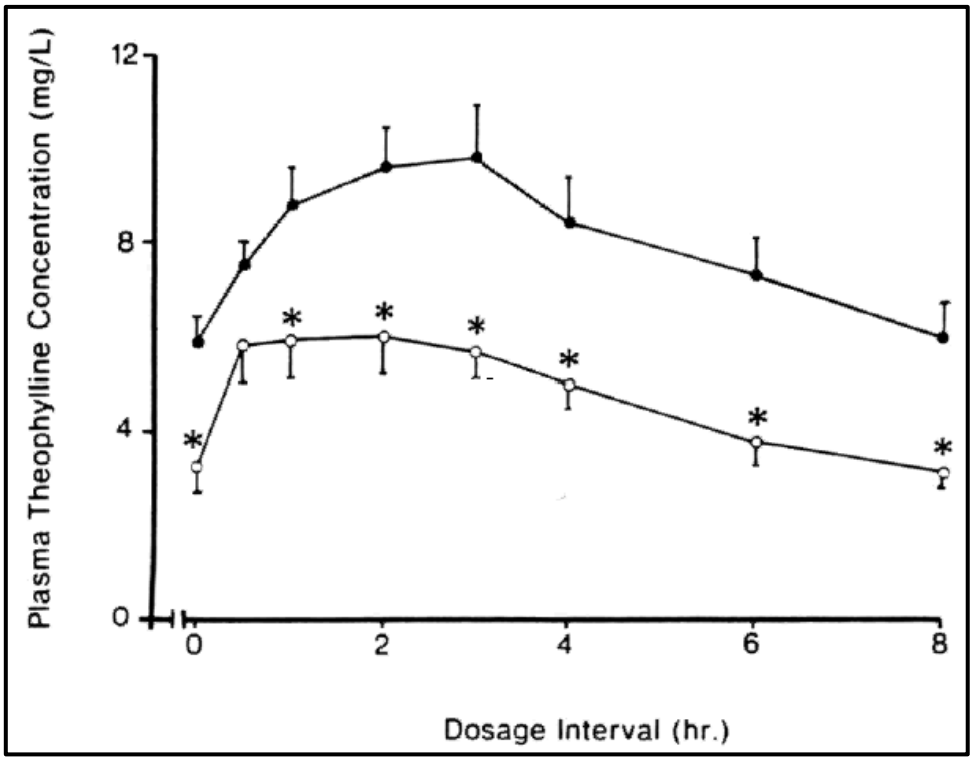
Induction of P-gp by rifampin

Greiner et al. (1999)
J Clin Invest

Consequences of enzyme induction

- Increase of drug's elimination,
  - Lower drug concentration
  - Decrease the drug's efficacy.

Theophylline



Induction of CYP1A2 by cigarette

Grygiel et al. 1981,
J Clin Pharmacol Ther

Consequences of enzyme induction

- Increase of metabolism
  - Increase of formation of reactive metabolites
  - Increase in toxicity

Regulatory guidances

- The potential for enzyme induction to cause adverse effects has been recognized by the FDA and EMA agencies, who consider this type of study as an important part of *in vitro* drug development.

EMA' guidance

April 2010

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

22 April 2010
CPMP/EWP/560/95/Rev. 1 – Corr.*
Committee for Human Medicinal Products (CHMP)

Guideline on the Investigation of Drug Interactions

Draft

Discussion in the Efficacy Working Party (EWP)	June/October 1996 February 1997
Transmission to the CPMP	March 1997
Transmission to interested parties	March 1997
Deadline for comments	September 1997
Re-submission to the EWP	December 1997
Approval by the CPMP	December 1997
Date for coming into operation	June 1998
Draft Rev. 1 Agreed by the EWP	April 2010
Adoption Rev. 1 by CHMP for release for consultation	22 April 2010
End of consultation Rev. 1 (deadline for comments)	31 October 2010

This guideline replaces guideline CPMP/EWP/560/95

*The correction concerns the reflection of the correct document number as well as the addition of the previous timelines on the cover page.

FDA' guidance

February 2012

Guidance for Industry

Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Comments and suggestions regarding this draft document should be submitted within 90 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register*.

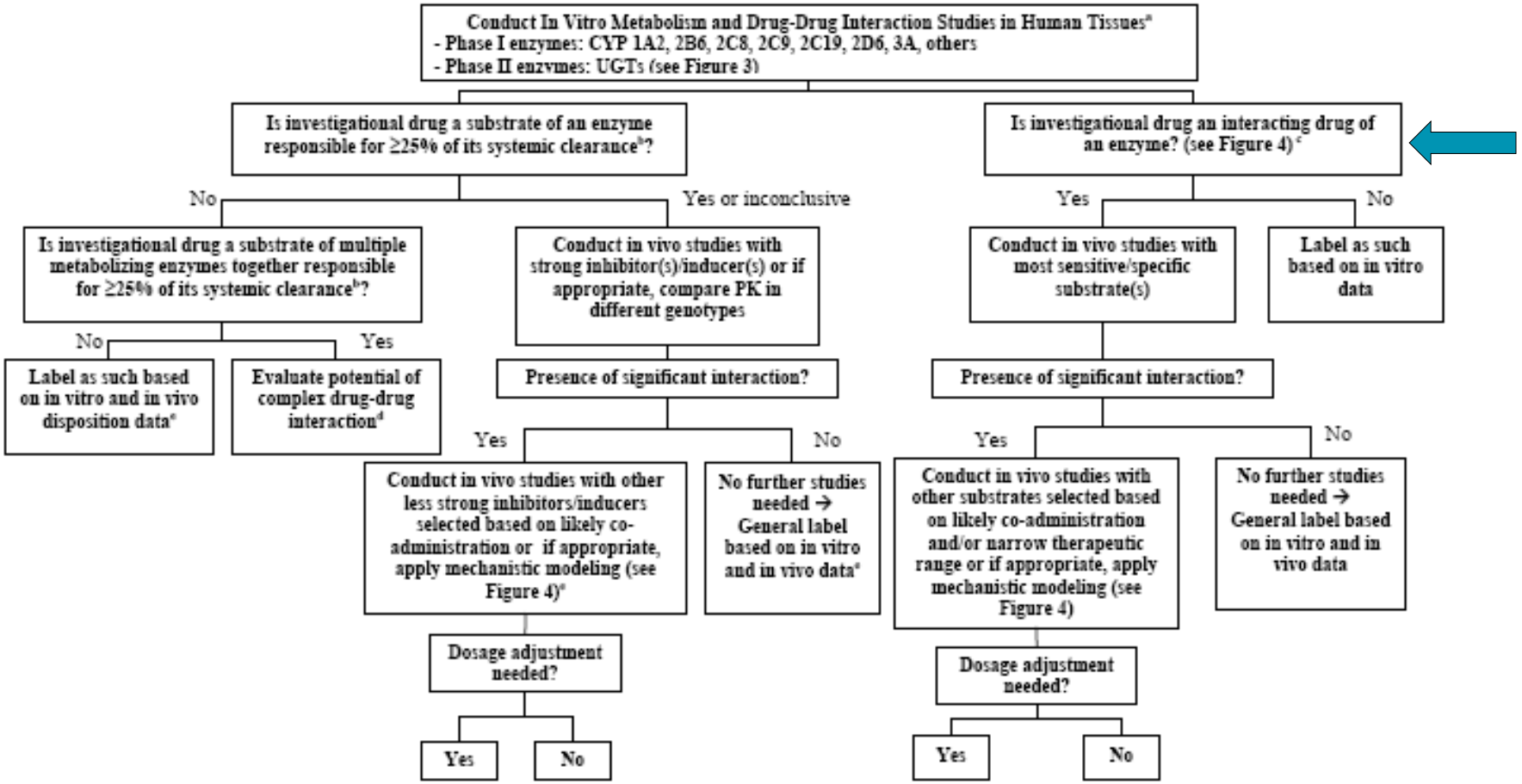
For questions regarding this draft document contact (CDER) Shiew-Mei Huang, 301-796-1541, or Lei Zhang, 301-796-1635.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

February 2012
Clinical Pharmacology

FDA' guidance

Figure 2. Metabolism-Based Drug-Drug Interaction Studies — Decision Tree



FDA' guidance

CYP induction

- Measure mRNA change by investigational drug in cultured human hepatocytes from ≥3 donors [a]
- Estimate DDI parameters

Is increase in mRNA > a predefined threshold[a]?
Or, is the calculated R value<1/1.1 (i.e., 0.9)?

$$R_3 = 1/(1 + d \times E_{max} \times [I]/(EC_{50} + [I]))^{[c]}$$

- The changes in the mRNA level of the target gene should be used as an endpoint
- Vehicle control, positive control (usually a known strong inducer), and negative control
- (usually a known non-inducer should be included in the experiment

Prototype inducers

Table 2. In Vitro CYP Inducers

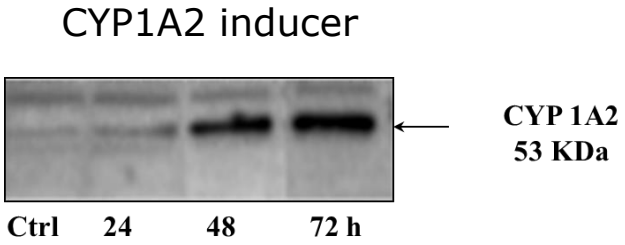
CYP	In Vitro Inducer * as Positive Controls	Recommended Concentration (μM) of the Positive Controls	Reported Fold Induction In Enzyme Activities
1A2	omeprazole lansoprazole	25-100 10	14-24 10
2B6	phenobarbital	500-1000	5-10
2C8	rifampin	10	2-4
2C9	rifampin	10	4
2C19	rifampin	10	20
2D6	none identified		
3A4	rifampin	10-50	4-31

