EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

Multi-study validation trial for cytochrome P450 induction providing a reliable human metabolically competent standard model or method using the human cryopreserved primary hepatocytes and the human cryopreserved HepaRG® cell line.

Validation project report

Abbreviations

ADME: Absorption, Distribution, Metabolism and Excretion
AoP: Adverse outcome Pathways
BBR: Between Batch Reproducibility
CAR: constitutive androstane receptor
CYP: cytochrome P450
Cryoheps: primary cryopreserved human hepatocytes
CryoHepaRG®: cryopreserved human HepaRG® cell line
DMSO: Dimethyl sulfoxide
EMA: European Medicines Agency
EURL ECVAM: European Union Reference Laboratory for Alternatives to Animal Testing
FDA: Food and Drug Administration
GLP: Good Laboratory Practice
HMM: hepatocyte maintenance medium
HPLC High Performance Liquid Chromatography
ICATM: International Cooperation on Alternative Test Methods
ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods
INST: Internal Standard
JaCVAM: Japanese Centre for the Validation of Alternative Methods
MoA: Mode of Action.
MS: Mass Spectrometry
MW: molecular weight
NICEATM: National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD: Organisation for Economic Co-operation and Development
PC: positive control
PXR: pregnane X receptor
SOP: Standard Operating Procedure
TD: toxicodynamic
TIM: Test Item Management
TK: toxicokinetic
VC: vehicle control
VMG: Validation Management Group
WLR: Within Laboratory Reproducibility

1 Summary

The main objective of this validation project is to assess the transferability, the reproducibility (within and between laboratories) and the predictive capacity of two Cytochrome P450 (CYP) induction *in vitro* methods, each of them evaluating the induction of enzymatic activity of four CYP enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A4).
The two CYP induction *in vitro* methods use two different metabolically competent *in vitro* Test Systems (TS):

(I) the cryopreserved human HepaRG® cells and

(II) the cryopreserved human primary hepatocytes

The predictive capacity is assessed using exclusively human CYP induction *in vivo* reference data.

This is the first project in its kind comparing cryopreserved human HepaRG® cells and cryopreserved human primary hepatocytes in their ability to predict *in vitro* human CYP1A2, CYP2B6, CYP2C9 and CYP3A induction. The project required reliable human *in vivo* data on the induction of the four CYPs for a proper evaluation of the predictive capacity of the *in vitro* results. As *in vivo* human data of sufficient quality for the four CYPs are only available for pharmaceuticals, all the substances (test items) used in this validation project were pharmaceuticals.

This project is a first response to the scientific community request of having reliable and relevant human hepatic *in vitro* metabolically competent test systems and transferable, reproducible and predictive *in vitro* methods to be used in integrated approaches for biotransformation and toxicological Mode of Action studies of substances and mixture/products of various industrial sectors. Therefore, the information/data produced in this validation project will also help to gain more insight into xenobiotic biotransformation and toxicological Mode of Action (MoA).

In this project data are being generated on the CYP1A2, CYP2B6, CYP2C9 and CYP3A4 Phase I biotransformation enzymatic activities with and without treatment with test items. The project used substances that induce the CYPs *in vivo* in humans but also used test items that are non-inducers.

The successful outcome of this project, coordinated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), responds to the EU

existing and future regulatory requirements (REACH Regulation (EC, 2006), Cosmetics Regulation EC 1223/2009 (EC, 2009), Animal Welfare Directive 2010/63/EU (EC, 2010), OECD (OECD, 2012)) and the scientific community expectations calling for toxicological testing methods delivering key information to complement hazard and risk assessments of substances within integrated approaches based on reliable and relevant non-animal methods.

Since the human *in vitro* CYP validation study is the first project in its kind the VMG could not set specific targets apriori for each of the modules. The VMG evaluated the obtained information and as such draw ex-post conclusion based on the data generated.

Having reviewed the information generated during the course of the validation project, the Validation Management Group (VMG) concluded **that the findings satisfy fully for the human cryoHepaRG CYP induction *in vitro* method and partially (results are batch dependent) for the human cryoHep CYP induction *in vitro* method the validation modules 1-4** (test definition, within laboratory reproducibility, transferability, between laboratory reproducibility) and contributes to assessment of module 5 (predictive capacity) of the EURL ECVAM modular approach to validation.

The VMG concluded that the CYP induction method relies on a complex experimental setup and thus requires a skilled and analytically well-resourced biochemical and cell biological laboratory. Frequent occurrences of irregularities in concentration response curves and uncertainties in their interpretations suggested that there are a number of critical points to be taken into consideration in the design and execution of the experiments, such as the selection of concentration range and delineation of solubility limit and potential cytotoxicity range of an unknown compound.

The above mentioned prerequisites fulfilled, the VMG concluded that the information generated in the study shows that the human *in vitro* CYP induction method is robust, reliable and relevant. Therefore, the VMG supports the use of the human *in vitro* CYP induction method in a weight-of-evidence approach to support regulatory decision making.

The VMG also considers the CYP induction method deserves further evaluation as part of an integrated testing strategy for the role it might play in the determination of xenobiotic exposure and potency predictions and its role in alternatives for systemic toxicity hazard identification. The CYP induction *in vitro* method can be considered as a candidate regulatory *in vitro* test method to gain insight in the toxicological MoA of substances in the context of the new safety assessment paradigm using exclusively *in vitro* approaches based on human cells and tissues in combination with the appropriate *in silico* approaches and overall systems biology knowledge.

2 Background

Following absorption, a xenobiotic is usually transformed to one or more metabolites by human body enzymes. This process, referred to as biotransformation, affects the transport and partitioning of a xenobiotic and/or its metabolites into and within the body, its toxicity and its rate and route of elimination (Coecke et al, 2006). Liver is the major site of biotransformation. In this organ, biotransformation is divided into two main phases: Phase I and Phase II. Phase I is usually oxidative (e.g. hydroxylation, hydrolysis) and has a major protective function in rendering lipophilic molecules more polar and more readily excretable. In Phase II, often referred to as detoxification, such oxidised moieties are subsequently conjugated with highly polar molecules (e.g. glucuronic acid), before they are excreted. Cellular disposition also involves also uptake transporters and efflux transporters, which are currently under intensive investigation.
CYPs are Phase I enzymes and have a high prevalence in biotransformation (oxidative metabolism) of both endogenous and exogenous xenobiotics. Amongst exogenous compounds, industrial chemicals, cosmetic ingredients, pesticides and food additives have been cited in scientific literature (Parkinson et al, 2010). CYPs are in high quantity found in liver cells but are also located in other cells throughout the body. Within cells, the CYP enzymes in the endoplasmic reticulum are the principal catalysts of exogenous compound metabolism, but CYP enzymes are found also in mitochondria and other subcellular organelles (Knockaert et al, 2011).

Exposure to xenobiotics can lead to the induction or the inhibition of biotransformation enzymes including CYPs. Due to the relatively broad substrate specificity of CYPs, many metabolic routes of elimination can be inhibited or induced by concomitant xenobiotic administration/exposure (i.e. mixtures, chemical-chemical, drug-drug).

Induction is defined as an increase in the amount and activity of a metabolising enzyme due to ***de novo*** **CYP protein synthesis or stabilisation of CYP enzymes**. It is a **longer-term** consequence of a xenobiotic exposure and as result the overall specific CYP catalytic activity increases.

Inhibition can be an **acute decrease** of metabolism of a particular substrate by another simultaneously present xenobiotic or a reactive metabolite that binds to the CYP or to the heme of the CYP (Pelkonen et al, 2008). Both CYP induction and inhibition may lead to a significant variation in the concentration of the xenobiotic and its metabolites at the target site, enhance clearance or toxic accumulation of the parent compound (or its metabolites) or produce toxic metabolites. CYP inhibition may cause toxic effects by increasing the concentration of the toxic parent chemical at the target site, while CYP

induction may lead to increased rates of metabolism and clearance or to the increased production of toxic metabolites. Metabolism of xenobiotics can also cause a time dependent inhibition because reactive metabolites formed may bind covalently to the enzyme or metabolites bind tightly but not covalently to the enzyme (Obach et al, 2007). Time dependent inhibition can thus confound induction results and may also be an indicator of reactive metabolites.

Due to the different underlying mechanism, different *in vitro* methods have been used to evaluate CYP induction and inhibition. The most widely used *in vitro* method to study inhibition is to measure the affinity of a xenobiotic for CYPs in CYP selective substrate assays in human liver microsomes or in recombinant enzyme-based systems.

To evaluate human CYP induction, *in vitro* continuous or repeated challenge with the test item (i.e. xenobiotic) is necessary and an *in vitro* human metabolically competent test system relatively stable for 2-3 days is needed since much of the induction involves increased gene transcription and subsequent elevated protein formation, which takes 2-3 days in time.

Industrial sectors, such as the chemical and pharmaceutical ones, are interested in understanding the kinetic interactions and the potential alteration of the metabolism of co-administrated compounds (e.g. mixtures) as part of a toxicokinetic evaluation. Recently, the European Food Safety Authority (EFSA) published draft guidance on metabolism and toxicokinetic studies for some food additives such as complex mixtures and botanical preparations (EFSA, 2011).

While since 1997the European Medicines Agency (EMA) and Food and Drug Administration (FDA) Guidelines (EMA, 2012; FDA, 2012) require CYP induction assessment for new pharmaceuticals, metabolism and CYP induction for safety assessment of a broad spectrum of chemical compounds (e.g. chemicals, cosmetics, food additives and pesticides) is currently not systematically addressed by standardised test methods. In the pharmaceutical sector, enzyme induction is often investigated by administering a probe drug with a metabolic pathway that is specific for the enzyme of interest. The probe drug is administered before and after repeated dosing of the investigated drug, and the metabolism of the probe drug is examined.

No OECD Guidelines exist for evaluating *in vitro* human CYP induction. According to the OECD TG 417 “Guidelines for the Testing of Chemicals: Toxicokinetics” (OECD, 2010), TK should be evaluated *in vivo* using the rat as a test system.

However, it is of critical importance using a human relevant *in vitro* test system to predict CYP induction *in vivo*. CYP induction by xenobiotics is a complex process

including receptor activation, stabilization of transcripts and proteins that eventually will lead to an increased capacity of the cell to metabolize xenobiotics including many pharmaceuticals and environmental toxicants.

The importance to use a human relevant system is based on the fact that **the AhR, PXR and CAR found in toxicological animal models such as mouse and rat, exhibit significant differences in specificity why rodent based models would not be predictive for the human situation.** There are several examples where a compound has strong effect on the human receptor and no effect on animal receptors or vice versa. In humans a prototypical CAR agonist CITCO (imidazo[2,1-b]thiazole-5-carbalde-hyde O-(3,4-dichlorobenzyl)oxime) has no effect on the rodent CAR receptor while TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene) a prototypical inducer of the rodent CAR receptor has no effect on the human receptor. Several examples can be given for the PXR receptor that is inducing the most important xenobiotic metabolizing enzyme in humans, CYP3A. Rifampicin the prototypical inducer of the human PXR, used in present evaluation, has no effect on rodent PXR. Other substances such as artemisinin, clotrimazole, nicardepin and nifidipin are all activators of the human PXR but have no effect on the rodent PXR (Summarized in Chapter 6, Casarett and Doull´s Toxicology, The Basic Science of Poisons, Eight Edition, Edited by C. Klaassen, 2013). The potency of compounds to activate receptors can also vary drastically between species. Dexamethasone and pregnenolone 16α-carbonitrileare are strong PXR activators and/or inducers of CYP3A in rodents but not in human (Martignoni et al, 2006). Differences of induction among species are explained by discrepancies in the ligand-binding domain of the receptors implying that their ligand specificities may differ dramatically between species. Therefore, extrapolation of animal data with respect to the inducibility of CYP enzymes in human is not reliable.