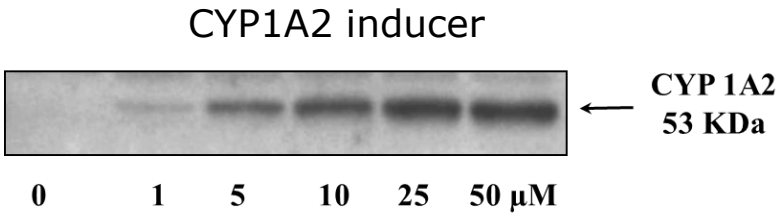
*Note that this is not an exhaustive list. For an updated list, see the following link

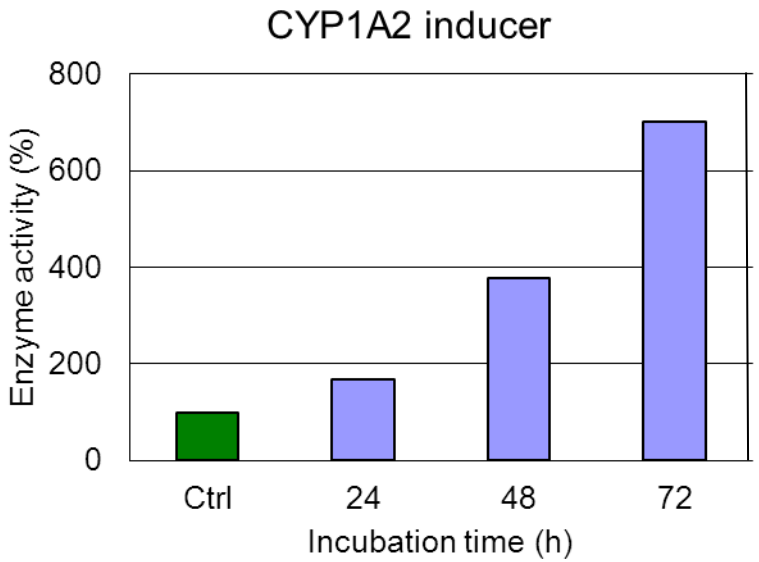
- **Some CYP are not inducible: CYP2D6**

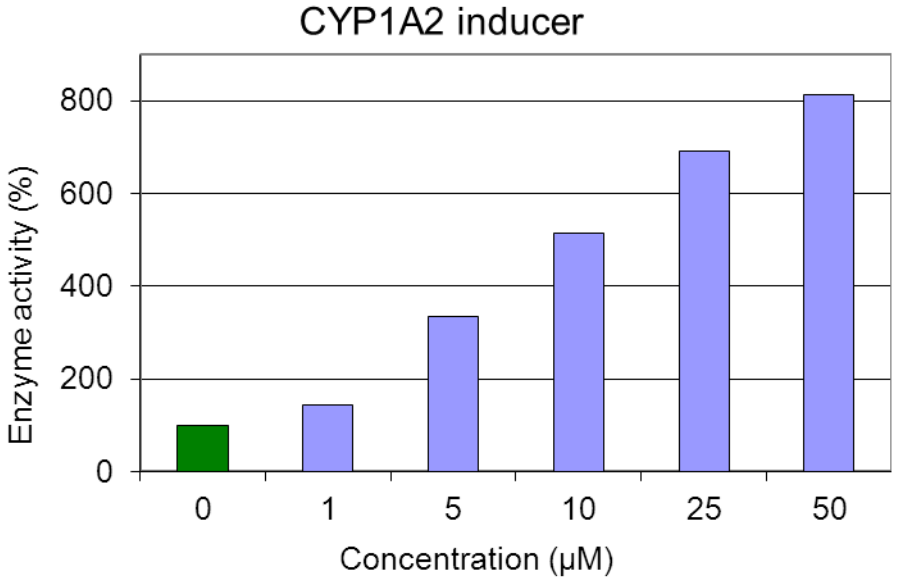
Time and dose effect

- Enzyme induction is a slow regulatory process, involving biosynthesis of mRNA and protein. Therefore, the CYP induction is a time- and concentration (dose)-dependent process.









In vitro analysis on human hepatocytes

Time and dose effect

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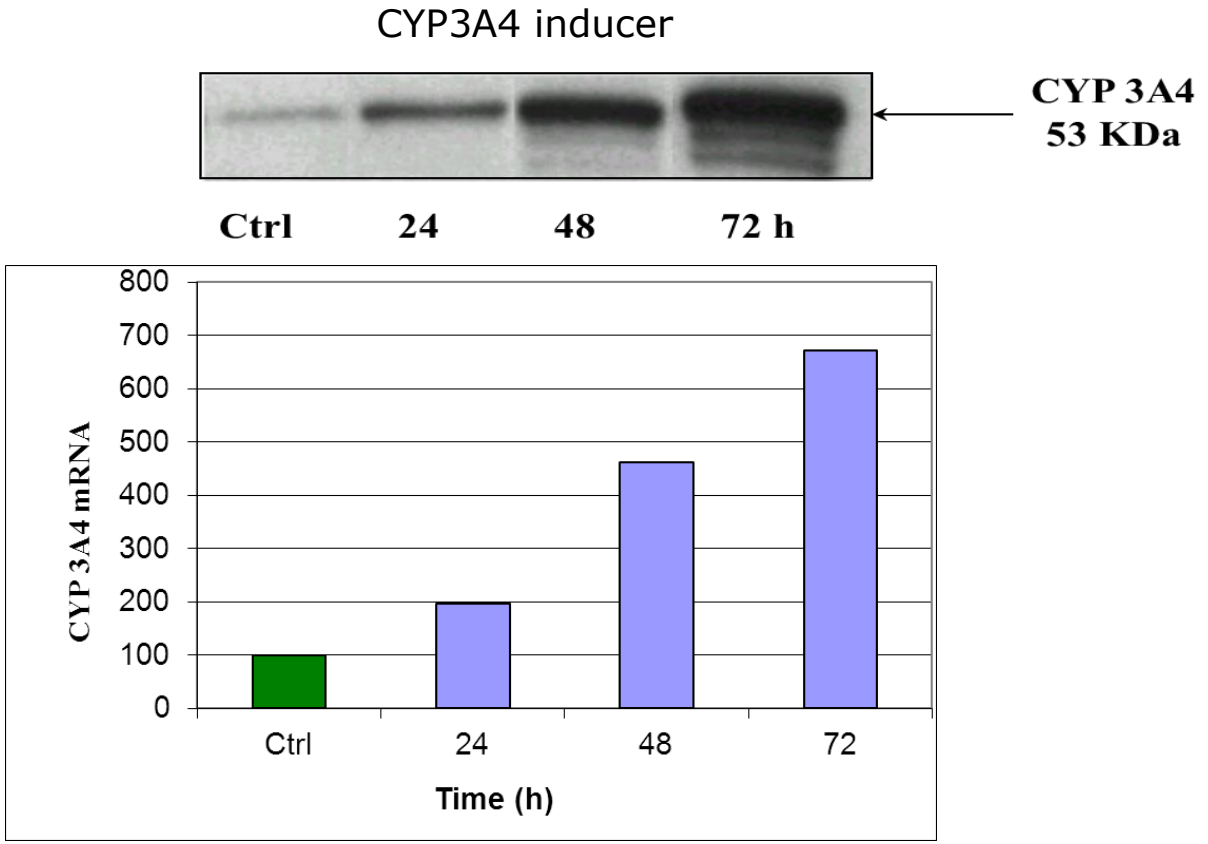
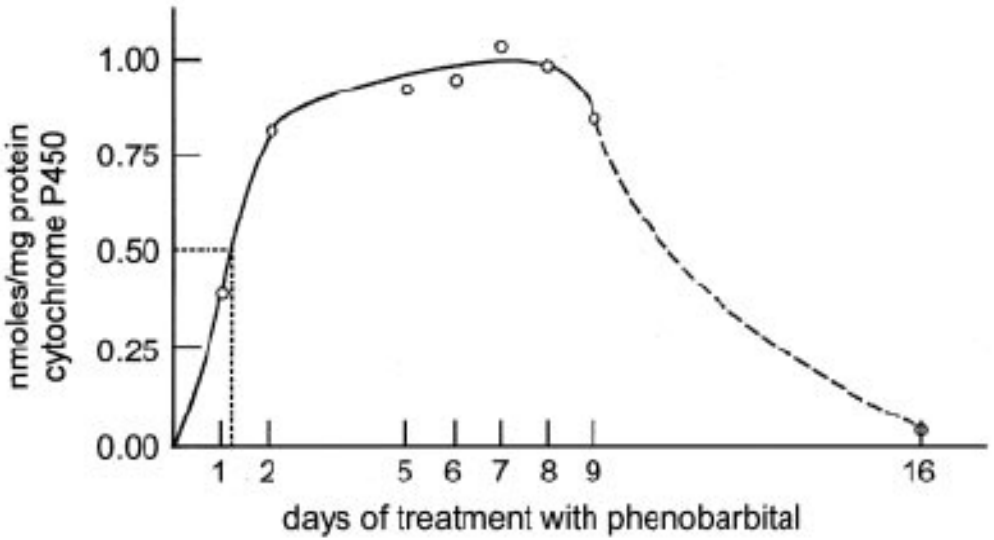


In vitro analysis on human hepatocytes

Time and dose effect

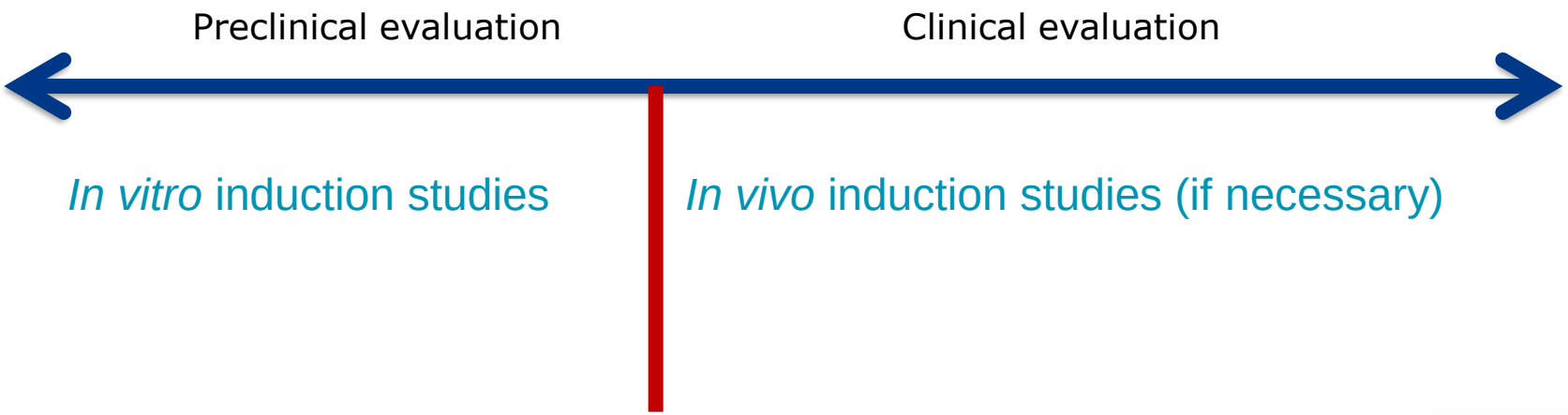
- *In vivo*, enzyme induction takes days (5 – 7 days) to reach a maximum enzyme level and to return to the enzyme basal level after discontinuing the treatment with inducer.



Handschin & Meyer, Pharmacol Rev 55:649–673, 2003

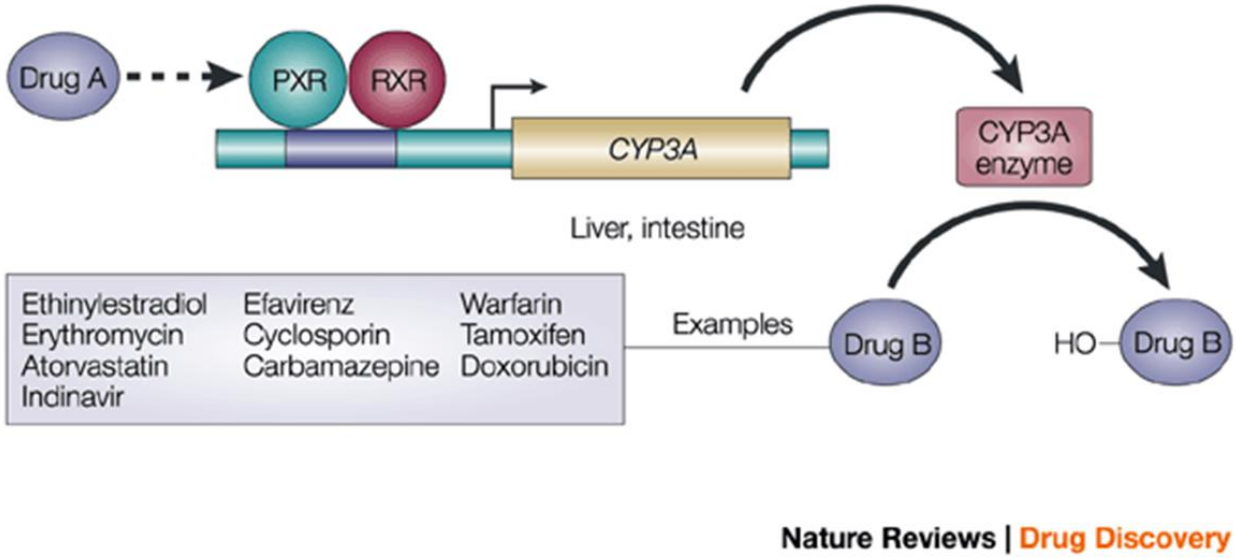
Enzyme induction studies

- Because CYP induction is a metabolic liability in drug therapy, it is highly desirable to develop new drug candidates that are not potent CYP inducer.
- Ideally, the information on whether a new drug candidate is a potent CYP inducer should be obtained at the drug discovery stage before the drug candidate is selected for clinical development.



Molecular mechanisms

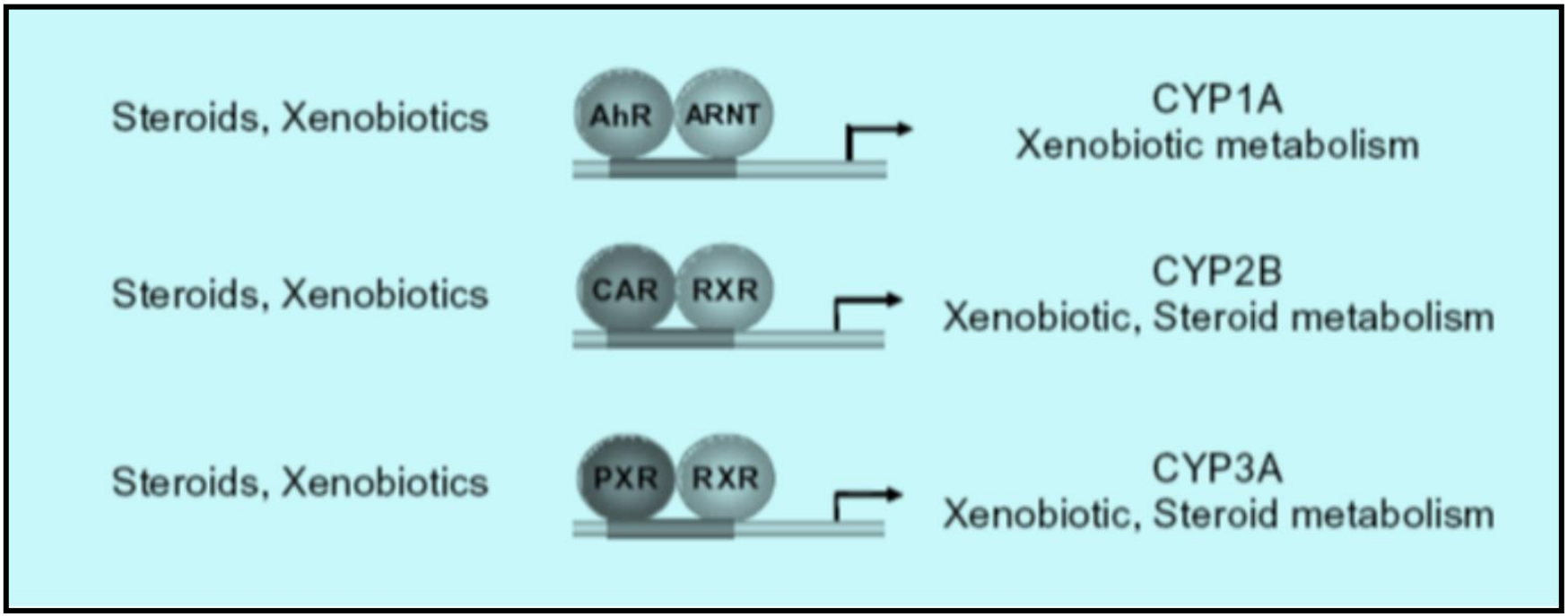
- Most CYP genes are induced through a ligand-activated nuclear receptor:
  - the pregnane X receptor (PXR)
  - the aryl hydrocarbon receptor (AhR)
  - the constitutive androstane receptor (CAR)



T.M. Wilson, S. A. Kliewer 2002:1, 259-266

Molecular mechanisms

- Nuclear receptors:



(Jacobs, 2004)

Molecular mechanisms

- Non-transcriptional mechanisms can be involved in enzyme induction
  - Decrease in the rate of protein degradation without increasing the rate of protein synthesis
  - Stabilization of the enzyme protein (CYP2E)
  - stabilization of mRNA .