In 1999, a EURL ECVAM task force report stressed the importance of validating metabolically competent test systems using CYP induction as a sensitive indicator to check their metabolic machinery (Coecke et al, 1999). In 2009 two *in vitro* methods for in vitro CYP induction using human metabolically competent systems were proposed to EURL ECVAM for evaluation. While **human primary hepatocytes** have been for a long time the gold standard test system for human CYP induction studies in the pharmaceutical sector, in 2008, Kanebratt and Andersson evaluated **human-derived HepaRG cells** as an *in vitro* model to predict CYP induction of drugs in humans by exposing the cells to prototypical inducers. Based on their results, HepaRG cells respond to PXR, CAR and AhR activators and are a promising human *in vitro* test system for investigating enzyme induction (Kanebratt et al, 2008b). A comparison of CYP3A4 induction in HepaRG cells and primary human hepatocytes has been published in 2009 (McGinnity et al, 2009).

The underlying biological mechanism (Tompkins et al, 2007) of CYP induction (xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene) is a very good indicator for the assessment of the functionality of the molecular machinery of any metabolically competent hepatic system proposed for regulatory uses. CYP induction *per se*, following the nuclear receptor-xenobiotic interaction, is suggested as an important biological event in several AoPs (Pelkonen et al, 2008; USEPA, 2011; Vinken et al, 2013).

The **Karolinska cocktail** was developed to investigate different CYP activities *in vivo* in humans (Kanebratt et al, 2008c). Similarly, a cocktail approach was developed to determine in the same experiment the induction of different important human CYP enzymes on *in vitro* human hepatic test systems (Kanebratt et al, 2008b). In this validation project, this *in vitro* methodology was used to determine the potential of selected test items to induce CYP1A2, CYP2B6, CYP2C9, and CYP3A4. The specific biotransformation CYP products were measured with an analytical method (LC/MS-MS), using four CYP selective probes (phenacetin, midazolam, diclofenac and bupropion).

The selection of three of these CYP isoforms was based primarily on their widespread use as target CYPs for classical model inducers: CYP1A2 for dioxins and PAHs, CYP2B6 for phenobarbital and CYP3A4 for rifampicin. The fourth isoform, CYP2C9 is less responsive to induction, but is of considerable importance in metabolising xenobiotics. Close to 17 % of pharmaceuticals are metabolised by CYP2C9 (Zanger et al, 2008). In primary human hepatocytes CYP2C9 has shown to respond with a large variability to well-known inducers from non-responder to significant induction (Yayima et al. 2014). The test items were tested at a wide range of concentrations in order to cover **human clinically (*in vivo*) relevant concentrations** of CYP inducers for comparison with the available human reference data.

The human CYP *in vitro* method addresses CYP induction but **does not provide information on CYP inhibition** since for inhibition other *in vitro* test systems are more appropriate (e.g. CYP affinity assays in human liver microsomes). The CYP induction *in vitro* method using human test systems provides information on cellular events (e.g. xenobiotic-nuclear receptor binding and its pleiotropic consequences) and it is conceivable that this *in vitro* method **is useful for a wide variety of xenobiotics**, **independent of their use class** (i.e. not only for new pharmaceutical ingredients but also for other compounds such as cosmetic ingredients, industrial chemicals, food additives, pesticides and mixtures). However, xenobiotics that form reactive metabolites or metabolites tightly bound to active site may confound CYP induction results, since xenobiotics that are time or mechanisms dependent inhibitors will remain inhibitors in the CYP induction experiment.

Following the evaluation of the information provided on the two human *in vitro* CYP induction methods and after reviewing the existing Standard Operating Procedures (SOPs), EURL ECVAM deemed that the availability of reliable and relevant human CYP induction methods is of importance and responds to current and future regulatory requirement of different industrial sectors. Therefore, this validation projects aims to issue an OECD performance-based test guideline on *in vitro* human CYP induction methods and is currently listed as a project on the OECD work programme.

After the decision that the SOPs were sufficiently developed and standardised, the EURL ECVAM initiated the multi-study validation ring trial.

3 Management of the project

Reference documents:

- *List of additional available documents filed for the study and available on request (Appendix 01)*
- *Project Plan (Appendix 02)*

3.1 Project objectives

3.1.1 Primary objective

The main objective of this validation project is to assess the transferability, the reproducibility (within and between laboratories) and the predictive capacity of two Cytochrome P450 (CYP) induction *in vitro* methods, each of them evaluating the induction of enzymatic activity of four CYP isoforms (CYP1A2, CYP2B6, CYP2C9 and CYP3A4). The two CYP induction *in vitro* methods use two different metabolically competent *in vitro* Test Systems (TS):

(I) the cryopreserved human HepaRG® cells and

(II) the cryopreserved human primary hepatocytes

The predictive capacity is assessed using exclusively human CYP induction *in vivo* reference data.

Therefore, owing to the nature of the measured effect, relevance was not assessed through analysis of predictions of adverse effects but by evaluating to which extent *in vitro* human CYP induction profiles reflected those derived from human clinical *in vivo* reference data.

3.1.2 Secondary objective

The information / data produced from this validation project will also help to gain insight into xenobiotic biotransformation and toxicological Mode of Action (MoA).

In this project data are being generated on the CYP1A2, CYP2B6, CYP2C9 and CYP3A4 enzymatic activities with and without treatment with inducers allowing gaining inside in xenobiotic biotransformation of the four CYP isoforms under investigation in the two human cryopreserved test systems used.

The CYP induction *in vitro* method can be considered as a candidate *in vitro* test method to gain insight in the toxicological MoA of substances in the context of the new safety assessment paradigm using exclusively *in vitro* approaches based on human cells and tissues in combination with the appropriate *in silico* approaches and overall system biology knowledge.

In this context the two human *in vitro* CYP induction methods will also contribute to knowledge gathering on:

(1) **CYP induction as a key event in a toxicity pathway in its own right**: CYP induction in itself can lead to adverse effects by affecting biotransformation of endogenous (=non-xenobiotics) substances and thus disturbing normal intermediary metabolism and physiological homeostasis. (Hodgson and Rose, 2007). Nuclear receptor-controlled processes do not only involve CYP induction, but wider pleiotropic phenomena, i.e. tens or even hundreds of genes responding and consequences spreading concerning many physiological functions.

(2) **The human *in vitro* CYP induction method as a novel *in vitro* platform gaining knowledge of toxicological MoA of other non-inducing xenobiotics contained in mixtures.** With modifications concerning analytical tools and sampling schedules, it is possible to enlarge the scope of the test system to encompass the metabolic details of a potential inducer itself and enable the first screen of potential biotransformation of xenobiotics in mixtures and their possible chemical-chemical/drug-drug interactions (e.g. inhibition, induction, etc; Zahno et al, 2011).

(3) **CYP induction as an alert and human biomarker for exposure to a chemical insults**.

The CYP test method can be used to assess a potential inducing capacity of substances irrespective of their use class, i.e. including pharmaceutical ingredients, pesticides, cosmetic ingredients, ingredients of household products etc. Since the CYP induction method is based on xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene, any class of compounds that can interact with such receptors is predicted to be qualified to be used in the two in vitro test subject of this validation. The projects provides an essential piece of information for future integrated approaches based on a suite of *in vitro* methods and other information sources providing predictions on absence or presence of a specific adverse effect (Bernus et al, 1994; Ward et al, 2003).

It should be noted that the human CYP induction *in vitro* method does not provide information about the number or nature of possible (reactive/non-reactive) metabolites neither about human hepatic clearance/stability of the substances investigated. To

cover this aspect, *in vitro* hepatic clearance/stability and metabolite identification and reactivity assessments would be needed. The two *in vitro* test systems used in the human *in vitro* CYP induction method might be used for some of these other applications. For instance for measuring human hepatic clearance both cryopreserved HepaRG cells and pooled cryopreserved human hepatocytes have been reported as test systems for this application (Houston et al, 2012; Zanelli et al, 2012).

As an example of potential applications, the human *in vitro* CYP induction method may also help in defining the AoPs related to cancer. Known carcinogens, pro-carcinogens, and chemotherapeutics have CYPs involved in their metabolic pathways (Baird et al, 2005; Shimada, 2006; Guengerich, 2011; Rodriguez et al, 2006; Ma and Lu 2007; Maronpot et al 2010).

3.2 Project plan

Prior to the start of the project, a *Project Plan* was approved and issued by the Validation Management Group. The document is annexed to this report (see **Appendix 01**). The Project Plan documents the objectives, coordination and sponsorship of the project; the nature and roles of the study director and personnel at each testing site; the minimum quality assurance systems required in the case of non-GLP laboratories.
Prior to start the project, the Project plan was sent to all laboratories for their information.

3.2.1 Structure of the validation project

This validation project was organised to generate information relevant to module 1-4 (1: test definition, 2: within-laboratory reproducibility, 3: transferability, 4: between laboratory reproducibility) of the EURL ECVAM modular approach to validation (OECD, 2005; Hartung et al, 2004). The experimental data generated during the project also contributes to predictive capacity (Module 5) following comparison with available *in vivo* human data.

Due to the specific objective of this project – to assess the transferability, the reproducibility (within and between laboratories) and the predictive capacity of two Cytochrome P450 (CYP) induction *in vitro* methods, each of them evaluating the induction of enzymatic activity of four CYP isoforms (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) – the evaluation was performed on a selected set of test items having sufficient evidence in terms of human *in vivo* reference data. The main selection criterion was the ability of the test item to induce one or more of the selected four CYPs in humans *in vivo*. Furthermore, it was essential to find *in vivo* human data of sufficient quality for the

four CYPs. In addition, also available *in vitro* data on inducibility of the four CYPs were gathered and evaluated, when available. **This basic prerequisite of available *in vivo* data was the reason why the test items are all pharmaceuticals**. However, the *in vitro* method could be useful for a wide variety of xenobiotics other than pharmaceuticals, independently of their use class (e.g. cosmetic ingredients, chemicals, food additives and pesticides) based on the underlying molecular mechanism of CYP induction (xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene) .

Each human *in vitro* CYP induction method was conducted according to the same agreed-upon SOP in the different laboratories.

The ICATM (International Cooperation on Alternative Test Methods) member, EURL ECVAM entirely coordinated and sponsored the study with participation from other ICATM members such as NICEATM (National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods) and ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) and JaCVAM (Japanese Center for the Validation of Alternative Methods) via the VMG with regard to the project design, chemical selection and test method SOPs.

Figure 01 illustrates how the validation project was organised with respect to the management, the test methods, the participating laboratories, the selection, coding and distribution of the test items and the data collection and the statistical analysis.

This validation project report includes a summary and the background on the project, the presentation of the results generated during the experimental work carried out in the course of the validation project and the conclusions and recommendations by the VMG. Conclusions are mainly based on the data generated in this project, but they also take into account the available human *in vivo* and *in vitro* data on the induction of the four CYP enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A) by the selected test items.