Species differences

- Inductive response to inducers is markedly different, both quantitatively and qualitatively, among Human and animal species
  - Omeprazole, a gastric-acid-suppressing drug, is a good CYP1A enzyme inducer in humans, but has little inductive effect in mice or rabbits
  - Rifampicin is a potent inducer for CYP3A enzymes in rabbits and humans, whereas it has little inductive effect on CYP3A enzymes in rats
  - TCDD induced predominately CYP1A1 in rat hepatocytes, whereas TCDD induced mainly CYP1A2 in human hepatocytes
  - Rat CYP3A enzymes are readily induced by PCN, whereas neither rabbit nor human CYP3A enzyme is induced by PCN

Species differences

- Species difference in CYP induction is due to structural difference in nuclear receptors (PXR-CYP3A).

- Although animal models may provide some useful information on the factors that affect CYP induction, it is difficult to use animal models for the assessment of human CYP induction for new drug candidates.

- Therefore, the use of *in vitro* systems is the only means by which the potential of human CYP induction can be assessed.

In vitro models to study enzyme induction

- *In vitro* models to assess the potential of CYP induction
  - PXR reporter gene assays (high-throughput screen at discovery stage, a supplement model and not a replacement model for assessment of CYP3A4 induction)
  - Liver slices
  - Immortalized cell line (limited predictive value)
  - Primary culture of human hepatocytes

In vitro models to study CYP induction

- How to assess the potential of CYP induction?

  - Enzyme induction can be measured by assaying for:
    - Activity of specific isoforms,
    - Immunodetection of isoform protein,
    - Quantification of mRNA.

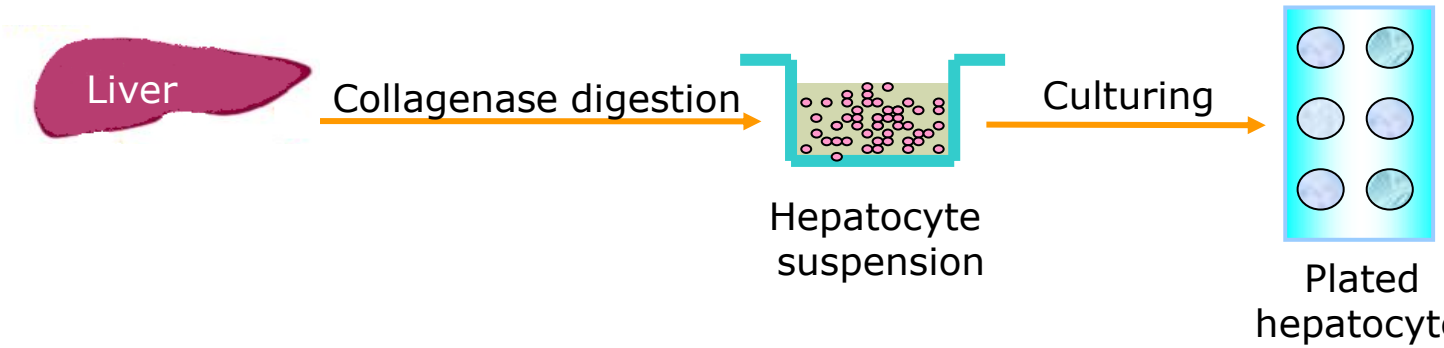
  - The use of known inducers as positive control agents is necessary to verify the sensitivity of these systems:
      - Omeprazole: CYP1A2
      - Phenobarbital: CYP2B6
      - Rifampicin: CYP3A4

Human hepatocyte model

- Primary culture of human hepatocytes to assess the potential of CYP induction
  - The most predictive model for evaluating CYP induction
  - The “gold standard” for *in vitro* testing of CYP induction and drug metabolism
  - But, optimal experimental conditions should be established to have a good prediction
  - Human hepatocytes are recommended by the U.S. FDA and EMA agencies
  - Cryopreserved hepatocytes (in suspension) could not be used.

Preparation of Human hepatocytes

- Human hepatocytes are obtained by collagenase digestion of liver biopsies



- Factors affecting the induction response:

Little effect
- *Extracellular matrix*
- *Medium formulation*

High effect
- Plating density
- Temporal changes in the CYP mRNA, protein level, and enzyme activity
- Solvent used in preparation of drug stock solution

GENERAL PROCEDURE:
In vitro induction study

Liver perfusion

↓

Fresh human hepatocytes in primary culture

Cryopreserved hepatocytes in suspension could not be used

↓

Incubation with reference inducers and test substance

↙ ↓ ↘

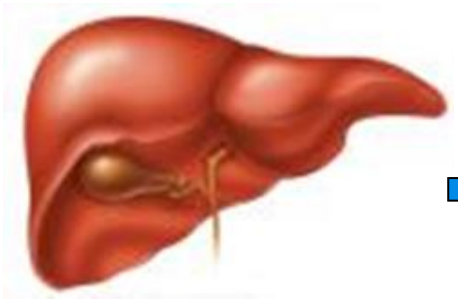
Measurement of CYP expression

Assessment of cytotoxicity

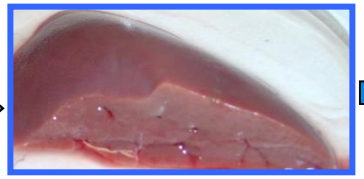
Measurement of CYP enzyme activities

GENERAL PROCEDURE:
Liver perfusion

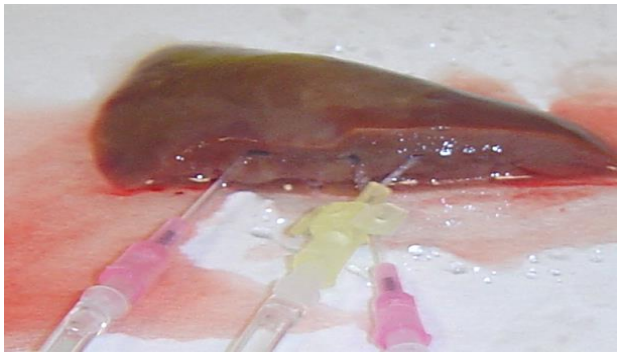
Liver perfusion



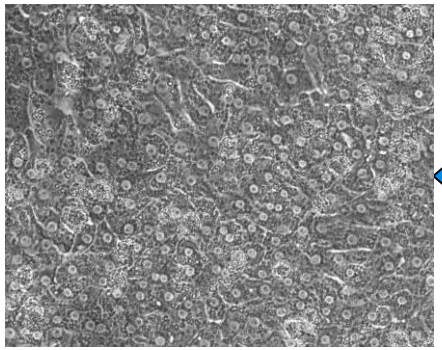
Human liver



Liver biopsy

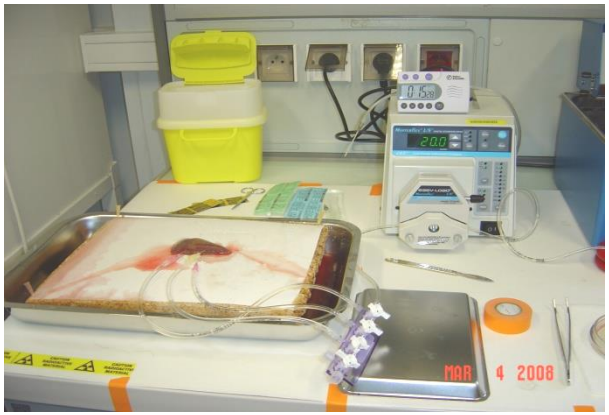


Perfusion with collagenase solution *(Life technologies)*



Hepatocytes in primary culture

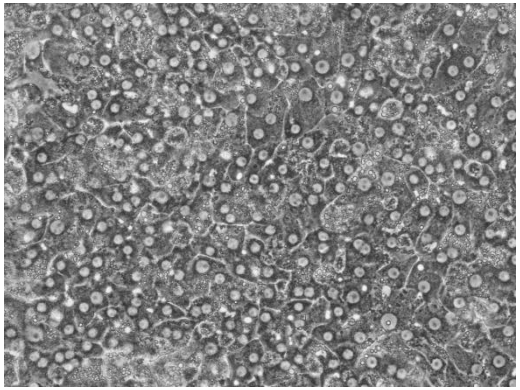
Dissociation of cells



Peristaltic pump

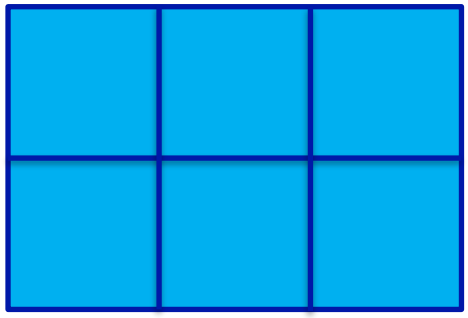
GENERAL PROCEDURE:
Primary culture of hepatocytes

Fresh human hepatocytes in primary culture



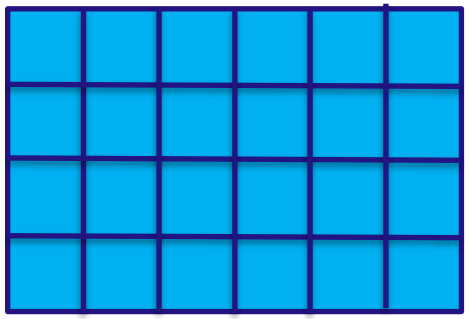
- Preparation from at least 3 donors
- Cell densities: 1 x 10^6 viable cells/mL
- Cell viability: > 80%
- Cell culture support: collagen coated
- Cell culture medium: Hepatozyme-SFM

CYP mRNA



6-well plate
1.8 x 10^6 cells/well
Incubation volume: 1 mL/well

CYP Enzyme activities



24-well plate
0.38 x 10^6 cells/well
Incubation volume: 325 µL/well

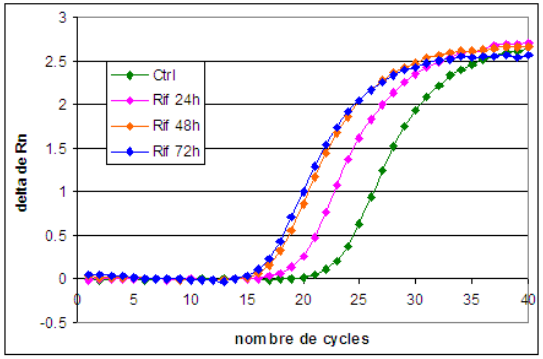
GENERAL PROCEDURE:
Hepatocyte incubation

Incubation with reference inducers and test substance

Omeprazole 50 µM	Phenobarbital 1000 µM	Rifampicin 20 µM	Test substance 0 - 100 µM	Control Vehicle
74 - 96 h	74 -9 6 h	74 - 96h	74 - 96h	74 - 96h
CYP1A2	CYP2B6	CYP3A4	CYPs	CYPs

Incubation duration: 72 or 96 hours.
Culture medium is renewed every 24 hours.
Culture medium is stored at -80°C for cytotoxicity assessment

GENERAL PROCEDURE:
Quantification of CYP mRNA



Quantification of CYP mRNA

Hepatocyte Lysis
350 µL lysis buffer

Promega's lysis buffer

Extraction of total RNA
final volume: 100 µL

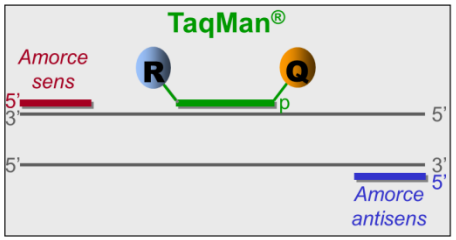
Promega's SV total RNA extraction

Quantification of RNA at 260 nm

Yield: about 100 ng/µL and 10 µg/well

Reverse Transcription reaction
in 100 µL with 500 ng RNA

High capacity cDNA reverse transcription kit, Life technologies



Real-time PCR
TaqMan primers & probes

5 µL RT product

CYP1A2, CYP2B6, CYP3A4

House keeping gene
Analysis performed in triplicate

GAPDH

GENERAL PROCEDURE:
Measurement of enzyme activity

Measurement of CYP enzyme activities

Phenacetin 20 µM	Bupropion 100 µM	Midazolam 5 µM
0 - 24 h	0 - 24 h	0 - 24 h
CYP1A2	CYP2B6	CYP3A4
Phenacetin O-deethylase	Bupropion hydroxylation	Midazolam hydroxylation
Acetaminophen	*hydroxybupropion*	*1' hydroxymidazolam*

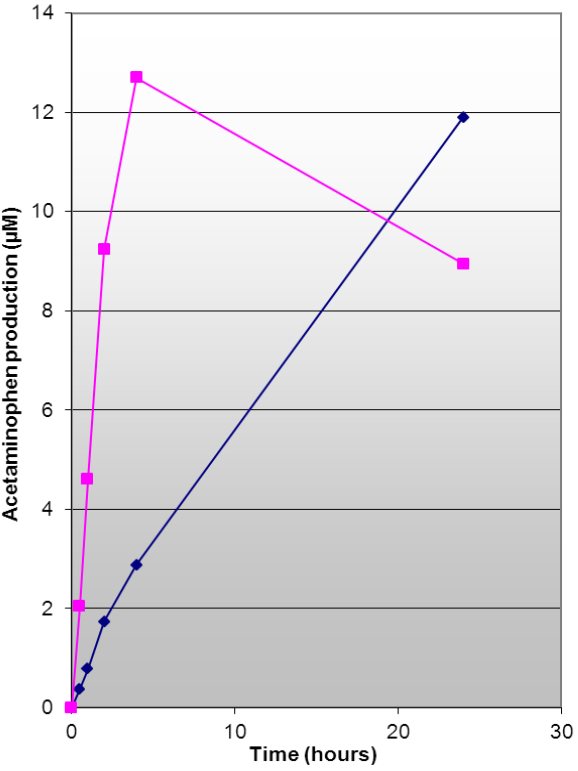
Validated LC/MS-MS methods

Incubation times: 0, 0.5, 1, 2, 4 and 24 hours

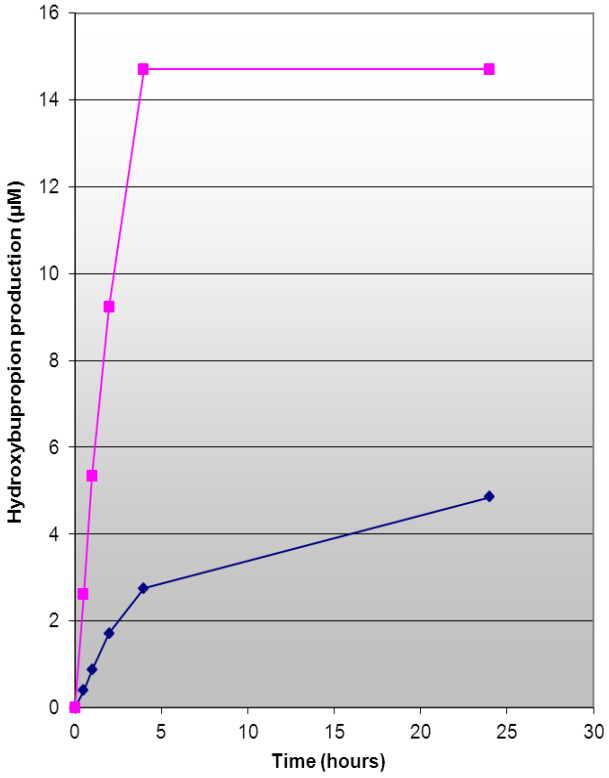
GENERAL PROCEDURE:
Measurement of enzyme activity

Kinetics of formation of specific CYP metabolites
Incubation time with reference substrates should be well validated

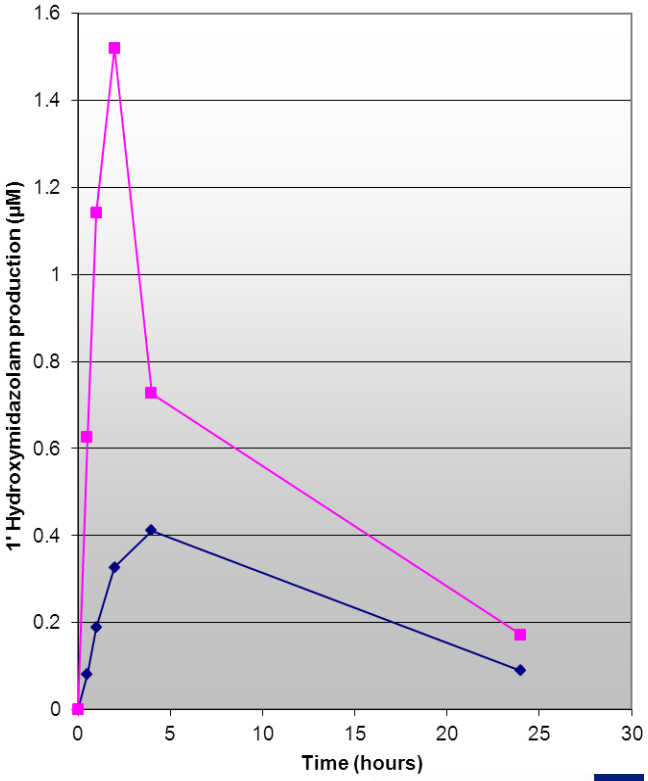
Acetaminophen (CYP1A2)



OH-Bupropion (CYP2B6)



OH-Midazolam (CYP3A4)



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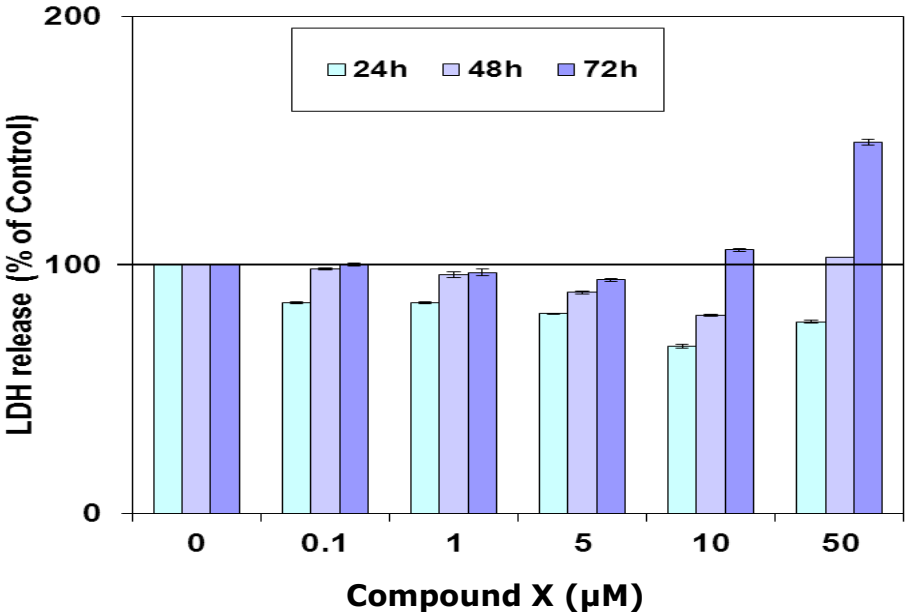
GENERAL PROCEDURE:
Cytotoxicity assessment