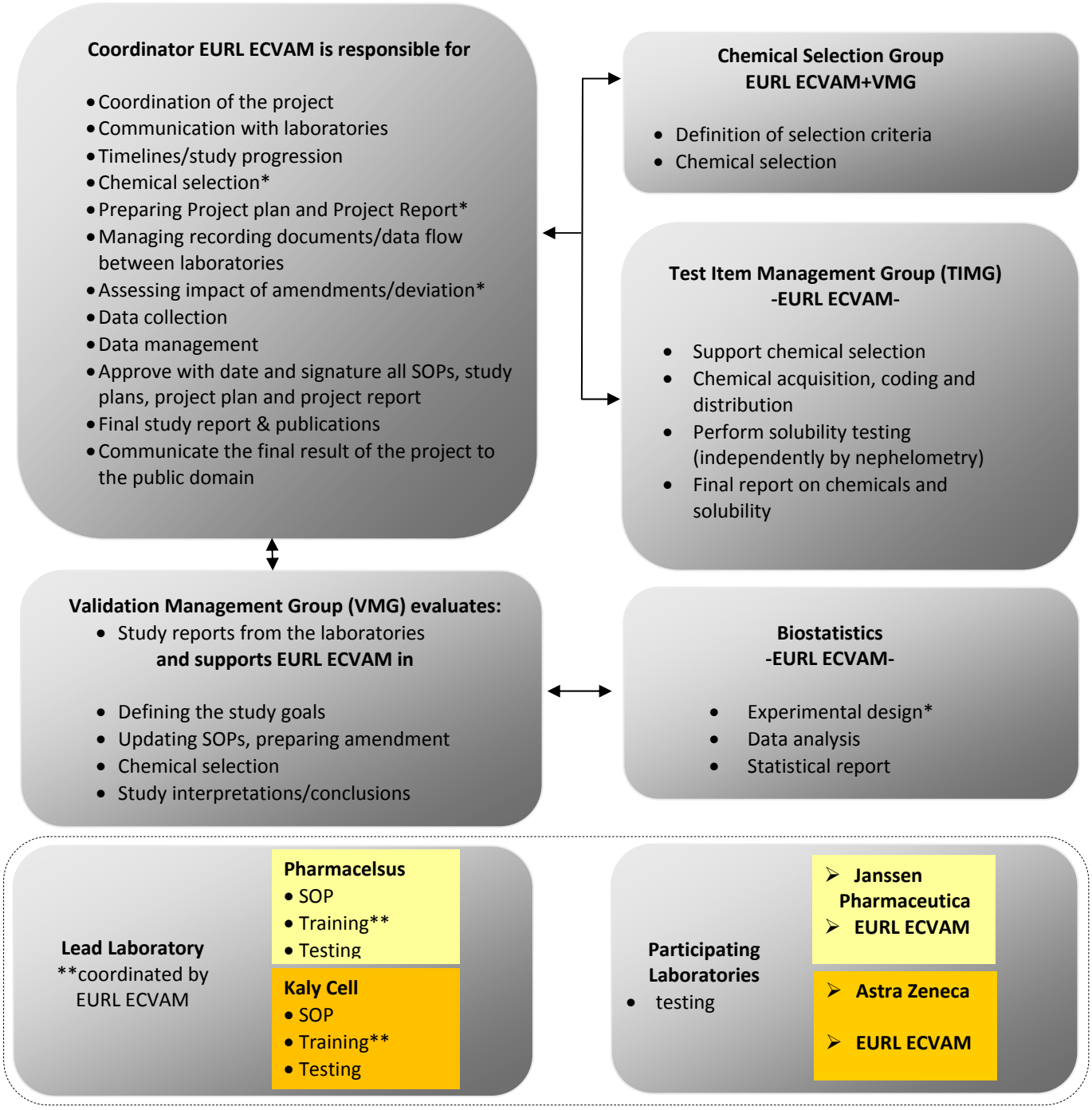


Figure 01 schematic representation of the study structure and organisation.

Kaly Cell was the lead laboratory for human cryopreserved primary hepatocytes and transferred the competences on this test system to Astra Zeneca and EURL ECVAM. Pharmacelsus GmbH was the lead laboratory submitting the CYP induction SOP on the cryopreserved human HepaRG® cell line and transferred the competences on this protocol to Janssen Pharmaceutica and EURL ECVAM.

Validation Management Group

Following the principles for the *in vitro* method validation (Hartung et al, 2004; OECD, 2005) a Validation Management Group (VMG) was established by EURL ECVAM. The role of VMG, a group of independent experts, is to overview the validation process, to evaluate the results, to provide comments at each critical stage, to make subsequent decisions during the progress of the project and to draw conclusions regarding the outcome of the project with respect to the project goals.

Representatives of other international validation organisations, ICCVAM and NICEATM (USA) and JaCVAM (Japan) are members of VMG.

A subgroup of VMG members (Chemical Selection Group) was responsible for the strategic decisions regarding the selection of the test items to be used in the project. The lead laboratories' representatives were not involved in discussions related to the selection of test items.

Validation Management Group members (alphabetical order)
EURL ECVAM members:
Sandra Coecke (validation project coordinator and meeting chair)
Camilla Bernasconi (day to day manager of the validation project since August 2012)
Tom Cole (test item acquisition, solubility testing, coding and distribution)
Andre Kleensang* (biostatistician till September 2010)
Ingrid Langezaal (day to day manager of the validation project until July 2012)

Roman Liska* (biostatistician since the 1st of October 2010)

External members:

Tommy B. Andersson	AstraZeneca R&D, Mölndal, Sweden
Sonja Beken	FAGG, Brussels, Belgium
Warren Casey	NICEATM/NIEHS, North Carolina, USA
Michael Cunningham	NIEHS, North Carolina, USA
Karen De Smet	FAGG, Brussels, Belgium
Magnus Ingelman-Sundberg	Karolinska Institutet, Stockholm, Sweden
Armin Kern	Bayer, Germany
Michael Paris	ILS/NICEATM/NIEHS, North Carolina, USA
Olavi Pelkonen	University of Oulu, Oulu, Finland
Erwin Roggen	Novozymes AS, Bagsvaerd, Denmark
Judy Strickland	ILS/NICEATM/NIEHS, North Carolina, USA
Momoko Sunouchi	National Institute of Health Sciences, Tokyo, Japan
Tamara Vanhaecke	Vrije Universiteit, Brussel, Belgium

The statistical analysis of the *in vitro* data was the responsibility of the independent biostatistician (*). The biostatistician was independent from the test method submitters and all the laboratories involved in the ring trial.

EURL ECVAM coordinated the whole validation project, selected the participating laboratories and was in charge of the management of the validation studies. It assisted the lead laboratories in improving the SOPs in terms of completeness, clarity, robustness and test definition and test description. It facilitated the harmonisation and standardization of the *in vitro* method to facilitate its translation into internationally recognised test guidelines and to ensure their acceptance for regulatory use. EURL ECVAM participated itself in the ring trail generating data in accordance with GLP principles.

3.2.2 Laboratories

Different laboratories listed below participated in the validation project. They were selected by EURL ECVAM on their competence and on the availability to participate into the ring trial on a **pro-bono** base.

IBET was initially a partner for the HepaRG® CYP induction *in vitro* method but subsequently replaced by Janssen Pharmaceutica. Pharmacelsus GmbH, IBET and EURL ECVAM were partners in an FP6 EU project (Vitrocellomics), aimed to establish and validate embryonic stem cell derived hepatocytes and to validate these cells for hepatotoxicity or other endpoints. However, due to difficulties in obtaining functionally active cells in sufficient amounts for supplying all partners, the consortium brought in the HepaRG® cell line. EURL ECVAM, Pharmacelsus and IBET started working on HepaRG® but, later on (May 2009), IBET had no financial resources to further participate in the project and Janssen Pharmaceutica entered the project being involved in all the formal validation Modules of the project.

UCB Pharma and Sanofi were initially partners in the cryopreserved hepatocyte CYP induction *in vitro* method. In 2010, both companies reported financial problems in continuing in the validation project. Following Kaly Cell's proposal, the VMG approved Astra Zeneca as the third laboratory working, on a pro-bono base, with human cryoheps throughout the validation project.

Due to their extensive experience with the *in vitro* method under validation, Pharmacelsus GmbH and KalyCell acted as scientific lead laboratories for the cryopreserved HepaRG® and the human cryopreserved primary hepatocyte test system, respectively.

As lead laboratories, they submitted the HepaRG® and cryoheps SOPs, respectively and were responsible for drafting the new updated versions of the SOPs and forms,

following EURL ECVAM and the VMG comments. They provided the training on the SOPs and transferred the necessary competences to the participating laboratories. They performed the complete Module 2 (with-in laboratory reproducibility) and participated in the ring trial of Module 4 (between-laboratory reproducibility).

For the two test methods, the only 100% naïve laboratory was EURL ECVAM, experimentally involved in both test methods (cryoheps and cryoHepaRG®), as the other laboratories performed similar methods on regular basis.

3.2.2.1 Laboratories working on human cryopreserved HepaRG® test system

Laboratory 1 Lead Laboratory:
Pharmacelsus GmbH (GLP)
Science Park 2
DE-66123 Saarbruecken (Germany)
Study director: Ursula Mueller-Vieira

Laboratory 2:
Janssen Pharmaceutica a division of Johnson and Johnson (GLP)
Turnhoutseweg 30
B-2340 Beerse (Belgium)
Study director: Jos Van Houdt

Laboratory 3:
EURL ECVAM (European Commission, the European Union Reference Laboratory for Alternatives to Animal Testing)
Via E. Fermi, 2749
I-21027 Ispra (Italy)
Study director: Iwona Wilk-Zasadna and Siegfried Morath*.

3.2.2.2 Laboratories working on human cryopreserved primary hepatocytes

Laboratory 1 Lead Laboratory:
Kaly Cell (non-GLP)
20, rue du Général Leclerc
F-67115 Plobsheim (France)
Study director: Lysiane Richert

Laboratory 2:

Astra Zeneca (non-GLP)
SE-431 83 Mölndal (Sweden)
Study director: Anna Lena Ungell. From the 25th of February 2013 on Helena Sjöberg.

Laboratory 3:

EURL ECVAM (European Commission, the European Union Reference Laboratory for Alternatives to Animal Testing)
Via E. Fermi, 2749
I-21027 Ispra (Italy)
Study director: Iwona Wilk-Zasadna and Siegfried Morath*.

*in September 2012 the responsibility of Study Director was transferred to Siegfried Morath. Siegfried Morath acted as Study Director for the LC-MS analysis of the induction experimental work of Module 4a (4 coded test items) and 4b (9 coded test items) for cryoheps and cryoHepaRG®.

3.2.3 Quality System of the participating laboratories

The validation project should be ideally carried out in accordance to GLP.
Pharmacelsus GmbH and Janssen Pharmaceutica are OECD Good Laboratory Practice (GLP) compliant and subject to inspections by relevant regulatory agencies; however the project was not conducted under full GLP compliance at these laboratories due to financial and human resource issues declared by both companies.
EURL ECVAM was in the process of requesting the OECD GLP compliance status during the conduct of the project. In May 2012 EURL ECVAM became an OECD compliant GLP test facility for the ***validation of vitro methods***. Therefore, EURL ECVAM could once its OECD GLP compliance status was confirmed conduct solubility and cytotoxicity parts of Module 4b with cryoHepaRG® under GLP. Since the LC-MS-MS equipment used by EURL ECVAM was hosted in an ISO 17025 accredited JRC facility all the other EURL ECVAM studies could not fully be compliant with GLP but were listed on the EURL ECVAM Test Facility Master Schedule and were carried out similarly as a GLP compliant study, but due to the equipment location could not be considered as a full GLP-compliant study.
Astra Zeneca is not GLP-compliant but follows a system termed GLS (general laboratory standard) which is internally audited by the research quality management group. This standard sets the minimum laboratory quality requirements for all R&D. It ensures that procedures and results are accurate, reliable, traceable, and reproducible and, where appropriate, comply with the appropriate regulatory authorities' legislation. For the non-GLP laboratories participating in the validation project, the VMG defined and requested the application of a minimum set of quality assurance requirements

considered essential for the acceptance of information and data produced in the validation process.

The minimum requirements were:

- Qualified personnel, and appropriate facilities, equipment and materials.
- Records of qualifications, training and experience, and a job description for each professional and technical individual, are available.
- For each study, an individual (Study Director) with appropriate qualifications, training and experience shall be appointed to be responsible for its overall conduct and for the report issued.
- Instruments used for the generation of experimental data shall be inspected regularly, cleaned, maintained and calibrated according to the established SOPs, if available, or to the manufacturers' instructions. Records of these processes shall be kept, and made available for inspection on request.
- All data generated during the project shall be recorded directly, promptly and legibly by the individual(s) responsible. These entries shall be attributable and dated.
- All changes to data shall be identified with the date and the identity of the individual responsible and a reason for the change shall be documented and explained at the time.
- Quality Assurance should be performed in accordance with the principles of GLP (for GLP compliant laboratories).
- After completion of the each study, study plans, study reports, raw data and supporting material should be archived.

3.3 Experimental design

3.3.1 Sample size

The VMG agreed that for both test systems' controls and internal standards should be **harmonised** as much as possible.

Based on the data generated at the lead laboratories and described in the paper of Kanebratt (Kanebratt and Andersson, 2008), VMG agreed to use omeprazole (a CYP1A2 inducer, selective for activation of AhR) and troglitazone (a CYP3A4 inducer, binding both constitutive androstane receptor (CAR) and pregnane X receptor (PXR)) as test items for Module 1 and the following Modules 2 and 3 (within laboratory reproducibility and transferability).