Assessment of cytotoxicity

- Cytotoxicity is monitored by measuring the level of LDH (lactate deshydrogenase) released in incubation medium
- Other cytotoxicity assays are available

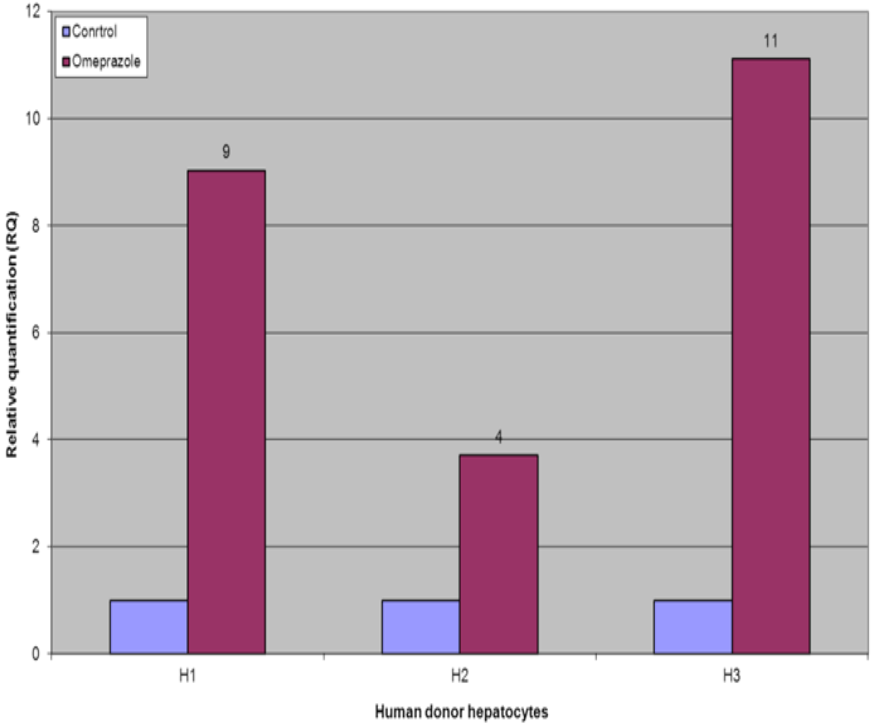


Slight toxicity at 50 µM

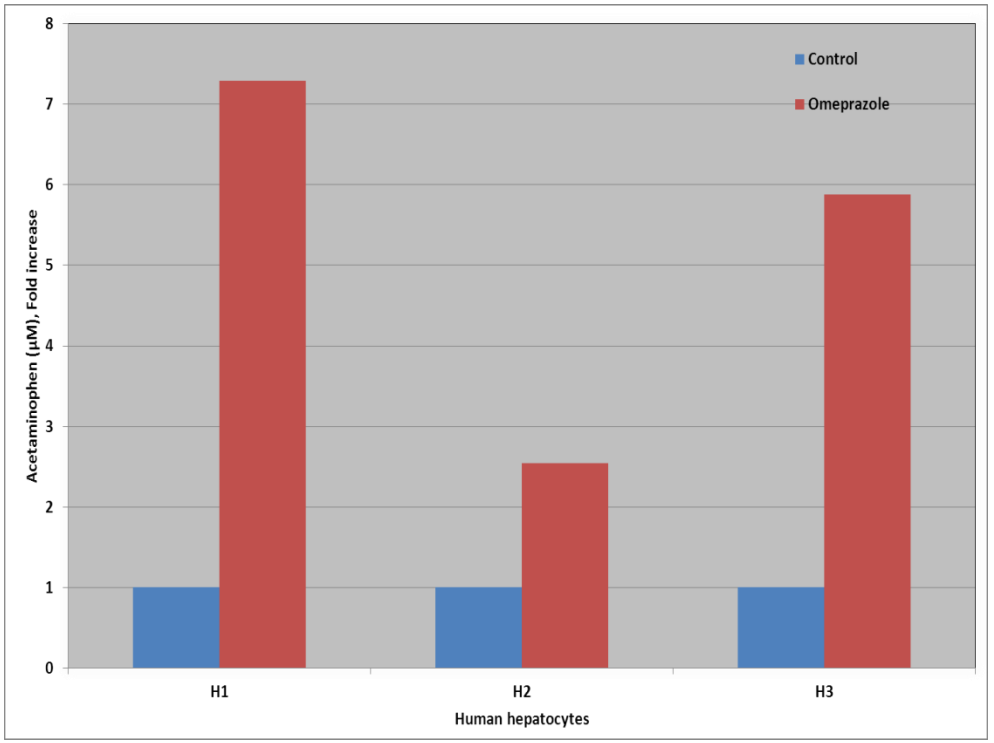
RESULTS: CYP1A2 induction by Omeprazole

Fresh human hepatocytes from 3 donors

CYP1A2 mRNA



CYP1A2 activity

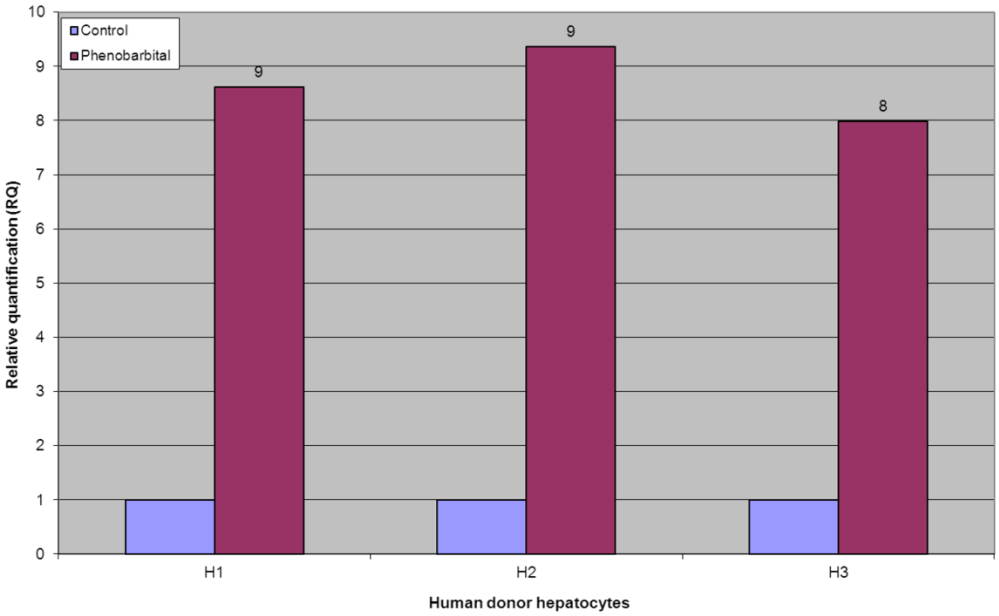


- In human hepatocytes, Omeprazole induces an important increase in CYP1A2 mRNA and enzyme activity.
- Good correlation between mRNA and enzyme activity
- In hepatocytes from human donor 2 (H2), CYP1A2 is less inducible, (steatosis?)

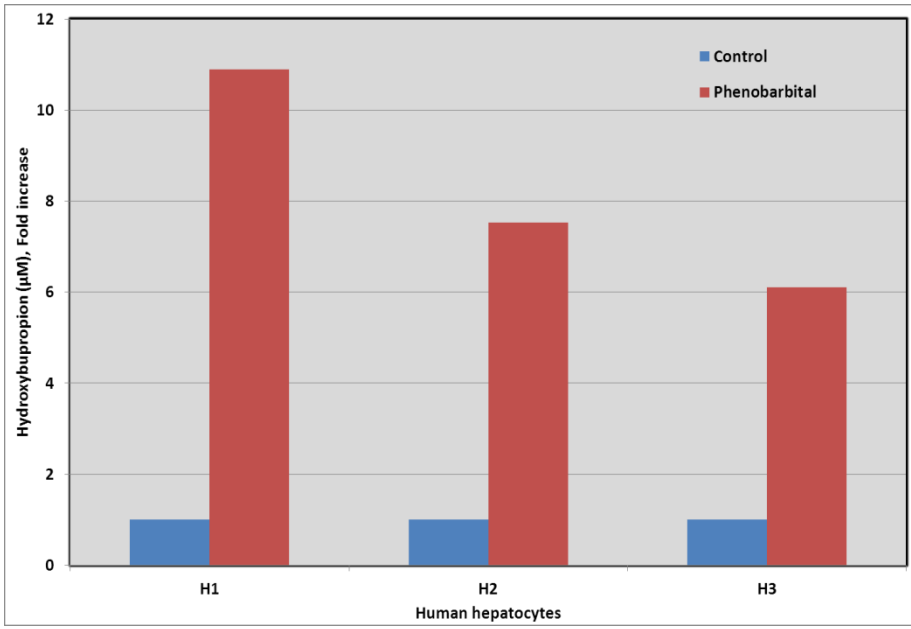
RESULTS: CYP2B6 induction by Phenobarbital