For blind coded testing, (Module 4 "between laboratory reproducibility"), **13 test items** were selected by VMG, in particular by the Chemical Selection Group, based on availability of human *in vivo* data of sufficient quality for the four CYPs.

On the basis of the above considerations, the following experimental design was approved by VMG:

- **Within Laboratory Reproducibility (WLR)**: for evaluation of the WLR (the aim of the WLR was to show the within-batch, within-laboratory and between-batch), in a first part of the validation project information was gathered on initially 2 chemicals (omeprazole and troglitazone) using the following experimental design:

  Module 2 for cryoHepaRG:

  I. **within-batch** reproducibility was tested by:
  - One batch cryoHepaRG (HPR116036)
  - Three consecutive assays in independent experiments
  - Two compounds (omeprazole, troglitazone)
  - Compounds not blinded, test concentrations given
  - First operator
  - Only in the lead laboratory

  II. **between-batch** and **within-laboratory** reproducibility was evaluated by:
  - Three batches cryoHepaRG (HPR116036, HPR116035, HPR116020)
  - Two compounds (omeprazole, troglitazone)
  - Compounds not blinded, test concentrations given
  - First operator
  - Second operator performs additional assay on the second and third batch

  As described in study plan for Module 2 for cryoheps:

  I. **within-batch** reproducibility was tested by:
  - One batch Cryoheps: HHC170407
  - Three consecutive assays in independent experiments
  - Two compounds (omeprazole, troglitazone)
  - Compounds not blinded, test concentrations given
  - First operator
  - Only in the lead laboratory

  II. **between-batch** and **within-laboratory** reproducibility was evaluated by:

- Three batches Cryoheps (HHC170407, B270808, S270407)
- Two compounds (omeprazole, troglitazone)
- Compounds not blinded, test concentrations given
- First operator performs assay on the second and third batch
- Second operator performs assay on each of the three batches

In a second part of the project additional data were generated on 12 compounds for cryoheps and on 10 compounds for cryoHepaRG®.

Test item	**cryoHepaRG** µg/ml	**Cryoheps** µg/ml
Omeprazole	40	40
Carbamazepine	40	40
Phenytoin sodium	30[1]	40[12]
Penicillin G sodium	40	40
Indole carbinol	Excluded for solubilty issues	
Rifabutin	cytotoxic	20
Sulfinpyrazone	40	40
Bosentan hydrate	40	10
Artemisinin	40	40
Efavirenz	cytotoxic	2.5
Rifampicin	40	40
Metoprolol	40	40
Sotalol hydrochloride	40	40

Data on the WLR were gathered when also the BLR were gathered.

- **Between Laboratory Reproducibility (BLR):** for evaluation of the BLR, **13 chemicals** were tested once (in triplicates) in every laboratory on 3 different cell batches for solubility, subsequently followed by cytotoxicity and then used for the induction assay those that were soluble and non-cytotoxic.

It was deemed that this experimental design would provide the information needed to perform a sufficiently robust assessment of the WLR and the BLR for the two test systems.

[1] The solvent to be used was a 1:1 blend DMSO:water

3.3.2 Project Modules

The project was structured and conducted in two sequential steps:

- Training of the participating laboratories, *in vitro* method transfer to the trained laboratories and verification of the SOP (Module 2 and 3).
- Assessment of the *in vitro* method performance by testing 13 test items, under blind conditions, in all the laboratories (Module 4).

Since all 13 test items would be tested in triplicate in each laboratory and on three different batches of Cryoheps and of cryoHepaRG®, VMG agreed to split Module 4 - between laboratories reproducibility - into two parts:

- **Module 4a**: the first four coded test items were tested in triplicate at each laboratory;
- **Module 4b** the remaining nine coded test items were tested in triplicate at each laboratory.

All the laboratories were requested to submit a study report at the end of module 4a, with the results being evaluated by VMG before giving the laboratories advice and the green light to proceed to Module 4b. This experimental design provided an additional review and control point, before the initiation of module 4b, in order to verify that no serious issues were arising before the bulk of the testing was performed.

VMG agreed to split Module 4 (4a and 4b) into three experimental steps:

1. **Solubility**: to assess the highest soluble test item concentration to be used in the cytotoxicity experimental part. The independent Test Item Management (TIM) group - at EURL ECVAM- ran solubility in parallel by means of nephelometer analysis and not visual inspection as stated in the SOPs,
2. **Cytotoxicity**: to assess the highest non-cytotoxic test item concentration to be used as a starting concentration in the induction step,
3. **Induction**: to assess the potential of test items to induce one or more of the four selected CYPs.

By applying the cocktail approach, which simultaneously measures the potential for induction of four selected CYPs isoforms (CYP1A2, CYP2B6, CYP2C9 and CYP3A4), **four data sets are generated for each test item**. Indeed the *in vitro* method informs on the potential of each test item to induce the specific CYP isoform/isoforms and provides data on how all four CYP isoforms are influenced by the test item.

3.4 Selection of test items (chemicals)

Reference documents:

- *Face-to-face CYP induction planning meeting before initiating the between-laboratory reproducibility work, 16-17 September 2010 (**Appendix 03**)*

The 13 blinded test items were selected by the Chemical Selection Group (CSG). Tommy Andersson, Thomas Cole, Michael Cunningham, Armin Kern, Ingrid Langezaal and Olavi Pelkonen were appointed by VMG and EURL ECVAM as member of the CSG (***Appendix 03***).

VMG agreed to **use the same set of test items for both test systems** (cryoheps and cryoHepaRG®).

The main **selection criterion** for the chemical selection **was the availability of** robust **human *in vivo* data** for a proper comparative evaluation of the *in vitro* results. VMG agreed that human *in vivo* data are essential to assess the reliability of the *in vitro* method and only xenobiotics for which human *in vivo* data exist could be selected.
This is the reason why **all the test items belong to the pharmaceutical sector**.

The CSG agreed that the test item should:

- be a confirmed inducer of CYP1A2 and/or CYP2B6, and/or CYP2C9 and/or CYP3A4 *in vivo* as demonstrated with key references or
- be a confirmed non-inducer of CYP as demonstrated with key references and not inhibit other CYPs
- be commercially available,
- be soluble in saline, DMSO or acetonitrile
- be stable after fresh preparation of a stock solution
- at least one test item should trigger CYP induction following the binding to one of the main nuclear receptors (PXR, CAR or AhR)

VMG agreed that all laboratories should use the **same solvent** and the **same test item starting concentration** and that the highest soluble and non-cytotoxic concentration of test item should be used to avoid missing the induction response.

Initially, the (commercial) database of the University of Washington and reviews of Pelkonen (Pelkonen et al, 2008) and Hukkanen (Hukkanen et al, 2012) were the main source of data. Additionally, a large number of original and review articles were referred to in compiling the data in tables 1-4.

The thirteen coded test items are listed in Table 01 with basic information on the use and physicochemical properties.

Table 02 reports human *in vivo* data on the potential of the selected test items for CYP induction as well as their possible autoinduction (i.e. inducing their own metabolism) and interactions with nuclear receptors. Information in this table constitutes the principal background and framework against which the *in vitro* studies performed during this validation process are compared.

A literature review was conducted to investigate the CYP isoforms involved in the metabolism of the test items. Data collected are summarized in Table 03. These data are directly applicable for the interpretation of the *in vitro* validation experiments, because the metabolism of the inducer itself by the enzymes it induces affects any long-term experiments in cells in culture. Consequently, knowledge of metabolism of the inducer itself should be part of the upfront package of the characterisation of compounds that are being tested for potential induction.

The literature review was also conducted to compile the relevant pharmacokinetic properties of the test items and the data are summarized in Table 04. The *in vivo* pharmacokinetic characteristics of the test items are naturally very important factors for their induction properties and constitute the essential basis for attempts in quantitative *in vitro – in vivo* extrapolation, but they are less important for the actual experimental *in vitro* work during the validation process.

Table 01: list of 13 test items used for coded testing. Properties and pharmacological application, molecular weight (MW), physical properties and water solubility, and recommended daily dosage are provided, based on data collected from literature, from the Drugs@FDA database, the DrugBank database (http://www.drugbank.ca), from Drugs.com (http://www.drugs.com), the Oregon State University database (http://lpi.oregonstate.edu/infocenter/phytochemicals) and the Finnish Drug Formulary (Pharmaca Fennica, 2013).

Test item	Use and Mode of Action	CAS #	MW g/mole	physical properties	solubility in water	Therapeutic dose mg
Omeprazole	proton pump inhibitor, acid reducer for treatment of active duodenal ulcer	73590-58-6	345.42	white to off-white powder	slightly	20-40
Carbamazepine	anticonvulsant and specific analgesic for trigeminal neuralgia	298-46-4	236.27	white to off-white powder	no	200
Phenytoin	anticonvulsant, antiepileptic drug	630-93-3	274.3	White crystalline	yes	300
Penicillin G	Narrow spectrum antibiotic for gram positive aerobic organisms (e.g. Streptococcus)	69-57-8	356.4	crystalline	yes	12-18 million units (depending on the type of infection)
Indole-3-carbinol	Anti-cancer	700-06-1	147.2	solid (beige)	Very slightly	200-400
Efavirenz	potent non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV-1 patients	154598-52-4	315.68	solid (white)	no	600
Sulfinpyrazone	uricosuric drug used to reduce the serum urate levels in gout therapy	57-96-5	404.5	white crystalline powder	slightly	200-400
Bosentan	endothelin receptor	157212-55-0	569.6	white to yellowish	Poorly in water and in	62.5-125

	antagonist used for treatment of pulmonary arterial hypertension			powder	aqueous solutions at low pH (0.1 mg/100 ml at pH 1.1 and 4.0; 0.2 mg/100 ml at pH 5.0). Solubility increases at higher pH values (43 mg/100 ml at pH 7.5).	
Artemisinin	antimalarial agent	63968-64-9	282.3	solid (white)	no	500-1000
Rifabutin	Broad spectrum antibiotic antimycobacterial by inhibition of DNA inhibition of DNA-dependent RNA polymerase in gram-positive and some gram-negative bacteria	72559-06-9	847.02	red-violet powder	slightly	300
Rifampicin	inhibits DNA-dependent RNA polymerase activity. It is a very broad spectrum antibiotic against most gram-positive and gram-negative organisms (including Pseudomonas aeruginosa) and specifically Mycobacterium tuberculosis	13292-46-1	822.9	red-orange powder	Very slightly	450-600
Metoprolol	cardioselective β1-adrenergic blocking agent used for acute myocardial infarction	51348-51-1	267.4	white crystalline powder	yes	23.75
Sotalol HCl	non-selective competitive β-adrenergic receptor blocker used as antiarrhythmic	959-24-0	308.8	white, crystalline solid	yes	160-600

Table 02 *in vivo* human data on the potentiality of the 13 selected test items to induce CYP1A2, CYP2B6, CYP2C9, CYP3A4. Possible autoinduction and the receptor involved in the specific mode of action of each test items are reported (Zhou et al, 2009; Preissner et al, 2010).