Fresh human hepatocytes from 3 donors

CYP 2B6 mRNA



CYP 2B6 activity

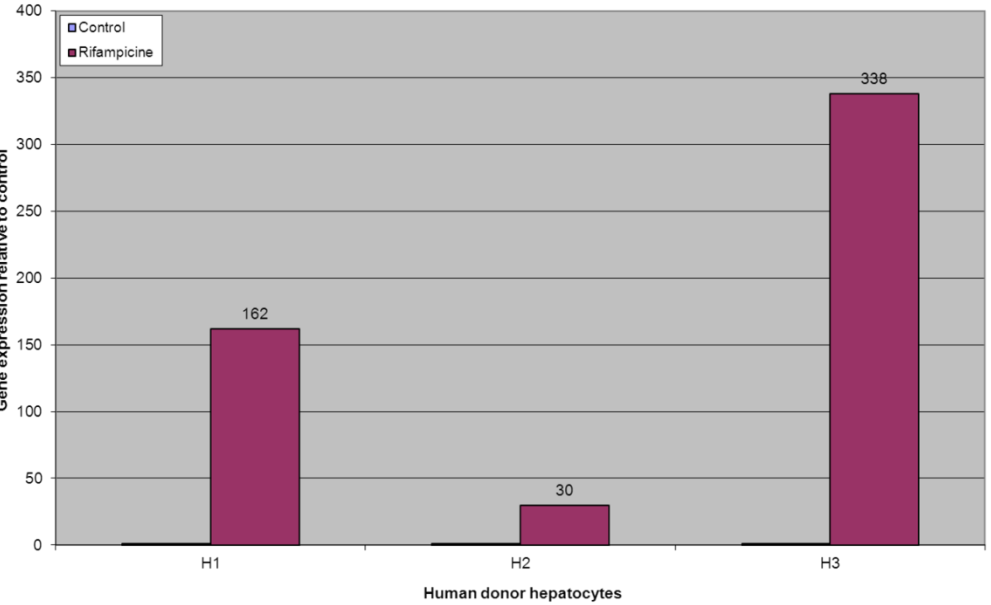


- In human hepatocytes, Phenobarbital induces an important increase in CYP2B6 mRNA and enzyme activity.
- Good correlation between mRNA and enzyme activity
- No inter-individual variability between the three donors

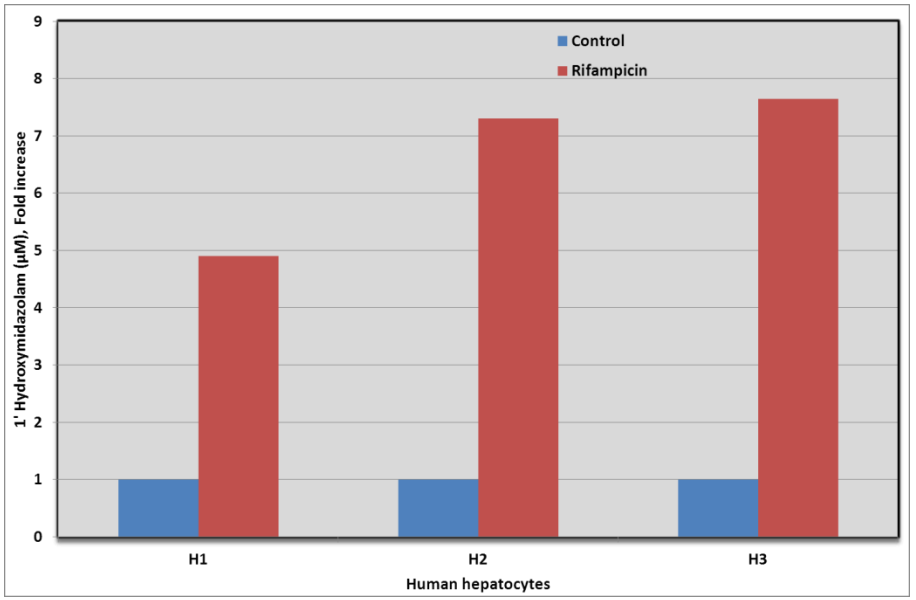
RESULTS: CYP3A4 induction by Rifampicin

Fresh human hepatocytes from 3 donors

CYP 3A4 mRNA



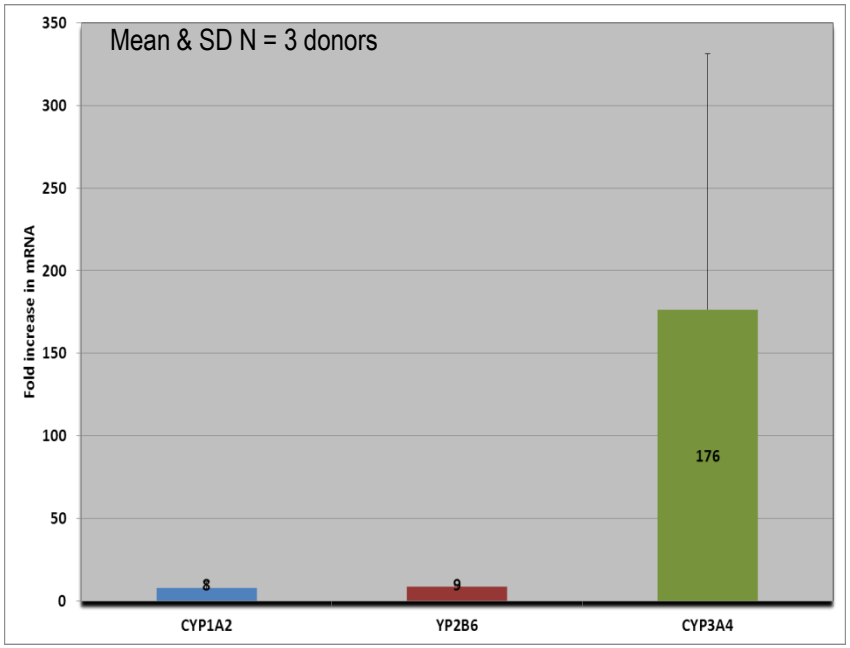
CYP 3A4 activity



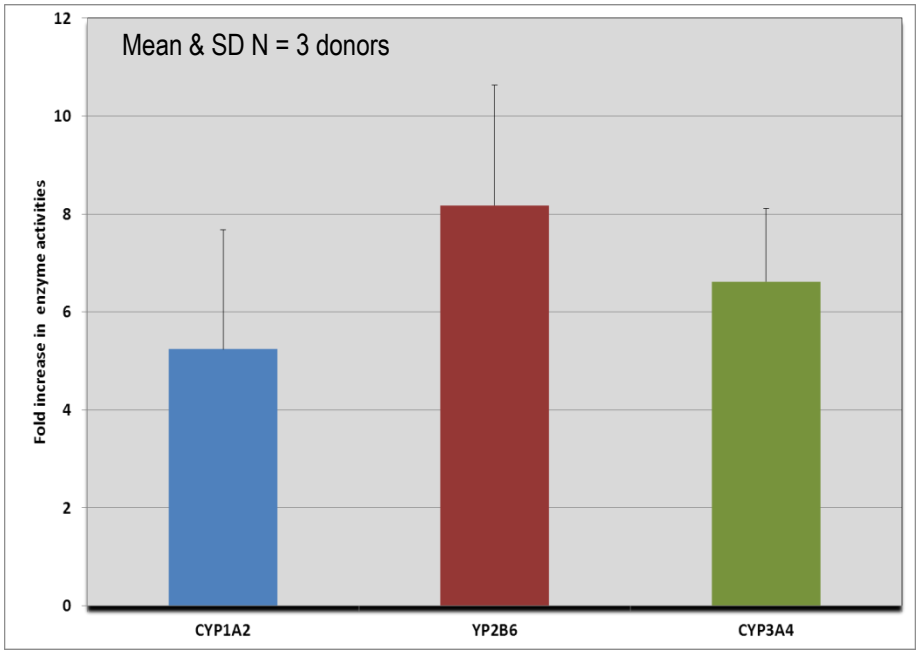
- In human hepatocytes, Rifampicin induces an important increase in CYP3A4 mRNA and enzyme activity.
- Poor correlation between mRNA and enzyme activity
- High inter-individual variability between the three donors, especially for gene expression (30 to 330 folds)

RESULTS: Comparison of induction response between mRNA and activity

mRNA



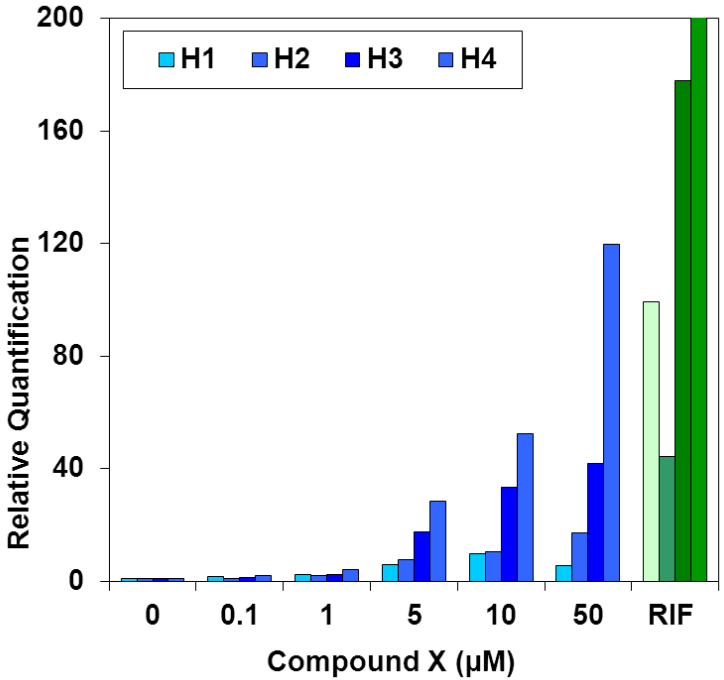
activity



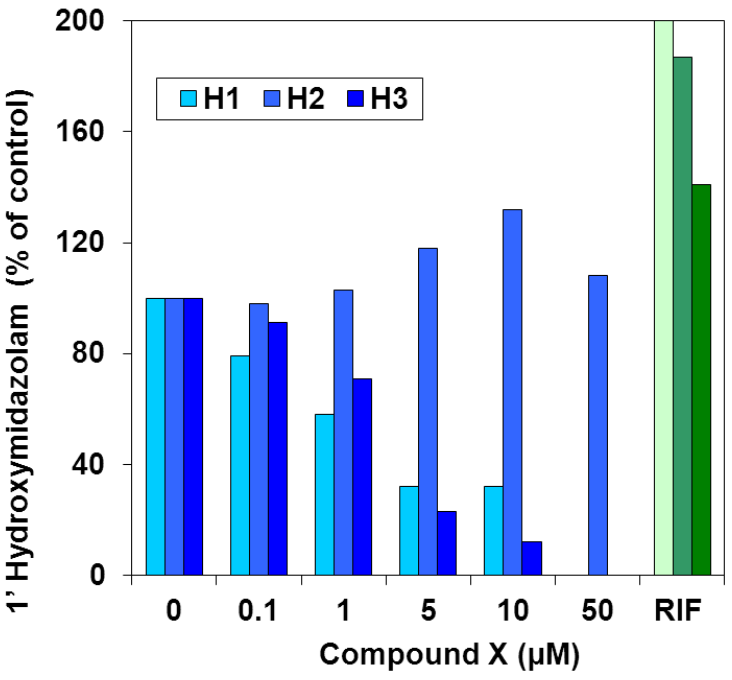
- Following incubation with prototype inducers:
  - CYP1A2, CYP2B6 and CYP3A4: same increase in enzyme activity level (2 – 10)
  - CYP1A2 and CYP2B6: same increase in mRNA & good correlation between mRNA and enzyme activities levels.
  - CYP3A4: Very important increase in RNA level compared to CYP1A2 and CYP2B6 & poor correlation between mRNA and enzyme activities levels.

RESULTS: Effect of compound X on CYP3A4 in human hepatocytes

CYP3A4 mRNA



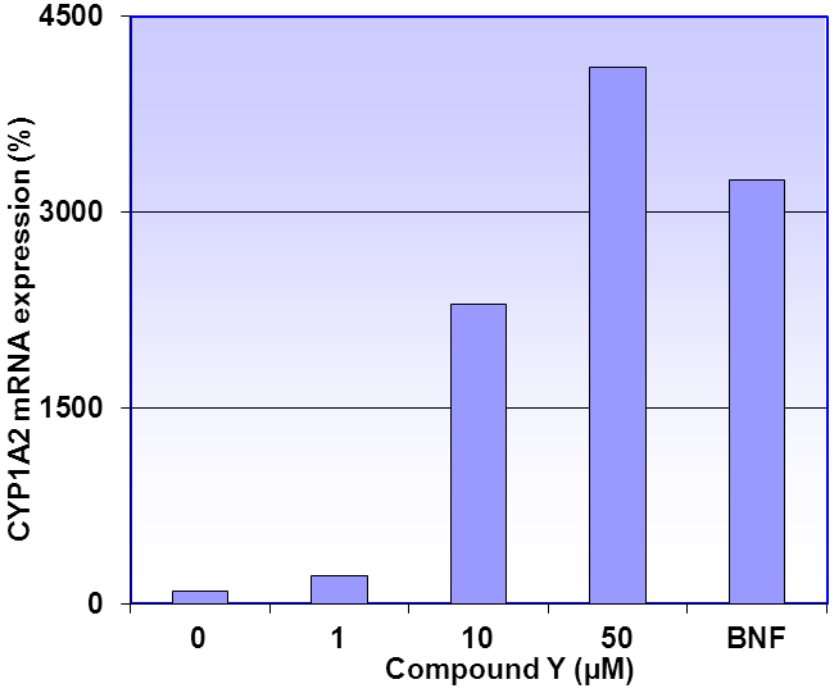
CYP3A4 activity



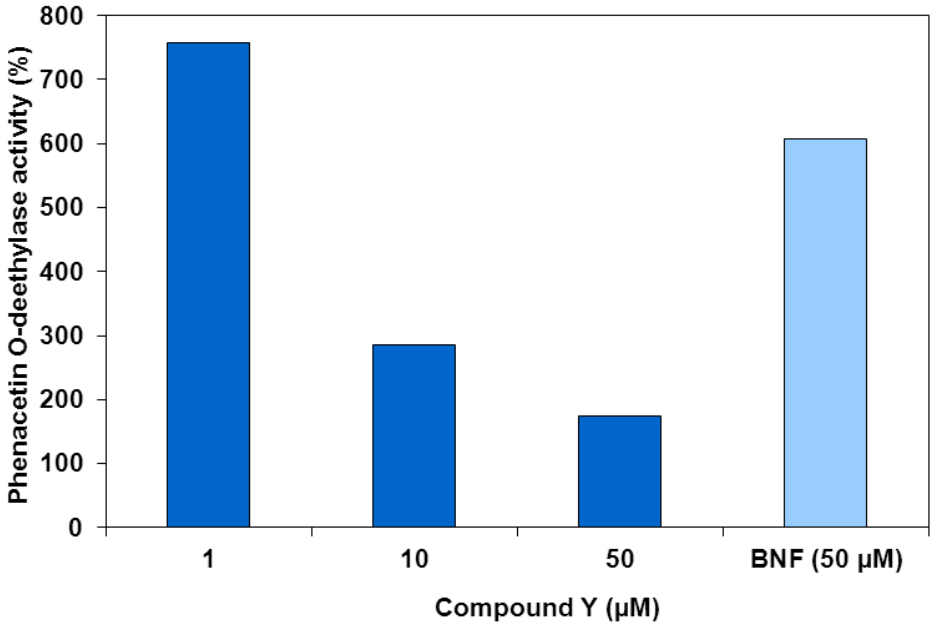
- Concentration-dependent increase in CYP3A4 mRNA, associated with concentration-dependent decrease in CYP3A enzyme activity
- Compound X is both inducer and inhibitor of CYP3A4 in human hepatocytes

RESULTS: Effect of compound Y on CYP1A2 in human hepatocytes

CYP1A2 mRNA



CYP1A2 activity



- Concentration-dependent increase in CYP1A2 mRNA, associated with concentration-dependent decrease in CYP1A2 enzyme activity
- Compound Y is both inducer and inhibitor of CYP1A2 in human hepatocytes

CONCLUSION

- Human hepatocytes are the “gold standard” for the assessment of human CYP induction for new drug candidates.

- Optimal experimental conditions should be established to ensure a good prediction of hepatocyte model.

Many thanks for :

Alexandre Gaborit
Karine Sevin
Magali Kouidhi
Pierre Comby

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GENERAL PROCEDURE: In vitro induction study

Measurement of CYP enzyme activities

Reference Substrates:	Final concentration	Vehicle
Midazolam: CYP3A4:	5 µM	DMSO
Phenacetin: CYP1A2:	20 µM	DMSO
Bupropion: CYP2B6:	100 µM	DMSO

Incubation duration: 0 to 24 hours.

At the end of incubation period with the inducers, hepatocytes are washed and then incubated with the reference substrates.

At the end of incubation period with the reference substrates, culture medium is collected and stored at -20°C.

Production of 1'hydroxymidazolam (CYP3A4), acetaminophen (CYP1A2) and hydroxybupropion (CYP2B6) is analyzed by LC/MS-MS with validated methods.

GENERAL PROCEDURE: In vitro induction study

Incubation with reference inducers and test substance

Reference inducers:	Final concentration	Vehicle
Rifampicin: CYP3A4:	20 µM	DMSO
Omeprazole: CYP1A2:	50 µM	DMSO
Phenobarbital: CYP2B6:	1000 µM	DMSO

Test substance: Several concentrations from 0 to 100 µM depending on the solubility and the Cmax (clinical studies)

Control hepatocytes: Incubation with the vehicle alone (0.5% DMSO)

Incubation duration: 72 or 96 hours. The culture medium is collected and replaced by fresh medium containing test and reference substances.

Collected culture medium is stored at -80°C for cytotoxicity assessment.