Test item	CYP isoform/probe reaction induced -human *in vivo* data- (reference)	Remarks	autoinduction	Receptor(s) involved
Omeprazole	**1A1:** Ethoxyresorufin O-deethylation and CYP1A1 mRNA in endoscopy biopsy samples in gut after treatment with omeprazole (McDonnell et al, 1992)	1A1 induction in gut epithelium may affect bioavailability of its substrates (Ma and Lu, 2007)	not observed	AhR
	1A2: caffeine 3-N-demethylation breath test (Rost, 1994) **1A2:** several specific activities in liver biopsies from omeprazole-treated patients before and after treatment (Diaz et al, 1990)	CYP1A2 induction in vivo dependent on CYP2C19 phenotype and omeprazole dose (Ma and Lu, 2007)	not observed	AhR PXR (mechanism of action uncertain; probably indirectly via upregulation of AhR)
	Warfarin (CYP2C9) and quinidine (CYP3A4 PK was not changed by Omeprazole (Andersson et al, 2001)	Omeprazole has not been shown to induce CYP2C9 and CYP3A4; no studies on CYP2B6 were found		
Carbamazepine	**1A2** caffeine breath test (Parker et al, 1998; Oscarson et al, 2006; Lucas et al, 1998) **2B6** efavirenz clearance (Ji et al, 2008); bupropion clearance and hydroxylation (Ketter et al, 1995) **2C9** warfarin 10-hydroxylation; clearance (Lai et al, 1992, Herman et al, 2006)	Inducers enhance carbamazepine clearance by CYP3A4	strong autoinduction within a week (Magnusson et al 2008)	CAR/PXR indirectly

	3A4 antipyrine kinetics, 6b-hydroxycortisol excretion (Moreland et al, 1982; Andreasen, 2012); ethinylestradiol and norgestrel kinetics (Crawford et al, 1990)			
Phenytoin sodium	**1A2** theophylline clearance (Wietholtz et al, 1989; Miller et al, 1990) **2B6** cyclophosphamide metabolite ratio (Slattery et al, 1996; Williams et al, 1999) **2C9** autoinduction (Miners and Birkett, 1998) **3A4** ethinylestradiol and norgestrel kinetics (Crawford et al, 1990); cortisol kinetics (Werk et al, 1964)	Inducers enhance phenytoin clearance by CYP2C9 (Miners and Birkett, 1998)	strong autoinduction within one-two weeks (Dickinson et al, 1985; Chetty et al 1998)	CAR/PXR indirectly
Penicillin G sodium	**Non-inducer**	renal clearance and partial hydrolysis	not observed	
Indole-3-carbinol	**1A2** caffeine metabolite ratio (Reed et al, 2005; Pantuck et al, 1979)(weak, Cui et al, 2002)		not known	AhR
Efavirenz	**2B6** bupropion hydroxylation (Robertson et al 2008); efavirenz 8-hydroxylation (Ngaimisi et al, 2010) **3A4** erythromycin breath test (Mouly et al, 2002); midazolam metabolic ratio (Fellay et al, 2005)	no effect on CYP3A4 in intestinal biopsy samples (Mouly et al 2002)	autoinduction on chronic therapy (Ngaimisi et al 2010)	CAR/PXR
Sulfinpyrazone	**1A2** theophylline clearance/metabolites (Birkett et al, 1983) **3A4** verapamil clearance ; (Wing et al, 1985) Walter et al, 1982; Staiger et al, 1983	No in vivo studies on CYP2B6, CYP2C9 found	No direct evidence available	PXR indirectly

Bosentan hydrate	**2C9** warfarin clearance (Weber et al, 1999b) (van Giersbergen et al, 2002b) **3A4** glyburide clearance (van Giersbergen et al, 2002b) (Weber et al, 1999c; Dingemanse et al, 2003)	No in vivo studies on CYP1A2 and CYP2B6 found	autoinduction within one week	PXR
Artemisinin	**2B6** S-mephenytoin N-demethylation (Simonsson et al, 2003; Elsherbiny et al, 2008) **2C19** S-mephenytoin 4-hydroxylation (Simonsson et al, 2003; Elsherbiny et al, 2008; Asimus et al,2007) **3A4** midazolam metabolite/parent ratio indicate induction (Asimus et al, 2007). No induction of CYP3A4 as reported by the omeprazole sulfone formation and cortisol metabolic ratio (Svensson et al, 1998)	No induction of 1A2, 2A6, 2D6, or 2E1 as measured by probe drug indices after cocktail administration (Asimus et al, 2007)	autoinduction on the basis of induction of CYP2B6 and CYP3A4 (Xing et al 2012)	CAR/PXR
Rifabutin	**2B6** efavirenz clearance (Hsu et al 2010) **3A4** ethinyl estradiol (LeBel et al 1998)(Perucca et al, 1988; Bartditch et al, 1999)	No induction of theophylline clearance (Strolin Benedetti 1995). No studies on CYP2C9	autoinduction (less than rifampicin) within 5 days (Blaschke and Skinner 1996)	PXR
Rifampicin	**1A2** (Robson et al, 1984; Wietholtz et al, 1995; Backman et al, 2006) **2B6** (Loboz et al, 2006; López-Cortés et al, 2002) **2C9** clearance of 7 drugs (Lin, 2006) (O'Reilly et al, 1974; Zilly et al, 1975; Williamson, 1998) **3A4** midazolam metabolism among increased clearance of >10 drugs (Lin,	An extensive review of Lin (Lin, 2006) CYP2B6: several studies showing no induction (Preissner et al, 2009)	autoinduction within 5 days; (main active metabolite participates?)	PXR

	2006)(see also Kanebratt et al, 2008c; Ohnhaus et al, 1979)			
Metoprolol	**Non-inducer (no in vivo studies focussed on potential induction of CYP enzymes by metoptolol were found in the literature)**	Principal metabolising enzyme CYP2D6 not inducible (except in pregnancy, Wadelius et al, 1997)	not observed	
Sotalol HCl	**Non-inducer**	Elimination by renal clearance	not observed	
Phenobarbital	Prototypical inducer of CYP2B6, but induces also variably CYP1A2, CYP2C9 and CYP3A4-associated activities (Perucca 1988)		autoinduction	CAR indirectly

Table 03 Metabolism of the 13 selected test items and principal metabolites.

Test item	Proportion of a parent metabolised (per cent)	Principal metabolic routes (isozymes catalyzing the conversion; the principal one in bold)	References
Omeprazole	99	5-hydroxy-omeprazole (**CYP2C19**) N-demethylomerazole (CYP2C19) omeprazole sulfoxide (CYP3A4) 3-hydroxy-omeprazole (CYP3A4)	Andersson et al, 1996 Ma and Lu, 2007
Carbamazepine	99	carbamazepine 10,11-epoxide (**CYP3A4**, CYP1A2 and CYP2C8) minor hydroxymetabolites	Magnusson et al, 2007
Phenytoin sodium	50	p-hydroxylation (**CYP2C9**, CYP2C19) minor metabolites	Chetty et al, 1998
Penicillin G sodium	40-60	hydrolysis non-enzymatically	
Indole-3-carbinol	?	dimerization and trimerization (stomach acid) hydroxymethylation oxidation to indole-3-carboxylic acid (I3-CA) and indole-3-carboxaldehyde (I3-CAL)	Hauder et al, 2011
Efavirenz	?	8-hydroxyefavirenz (**CYP2B6**, CYP3A)	Ward et al, 2003
Sulfinpyrazone	60-75	sulfinpyrazone sulfide (CYP2C9 and 3A4) sulfinpyrazone sulfone (CYP2C9 and 3A4)	He et al, 2001
Bosentan hydrate	>90	Ro 48–5033 hydroxymethyl (CYP3A4 and CYP2C9)(active) Ro 47–8634 phenol (CYP3A4) Ro 64–1056 hydroxy/phenol (CYP2C9 and 3A4)	Dingemanse et al, 2004; Weber et al, 1999a; van Giersbergen et al, 2002a
Artemisinin	?	hydroxylation (CYP2B6, 3A4)	Medhi et al, 2009; Giao and Vries, 2001
Rifabutin	90-95	25-O-desacetylrifabutin (active) 31-hydroxyl rifabutin (active)	Blaschke and Skinner, 1996

Rifampicin	50	25-O-desacetylation (active) Oxidative N-dealkylation	Blaschke and Skinner, 1996
Metoprolol	95	O-demethylation (**CYP2D6**) oxidative deamination α-hydroxylation (**CYP2D6**)	Blake et al, 2013
Sotalol	0	no metabolism	
Phenobarbital	33-75	p-hydroxylation and further N- glucosidation (CYP2C9)	Nelson et al, 1982

Table 04 Pharmacokinetic characteristics of the 13 selected test items.

Test item	Bioavailability	Maximum concentration (µM)	Time to maximum concentration (T_{max} hr)	Half-life (h)	Volume of distribution (L/kg) (plasma protein binding)	Clearance (L/h)	AUC (h x µM)
Omeprazole	0.4-0.6	0.68 ± 0.43 (20 mg; 2C19 EMs) 3.5 ± 1.4 (20 mg; PMs)	(1-2h)	<1 (0.5-1.5)	0,3 (97%)		1.11
Carbamazepine	>0.7	39 (8.4-76) 18.4 mg/kg/day oral	(6-24h)	36h (16-24h)	0.8-1.9 (54-80%)	0.8	1248
Phenytoin sodium	0.85-0.95	40-80		22 (7-60)	0.5-0.7 (90-93%)	0.50-3.3	468
Penicillin G sodium	0.3	36	(0.5h)	0.5-1.0	0.2-0.7 (50-65%)	30	
Indole-3-carbinol							
Efavirenz	<0.8	9.1 – 12.6	(5h)	(52-76)	nk (>99%)	12	184
Sulfinpyrazone	1.0	45	(1-2)	3 (1-9)	0.06 (98%)		287
Bosentan hydrate	0.5	500 mg/day 5.8 uM	(3-5 h)	5,4	0.3 (99%)	8.2	29
Artemisinin	0.5	1-2	(3h)	3	na		7.5
Rifabutin	0.2	0.44	(3 h)	37	9.3 (70%)	8.6	2.4
Metoprolol	0.12-0.8	0.14 uM (EMs); 0.38 uM (PMs)	(2-3)	3.5 (1-9)	3-6 (12%)	1	2.3
Sotalol	1.0	2.0	(2,5-4h)	12 (7-18)	1-2 (0%)	6	
Phenobarbital	>0.9	56 - 120	(2-4)	100 (50-150)	0.7-1.0 (50%)	0.24	1497
Rifampicin	>0.8	8 - 12 (600 mg)	(2-4)	3.4 (1-6)	1.0 (60-80%)	12.6	34.1

Data mainly from drug monographs (European Medicines Agency (EMA) and National Agencies e.g. in Finland: Pharmaca Fennica), Goodman-Gilman The pharmacological Basis of Therapeutics (editions of 2011 and 2003), and Dollery's Therapeutic Drugs and Kirchheiner et al, 2004.

CYP induction by nuclear receptors

Molecular mechanisms of induction of CYP enzymes have been elucidated to a considerable degree over the last two decades, although there are still gaps in the knowledge. Here only a short description of the major factors are described for the background, but it has to be remembered that other nuclear receptors such as RXR, ER, GR etc. may have similar actions as the receptors described here. This description is based on a number of review articles (Lin, 2006, Hukkanen, 2012, Masahiko et al, 2000)

Aryl hydrocarbon receptor (AhR)

Strictly speaking, AhR does not belong to the nuclear receptor family, but is a ligand-activated transcription factor belonging to the Per-Arnt-Sim family of transcription factors. AhR is expressed to a variable extent in a large number of tissues and cell lines. Regarding CYP enzymes, its induction spectrum is narrower than those of PXR or CAR, because its activation results in transcriptional expression of CYP1A1, CYP1A2, CYP1B1 and CYP2S1 enzymes. Typical ligands are PAHs, PCBs, PBBs, other halogenated aromatic hydrocarbons, and dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin. The inducing effect of tobacco smoking and eating charcoal broiled meat is mediated by AhR.

Pregnane X receptor (PXR; NR1I2)

PXR belongs to the nuclear receptor superfamily and it is mainly expressed in the liver and small intestine. The ligand-bound PXR forms a heterodimer with retinoid X receptor (RXR) and the resulting PXR/RXR heterodimer binds xenobiotic response elements (XRE) in the 5'-promoter region of its target genes to cause the induction of CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5 and CYP3A7. There are a large number of PXR ligands identified pharmaceuticals and environmental contaminants dominating the list. The induction spectrum is actually much wider than the above mentioned CYP enzymes and it contains several phase II xenobiotic metabolising enzymes and transporters.

Constitutive androstane receptor (CAR; NR1I3)

CAR is functionally related to PXR and this relationship is reflected in the functional concept of 'receptor cross-talk'. CAR is mainly expressed in the liver and kidney. The induction spectrum is wide, possibly due to cross-talk with PXR, and contains CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP3A5. A peculiar feature of CAR is its transactivation in the absence of a ligand, i.e. constitutive activity. There are also indirectly acting activators, compounds that do not directly bind to CAR, but still are

able to induce transcription via CAR. Ligand spectrum overlaps with that of PXR, but there are also differences.

3.5 Test items purchase, coding and distribution

Reference documents:

- *CYP induction validation study: test item chemical aliquot coding and distribution (**Appendix 04**)*
- *Report "Solubility by Nephelometry: compatibility of test chemicals with in vitro assays for pharmacokinetic CYP enzyme induction" (**Appendix 05**)*

The 2 test items used in the training and transfer module were not supplied by EURL ECVAM but purchased by the trained laboratories.

The TIM group was responsible for purchasing, additional solubility testing, coding and distributing the 13 test items to the laboratories as identity coded aliquots (name and molecular weight not disclosed). The selected chemicals were purchased at Sigma, Chemos and Watson International.

3.5.1 Solvent compatibility assessment and test item concentrations

The SOPs require that the test items are dissolved in DMSO. The final DMSO concentration during the induction experiments should be ≤ 0.1% v/v, achieved by 1000-fold dilution of a stock solution in incubation medium (for CryoHepaRG®: GlutaMAX with serum-free supplement; for Cryoheps: HMM (hepatocyte maintenance medium)), with test item concentrations correspondingly lowered to μg/ml range.

The test items were tested at a range of concentrations in order to cover clinically (human *in vivo*) relevant concentrations of CYP inducers for comparison with human reference data. The 13 test items have a molecular weight (MW) in the range of approximately 150-850 g/mole (average MW 400). As the molecular weight was unknown to the laboratories, the VMG agreed to adopt a simplified and relevant starting concentration of 40mg/ml for the stock solution in DMSO.
Laboratories were required to provide study reports.

It is important to ensure the compatibility of test items with the *in vitro* method under evaluation. Thus, in order to avoid possible problems with the subjective evaluation of solubility by visual, the independent TIM group at EURL ECVAM assessed the solubility of test items with the relevant and accurate nephelometer-based method (see

Appendix 05). The nephelometer results were not communicated to the laboratories, as the SOP required that they performed their own determination by visual inspection. However, these experiments were the reference standard to guide the laboratories with in the starting concentration for the cytotoxicity experiments in order to avoid that the validation project would be jeopardised by solubility differences between the participating laboratories.

3.5.2 Coding and decoding

The TIM Group assigned an identification letter (i.e. A, B, C) to each laboratory and generated a random code for each test item aliquot, unique for each *in vitro* method, laboratory and experiment. For the assessment of the BLR Module 4a -induction (four test items) three independent runs were foreseen for three different cell batches and for each test item (n=9). For this reason, 3 vials of each test item were sent to the laboratories, each assigned a different two-letter code. A number (1, 2 or 3) was added between the letter identifying the laboratory and the two-letter code to distinguish the three sets of test items and the laboratories were instructed never to mix chemicals labelled with different numbers in the same run/experiments. This ensured that the three evaluations of the corresponding test items were performed in different experiments in order to provide data suitable for a proper evaluation of BLR.

However, due to financial, time and personnel shortage at all the laboratories, VMG agreed to perform Module 4a by testing each test item once (n=3) per cell batch. VMG instructed the laboratories to combine the 3 aliquots as single aliquots.
For Module 4b – induction (9 test items) each laboratory received a vial per test item. Each vial was labelled with a letter identifying the laboratory, a hash and a two-letter code, unique for each method, laboratory and experiment.
The codes for all test item aliquots were recorded in a database (Excel spreadsheet format) prepared and maintained by the TIM Group. The identity of the test items was not disclosed to the laboratories.

The TIM group provided the laboratories with the final decoding list for the test items only after all the experimental data had been generated by the laboratories, quality checked and analysed by the biostatistician for the assessment of the BLR.
The decoding list was used by VMG and the biostatistician to analyse and assess the information generated in this project on the predictive capacity of the CYP induction *in vitro* method.

3.5.3 Emergency procedure implemented at the laboratories during the blind testing module

An emergency procedure was established to allow the laboratories to obtain the necessary chemical safety information in case of an accident. Individual sealed envelopes, each containing a Material Safety Data Sheets (MSDS) related to one specific test item and labelled with the corresponding code, were sent with the test items to a named recipient at each laboratory not associated with the testing (typically the Safety Officer at each participating laboratory) with the instruction to return the unopened MSDSs to EURL ECVAM upon completion of the testing modules.
During the validation project, no such incident was reported and none of the envelopes had to be unsealed. At the end of the project all sealed envelopes were returned to EURL ECVAM.
All the laboratories were instructed to treat all coded test items as potential carcinogens and toxic compounds.

3.6 Data Management

Prior to start the study, the SOP and the related data collection forms were distributed to the laboratories. The forms were developed by the lead laboratories (Kaly Cell and Pharmacelsus) and contained formulae tested by the lead laboratories.

There are two kinds of forms:

I. forms for raw data collection (pdf format): they are pdf files and raw data are entered manually;
II. processing spreadsheets for data analysis (Excel spreadsheet): raw data are entered manually, or via copy-and-paste into the processing spreadsheet. In this case, the raw data resides in the computerized system or is paper-based. If the data is copied into the processing spreadsheet, the operator should always check whether the raw data has been transferred completely and correctly.

At EURL ECVAM, solubility and cytotoxicity with CryoHepaRG test method were performed GLP-compliant. For this reason, the forms FRM01-ASY02 (Solubility of test items), FRM02-ASY02 (Cytotoxicity testing), FRM03-ASY02 (Preparation of test items for cytotoxicity) and FRM04-ASY02 (HepaRG cell culture preparation, cell counting and determination of viability) were validated and secured. Computerised systems validation is the process of establishing a high degree of assurance that a specific process, activity or system, will consistently and reliably produce a product meeting

predetermined specifications and quality characteristics. The supporting evidence is required to be appropriately documented.

The laboratories performed the calculations with the processing spreadsheets and, at the end of each experimental part (e.g. solubility, cytotoxicity, induction), they provided a study report and the related filled in forms to the trial coordinator.

For each module, before starting any experimental work (e.g solubility, cytotoxicity, induction), study directors from all the laboratories involved had to provide the trial coordinator with a study plan. At the end of the specific study, they had to provide a study report and the respective completed in forms. The study plans and the forms were submitted to VMG and to the independent biostatistician.

3.7 Statistical analysis of experimental data

On the data and reports from participating laboratories, an independent statistician made an evaluation of between-batch and between-laboratory reproducibility of both test methods. Pooled data were evaluated using the statistical software Matlab.

The experimental data were stored and analysed in appropriate data forms by participating laboratories and by the statistician and followed the Good Practices for the Computerised systems in regulated "GXP" environments available at http://www.labcompliance.com/info/links/international/computers.aspx.

Data quality: A laboratory performing the experiments made the first decision about the technical quality of the incubations and the raw results, e.g. about outliers. This information was taken into consideration in the statistical analysis.

4 Module 1: Test Definition

Reference documents:

- *SOP ' Cytochrome P450 induction in CryoHepaRG® cells (n-in-one incubations on 96-well plates)' Version 02 (**Appendix 06**)*
- *SOP "Cytochrome P450 induction in human cryopreserved hepatocytes (n-in-one incubations on 48-well plates)"version 08 (**Appendix 07**)*
- *Revisions of the Standard Operating Procedure "Cytochrome P450 induction in CryoHepaRG® cells (n-in-one incubations on 96-well plates)" (**Appendix 08**)*
- *Revisions of the Standard Operating Procedure "Cytochrome P450 induction in human cryopreserved hepatocytes (n-in-one incubations on 96-well plates)" (**Appendix 09**)*
- *Comments on Kaly Cell report "Assessment of chlorpromazine as positive control for cytotoxicity: additional results to Module 1 test definition" by Roman Liska 27 September 2011 (**Appendix 10**)*
- *Comments on Kaly Cell report "Positive control (chlorpromazine) concentration finding by evaluation of dose dependent cytotoxicity towards cryoheps – ECVAM validation follow up study AMD2011ECV004 (Module 4a) and ECVAM validation follow up study AMD2012ECV001 (Module 4a)" by Roman Liska 27 February 2012 (**Appendix 11**)*
- *Amendment to Comments on Kaly Cell report "Positive control (chlorpromazine) concentration finding by evaluation of dose dependent cytotoxicity towards cryoheps – ECVAM validation follow up study AMD2011ECV004 (Module 4a) and ECVAM validation follow up study AMD2012ECV001 (Module 4a)" by Roman Liska 02 April 2012 (**Appendix 12**)*

4.1 Intended purpose of the *in vitro* method

This project is a first response to the scientific community request of having reliable and relevant human hepatic *in vitro* metabolically competent test systems and transferable, reproducible and predictive human CYP induction *in vitro* methods to be used in integrated approaches for biotransformation and toxicological Mode of Action studies of substances and mixture/products of various industrial sectors.

Therefore, the information/data produced in this validation project will also help to gain more insight into xenobiotic biotransformation and toxicological Mode of Action (MoA). The above intended use cases were also described in the OECD Test Guideline programme project proposal description related to this project.

4.2 Evidence demonstrating the need of the test method

The CYP induction *in vitro* method was proposed as a pilot project and candidate for regulatory use by issuing a performance-based OECD test guideline for the human *in vitro* CYP induction methods. After evaluation by all OECD member countries the human CYP induction methods project was accepted and considered by all member countries as a project to put on the OECD work programme.

The successful outcome of this project, coordinated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), responds also to the EU existing and future regulatory requirements (REACH Regulation (EC, 2006), Cosmetics Regulation EC 1223/2009 (EC, 2009), Animal Welfare Directive 2010/63/EU (EC, 2010), OECD (OECD, 2012)) and the scientific community expectations calling for toxicological testing methods delivering key information to complement hazard and risk assessments of substances within integrated approaches based on reliable and relevant non-animal methods (Adler et al, 2011; OECD Draft Guidance Document 151, 2012).

Animal data do not reliably and consistently predict CYP induction in humans due to the large inter-species variability in different steps of the induction process (nuclear receptors, signal transduction pathways, expression of *enzymes, etc). This project provides a human based in vitro CYP induction in* vitro method avoiding species extrapolation in human safety assessment.

Therefore, it is of critical importance using a human relevant *in vitro* test system to predict CYP induction *in vivo*. CYP induction by xenobiotics is a complex process including receptor activation, stabilization of transcripts and proteins that eventually will lead to an increased capacity of the cell to metabolize xenobiotics including many pharmaceuticals and environmental toxicants. The importance to use a human relevant system is based on the fact that **the AhR, PXR and CAR found in toxicological animal models such as mouse and rat, exhibit significant differences in specificity why rodent based models would not be predictive for the human situation.** There are several examples where a compound has strong effect on the human receptor and no effect on animal receptors or vice versa. In humans a prototypical CAR agonist CITCO (imidazo[2,1-b]thiazole-5-carbalde-hyde O-(3,4-dichlorobenzyl)oxime) has no effect on the rodent CAR receptor while TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene) a prototypical inducer of the rodent CAR receptor has no effect on the human receptor. Several examples can be given for the PXR receptor that is inducing the most important xenobiotic metabolizing enzyme in humans, CYP3A. Rifampicin the prototypical inducer of the human PXR, used in present evaluation, has no effect on rodent PXR. Other

substances such as artemisinin, clotrimazole, nicardepin and nifidipin are all activators of the human PXR but have no effect on the rodent PXR (Summarized in Chapter 6, Casarett and Doull´s Toxicology, The Basic Science of Poisons, Eight Edition, Edited by C. Klaassen, 2013). The potency of compounds to activate receptors can also vary drastically between species. Dexamethasone and pregnenolone 16α-carbonitrileare are strong PXR activators and/or inducers of CYP3A in rodents but not in human (Martignoni et al, 2006). Differences of induction among species are explained by discrepancies in the ligand-binding domain of the receptors implying that their ligand specificities may differ dramatically between species. Therefore, extrapolation of animal data with respect to the inducibility of CYP enzymes in human is not reliable.

The toxicity of a chemical may be assessed by parsing understanding the toxicity pathways and the related key molecular and/or cellular events (MoA), each of which can be identified and quantified with appropriate test methods. Notably, the human *in vitro* CYP induction method addresses cellular and molecular events (e.g. xenobiotic-cellular interactions/uptake, xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene) and the CYP induction test method could therefore prove useful for any xenobiotic, including the evaluation of drugs and a wide range of other use classes (i.e. cosmetic ingredients, household products, chemicals, carcinogens, pro-carcinogens and chemotherapeutics).

Furthermore, the underlying biological mechanism (Tompkins et al, 2007) of CYP induction is a very good indicator for the assessment of the functionality of the molecular machinery of any metabolically competent hepatic system proposed for regulatory uses. CYP induction *per se*, following the nuclear receptor-xenobiotic interaction, is suggested as an important biological event in several AoPs (Pelkonen et al, 2008; USEPA, 2011; Vinken et al, 2013).

4.3 Status of development of the test systems

Today, the routine evaluation of the human CYP inducing potential of a given xenobiotic on different toxicity pathways (e.g. endogenous hormonal disturbance, drug-drug interaction, toxic effects exacerbation) is described by FDA, EMA and the Japanese Ministry of Health, Labour and Welfare and is considered of paramount importance for human safety assessment (Silva et al, 1999; FDA, 2012; EMA, 2012).
The *in vitro* methods for determining CYP induction in human primary cryopreserved hepatocytes and in human cryopreserved HepaRG® cells using the CYP enzyme activity as phenotypic endpoint has been fully defined and described during this validation project. The method is considered medium-throughput as, by applying the substrate

cocktail approach, it allows the analysis of the induction of four important metabolic active cytochrome P450 enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) at once.
Such procedures have been used (with different variations of the SOP non-standardised and non-validated at international level) in the pharmaceutical sector for the purpose of drug-drug interaction related studies.
In addition, the proposed *in vitro* method comprises of a defined solubility test and a cytotoxicity test to define the concentration range of each test compound and avoid precipitation of the compound and cell damage during the induction experiment.
During the validation project the applicability and transferability of the *in vitro* method has been demonstrated successfully and comprehensive SOPs are the product of this validation project.

4.4 Primary human cryopreserved hepatocytes

The differences between humans and animals in xenobiotic biotransformation and induction of cytochromes decrease the relevance and reliability of animal-based models. For this reason human primary hepatocytes in culture have become the most important promising tool for studying xenobiotic metabolism and toxicity with respect to human risk assessment.
In vitro CYP induction data, based on primary human hepatocytes, correlate well with the human clinical data, as long as experiments are performed at clinically relevant concentrations (Chu et al, 2009). Many liver specific functions are retained in freshly isolated hepatocytes for 24-72 hours, but most of them (e.g. CYP functions) are lost when further maintained in culture (Silva et al, 1999; Roymans et al, 2005). Recently Yajima et al., (2014) showed a large variation in induction response in different lots of cryopreserved primary human hepatocytes. In addition several lots exhibited non-detectable enzyme activity in non-treated cells which makes evaluation of an induction response by a potential inducer difficult.
Progress in cryopreservation techniques has greatly improved the utility of human hepatocytes, allowing (1) the timing of experiments not to be dictated by the availability of fresh tissue and (2) to pool or compare hepatocytes from several donors to address the inter-donor variability. Culture conditions have also been optimised in order to retain the ability to respond to inducers for a longer time.
Nowadays, cultured human primary hepatocytes (fresh or cryopreserved) are the most accepted (industry, academia) *in vitro* test system for assessing the potential for xenobiotics to induce human CYP isoforms and are still the gold standard for FDA Guidelines on drug-drug interaction studies (Chu et al, 2009; FDA, 2012).
Although nearly all pharmaceutical companies use primary human hepatocytes to characterise CYP induction potential of compounds in drug discovery and development,

costs, the sparse availability of human liver tissue and the limited number of sources of healthy tissue, the rapid de-differentiation in culture, the phenotypic variation and the pre-medication, age, sex and disease status of the donor are the main limitations in the use of freshly human hepatocytes. However, the advantage of using human hepatocytes is that one can get information on CYP induction in different donors. Therefore, EMA and FDA guidelines ask for information generation for human CYP induction using 3 different donors.

Many researchers use pools of hepatocytes for other application such as human hepatic metabolic stability/clearance. The use of separate hepatocyte donors or pools depends on the purpose of the study. So, individual donors or pools could be used for the study of a phenomenon of human CYP induction, but the use of individual preparations enrich the information by providing at least some idea about variability.

The human hepatocytes used in this study have been evaluated **freshly** on their reliability in specific designed studies for harmonising the isolation procedures between three laboratories by performing independent isolations and cultures of human hepatocytes and to assess their responses to the prototypical CYP enzyme inducers, β-naphthoflavone (BNF), rifampicin (RIF) or phenobarbital (PB) (Richert et al, 2010; LeCluyse et al, 2005; Richert et al, 2002).
Furthermore, experimental condition-related variables, such as seeding density, culture matrix and medium, start and duration of treatment, affecting the response of plateable thawed cryopreserved human hepatocytes to cytochrome P450 inducers have been optimised allowing the use of more reliably human **cryopreserved** hepatocytes for this validation project (Gerin et al, 2013; Alexandre et al, 2012; Abadie-Viollon et al, 2010; Desbans et al, 2014).

4.5 Human cryopreserved HepaRG® cells

The limited supply in primary human hepatocytes underlines the need of human hepatocyte-like cells that provide a continuous supply while maintaining stable expression of liver-specific functions (e.g. transporters, nuclear receptors) for routine screening and characterisation of human CYP induction. The SME and the pharmaceutical companies enrolled as participating laboratories underlined the need for an alternative to the FDA and EMA recommended test systems. Several lines originating from hepatocarcinoma have been evaluated as an alternative to the *in vitro* CYP induction method using primary hepatocytes from 3 donors. Most of these cell lines, however, show low basal expression of biotransformation enzymes (e.g. HepG2),

transporters and nuclear receptors (Fa2N-4) and little to no CYP induction in response to inducers.
HepaRG® cells were first described in 2002 by Gripon *et al*. (Gripon et al, 2002) as a human hepatocyte-derived cell line that supports the full replication cycle of HBV. HepG2 cell (hepatocellular carcinoma), Hela cells (cervical cancer) HuH7 (hepatocarcinoma cell line) are routinely used in cell toxicity assays. The HepaRG® cell line is HCV negative and has been isolated from a HCV positive patient suffering of grade I well differentiated hepatocarcinoma (Gripon et al, 2002). Since 2007, Biopredic granted a worldwide exclusive license. HepaRG is the only cell line that is considered by the current industrial end-users of this cell line, a line with functions more like an adult liver cell and with some unique characteristics like supporting the whole Hepatitis C cycle and the ADME gene expression.

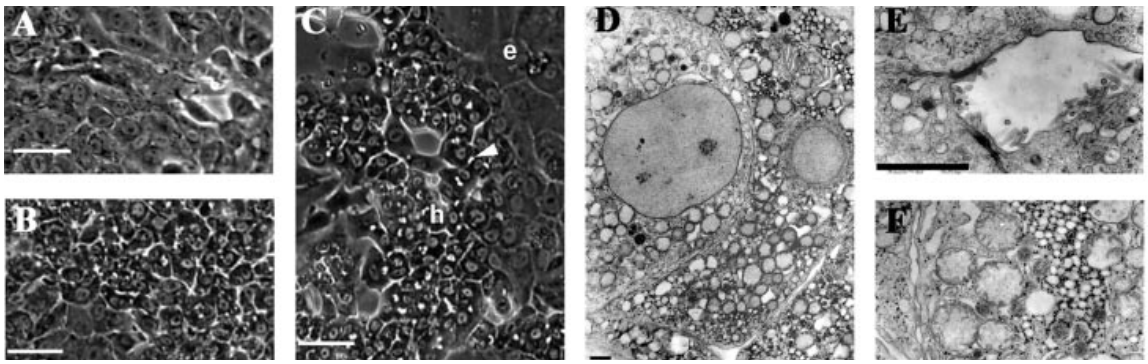
When passaged at low density, HepaRG® cells acquire undifferentiated elongated cell morphology and are able to actively divide and reach confluence within 1 week. At that time, two morphologically different cell types appear: one forms clusters of granular epithelial cells resembling hepatocytes while the second surrounding the former is more flattened and retains a clear cytoplasm (Table 05 and Figure 2). Addition of 2% dimethyl sulfoxide (DMSO) and 50 μM hydrocortisone hemisuccinate induces differentiation of the hepatocyte-like cells into more granular cells, closely resembling typical adult primary hepatocytes and bile canaliculi-like structures (Gripon et al, 2002).

Table 05 HepaRG cell line

characteristic		
Doubling time		24h
Karyotype		pseudodiploid
Hepatocyte-like cells		50-55% (Cerec et al., 2007)
Morphology	proliferation phase	epithelial phenotype with no regular structural organisation (Fig 2A)
	confluence	granular hepatocyte-like cells (Fig 2B)
	+2% DMSO to the medium, 2 weeks after plating	granular hepatocyte-like cells organise in well delineated trabeculae resembling those in primary human hepatocyte culture in which many bright canaliculi-like structures could be recognised. Few flat epithelium-like cells filled the empty spaces around.
	after 2 weeks with2% DMSO	trabeculae organisation completed. granular cell morphology closely

	resembled hepatocytes (Fig 2C)
CYP expression	DMSO required

Figure 02 Morphology of HepaRG® cells (Gripon et al., 2002). Phase contrast micrographs of HepaRG® cells under proliferating conditions (A), maintained in culture for 30 days without DMSO (B), and maintained for 15 days without DMSO, then treated with 2% DMSO for 15 days (C). Hepatocyte-like cells and epithelium-like cells are indicated, respectively, by ''h'' and ''e.'' A bile canaliculus is indicated by a white triangle. (Bars ⌟ 50 μm.) Electron micrographs of HepaRG cells: low magnification view of HepaRG® cells (D) and higher magnification views (E and F), showing a typical bile canaliculus-like structure and glycogen accumulation, respectively.



Compared to other cell lines, HepaRG® has two important hepatic functional features: (1) maintenance of an efficient proliferation differentiation interplay accompanied by morphological changes leading to hepatocyte-like cells, and (2) maintenance of stable expression of P450 enzymes, phase II enzymes, transporters, and nuclear transcription factors for up to 6 weeks in culture (Aninat et al, 2006; Cerec et al, 2007; Gripon et al, 2002; Guillouzo et al, 2007; Josse et al, 2008; Kanebratt et al, 2008a; Le Vee et al, 2006). Due to these features, the HepaRG® cell line is considered a valuable human-relevant *in vitro* model for investigating P450 induction properties of drug compounds (Kanebratt et al, 2008a).

In contrast to other hepatoma cell lines like HepG2, Fa2N-4 and HuH7, differentiated HepaRG® cells respond to prototypical inducers of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 at levels similar to those found in cultured human primary hepatocytes and the responses are stable over one month when cultured in DMSO. When DMSO is withdrawn from the medium, both CYP mRNAs and enzyme activity decrease but remain constant for two weeks whereas transporters and

liver-specific factors are unaffected (Kanebratt et al, 2008a). The functionality of CYPs in HepaRG® cells is supported by the demonstration of their specific activities using specific substrates and responsiveness to inducers (Andersson et al, 2012; Josse et al, 2008; Turpeinen et al, 2009). Responsiveness to prototypical inducers of CYPs is reported to be similar in different passages of HepaRG® cells. Kanebratt (Kanebratt et al, 2008b) reported a good inter-batch reproducibility with respect to induction studies. Kanebratt and Andersson (Kanebratt et Andersson 2008) recommended the removal of DMSO minimum for one day to decrease CYP activities before treatment with test compounds and prototypical inducers.
Whenever HepaRG® cells are used for metabolism studies, it should be taken into account that these cells originate from one individual with genotypic variant alleles for CYP2C9 and CYP2D6. Compared to the wild type enzymes, these variants have a slower metabolic capacity as demonstrated by the low CYP2C9-dependent activity of diclofenac and CYP2D6-dependent activity of dextromethorphan (Andersson et al, 2012).

The HepaRG® cells are nowadays available as cryopreserved differentiated cells (i.e. they are differentiated in 2% DMSO and then frozen as a suspension). The freeze/thaw process does not alter their functional activities and the inter-batch reproducibility is excellent; therefore, these cells are ready to use in different applications such as metabolism studies and enzyme inhibition/induction.

A cell model that has reproducible and marked CYP induction responses allows comparing data on different compounds tested at different times, and testing inducers with low induction responses avoiding false negative results.

Although primary human hepatocytes derived from 3 separate donors are still the gold standard for CYP induction studies for regulatory purposes, the loss of chemical-metabolising and chemical-transporter capacity when they are maintained in culture and their limited availability underline the need of alternative models. HepaRG® cells represent a promising alternative model to primary human hepatocytes as they combine long-term stability of chemical-metabolising enzymes and transporters with the correct plasma membrane polarisation. HepaRG® cells have also been used to study:

- regulation of lipid metabolism as CYP3A family and transcription factors are expressed (Guillouzo et al, 2007) in these cells,

- *in vivo*-like uptake-metabolism-secretion of parent compound and metabolites formed within the liver, as they are polarised cells with tight-junctions and canaliculi (Kanebratt et al, 2008a), and as they express uptake and efflux transporters in a polarised fashion.

- *In vitro* CYP induction, as they respond to prototypical inducers at mRNA and enzyme activity level and they retain receptors and factors involved in the CYP induction process (Andersson et al, 2012).

Table 06 Comparison of the two test systems used in this validation project

Human primary hepatocytes	Human HepaRG® cell line
gold standard for *in vitro* CYP induction (especially by pharmaceuticals)	from hepatocarcinoma of a female patient => one donor
donor variability both in basal P450 levels and in the extent of induction (low basal-high induction)	metabolic competent cells
need of multiple donors (EMA & FDA require 3 donors)	express nuclear receptors and drug transporters
need of successful and consistent isolation	stable cell line
loss of drug metabolising/transporter capacity in culture	easy to handle, stable expression of many phase I and II enzymes which are inducible
quality and metabolic/functional activity of the cells is variable	good recovery after thawing (with respect to cell number and viability)

In Japan, the use of the primary-cultured human hepatocytes (fresh or cryopreserved) is recommended for the evaluation of CYP induction. On the other hand, the data from HepaRG® cells are used only to supplement data form the primary-cultured human hepatocytes. In japan, the HepaRG® test system is recognised not only as supportive tool but also as a tool for the evaluation of CYP induction and cytotoxicity. Therefore, food- and chemical-related Japanese industrial sectors seem to be interested in the HepaRG® test system.
If the evaluation system using HepaRG® for CYP induction is established based on the validation data, companies from pharmaceutical or any other industrial sector will be able to employ the data of HepaRG® as new-xenobiotic application material in the future.

4.6 Scientific basis of CYP induction

Biotransformation

One of the most important functions of hepatocytes is the biotransformation of both endogenous and exogenous compounds. These liver parenchymal cells are very rich in smooth endoplasmic reticulum which incorporates a large amount of biotransformation enzymes. The characteristically polarised structure of hepatocytes allows the excretion of the biotransformation products into the bile or the blood (Sevior et al, 2012).

Biotransformation in the liver is accomplished by two classes of enzymes: phase I and phase II biotransformation enzymes (Coecke et al, 2006). Cytochrome P450s (CYP) are pivotal phase I mono-oxygenase enzymes involved in the synthesis and degradation of endogenous steroid hormones, vitamins and fatty acid derivates, but also in the transformation of xenobiotics, such as drugs, environmental pollutants and carcinogens into more hydrophilic molecules, facilitating their excretion.

Enzyme inhibition/induction may lead to a significant variation of the compound or its metabolite concentration at the target site. These mechanisms might lead to enhanced clearance or toxic accumulation of the parent compound (or its metabolites) or production of toxic metabolites. Induction is defined as an increase in the amount and activity of a metabolising enzyme due to *de novo* CYP protein synthesis or stabilisation of CYP enzymes. It is a long-term consequence of a xenobiotic exposure and as a result the overall CYP catalytic activity increases. Inhibition can be an acute decrease of metabolism of a particular substrate by another simultaneously present xenobiotic or a reactive metabolite that binds to the CYP or to the heme of the CYP (Pelkonen *et al*, 2008). CYP inhibition may cause toxic effects by increasing the concentration of the parent chemical at the target site, while CYP induction may lead to increased metabolism rate and clearance or to the production of toxic metabolites. From the toxicological point of view, CYP induction plays a crucial role in accelerating the metabolism of the chemical being exposed to, leading to inactivation or detoxification of these chemicals (e.g. clearance).
Due to the different underlying mechanisms, different in vitro methods have been used to evaluate CYP induction and inhibition. The most widely used *in vitro* method to study CYP inhibition is to measure a potential inhibitory activity of a xenobiotic in CYP selective substrate assays in human liver microsomes. To evaluate CYP induction *in vitro*, there is the need for a plateable *in vitro* metabolically competent test system stable for 2-3 days which is **repeatedly challenged** with the test items (i.e. xenobiotic). Indeed, unlike CYP inhibition, CYP Induction is a longer (hours to days) process.

Molecular mechanism of CYP induction

At the molecular level, CYP induction is a process involving several cellular machineries and due to its complex biological mechanism (Tompkins et al, 2007; Aguiar et al, 2005; Bao, 2010), it has been used, in this validation project, as a biological tool or biomarker

to assess the relevance of the two metabolically competent test systems. Furthermore, CYP induction is a key building block in defining adverse outcome pathways based on nuclear receptor interactions as key events (Pelkonen et al, 2008; Vinken et al 2013; USEPA, 2011).

The biological process of a selective isoform induction by a xenobiotic inducer can be summarised as follows:

- The chemical binds to a specific intracellular nuclear receptor,
- The activated receptor forms heterodimer with factors, such as Ahrnt (Ahr nuclear translocator) and retinoid X receptor (RXR for both PXR and CAR) and migrates into the nucleus,
- The heterodimer binds to the target xenobiotic response elements (XRE) located in both the proximal and distal P450 gene promoters,
- The transcription of the respective CYP gene is enhanced, which is followed by the *de novo* protein synthesis and post-translational modification to a functional CYP enzyme.

Among the nuclear receptors the Constitutive Androstane Receptor (CAR), the Pregnane X Receptor (PXR) and the Aryl Hydrocarbon Receptor (AhR) are involved in CYP-mediated metabolism. These receptors control the expression of CYP1A (AhR), CYP2, and CYP3A (PXR and CAR) families (Denison et al, 2003; Lehmann et al, 1998; Gibson et al, 2002; Chen et al, 2004; Wang et al, 2004; Sueyoshi et al, 1999; Goodwin et al, 2002; Wang et al, 2012), as well as UGTs and glutathione-S-transferases and the transporters MDR1 and MRP2 (Hewitt et al, 2007).

CYP induction: mRNA versus enzymatic activity

There are two general mechanisms by which enzyme induction occurs: (1) stabilisation of enzyme or mRNA and (2) increased gene transcription (Zahno et al, 2011).
Unlike CYP inhibition, CYP induction is a slow process and, before the increase in enzyme activity can be observed, a lag period elapses (Honkakoski et al, 2000).

Xenobiotics that are both time-dependent inhibitors and CYP inducer may result in no net effect of the enzyme activity, but a clear increase in mRNA levels (Einolf et al. 2014). Several protease inhibitors are CYP3A inducers, but also time-dependent CYP3A inhibitors and the net effect on CYP activity is less than expected from mRNA measurements (Ernest et al. 2005).

The FDA and EMA Guidelines (Table 07) recommend using primary human hepatocytes and data from other cell systems are considered as complementary or supportive information. The recommended endpoints for CYP induction are measurement of mRNA (FDA) and CYP enzyme activity (EMA). In the EMA Guidelines catalytic activity measurement is recommended especially if induction is suspected to be due to protein stabilisation; therefore no complete information can be obtained unless CYP enzymatic activity is measured. The contents of draft Japanese Guidance on the investigation of drug interactions (in Japanese, December 2013) under the initiative of Ministry of Health, Labour and Welfare are similar to those of the Guideline/Guidance for the drug interactions suggested by EMA and FDA. mRNA levels of CYP1A2, CYP2B6 and CYP3A4 are mainly measured for the quantitative evaluation of the CYP induction. It is possible to assay CYP activities in hepatocytes, if there is no inhibition of CYP, especially time-dependent inhibition, by drugs.

In this validation project, CYP induction is measured at the level of activity, i.e. measuring the enzymatic transformation of substrate xenobiotics into known identifiable products and not mRNA level. Indeed, it is well documented that there is an apparent **discrepancy between mRNA induction and catalytic activity** and the lack of positive correlation between CYP activity and the specific CYP mRNA level (Mwinyi et al, 2011; Choi et al, 2013; Surapureddi et al, 2011). Nakajima (Nakajima et al, 2011) reported no positive correlation between CYP2E1 activity and CYP2E1 mRNA levels due to post-transcriptional regulation. Abass (Abass et al, 2012) observed that in HepaRG® cells, phenobarbital induced the CYP activity in a dose dependent manner, in contrast with mRNA.

The lack of correlation between mRNA and catalytic activity has been ascribed to several different kinds of **post-transcriptional control mechanisms** including microRNA (e.g. for CYP3A4; CYP2B6, CYP2E1), factors controlling translation and post-translational insertion in the membranes and phosphorylation (Takagi et al., 2008; Nakajima et al, 2011; Wang et al, 2009; Aguiar et al, 2005). Although the understanding of the mechanism of transcriptional regulation of CYPs has progressed, the post-transcriptional regulation is still largely unclear.

Nowadays many high throughput *in vitro* methods are available for measuring the activation of nuclear receptors by xenobiotics. However, the observed activation of a nuclear receptor in an *in vitro* method does not necessarily indicate induction of the CYP enzyme activities (Abass et al, 2012).

In contrast to nuclear receptor activation and CYP mRNA level, **CYP activity, covering both *de novo* protein synthesis and protein stabilisation**, is the functional endpoint of CYP induction and the basis of potential chemical-chemical interactions in humans.

Table 07 Comparison of the main parameters cited in the FDA and EMA drug-drug interaction Guidelines concerning *in vitro* CYP induction assessment (n.s.: not specified).

	FDA (draft)	EMA (final)
Test system	Cultured fresh or cryopreserved human hepatocytes. Other cell types are considered as complementary.	Human hepatocytes (fresh or cryopreserved) are the preferred *in vitro* system. Cell lines (e.g. HepaRG), nuclear receptor binding assay, or reporter gene assay are considered as supportive data.
Fresh or cryopreserved	Cultured (fresh or cryopreserved)	Cultured (fresh or cryopreserved)
Number of donors	At least 3 different donors	Due to the inter-individual and cell batch variability in induction response, it is recommended to use hepatocytes from at least 3 different evaluable donors for the "basic method" evaluation
CYP enzymes to be investigated	CYP1A2, CYP2B6, and CYP3A; if CYP3A positive, CYP2C is required	CYP1A2, CYP2B6, and CYP3A4 should always be included as markers of induction mediated via PXR/CAR (CYP3A4, CYP2B6) and the Ah-receptor (CYP1A2).
Other enzymes	To be considered if important for the drug	A number of enzymes could be investigated
Number of test item concentrations to be tested	3 or more	3 or more
Concentrations of test item	n.s.	The studied exposure range should cover the worst case concentrations expected in the hepatocytes *in vivo*.
Duration of treatment	n.s.	3 days. Shorter durations should be well justified
Endpoints recommended	mRNA	mRNA. Catalytic activity recommended if induction due to protein stabilisation

		suspected. Concentration of parent drug in the medium should be measured at several time points on the last day of incubation unless shown previously that loss is negligible or medium change interval compensates for loss. The degree of protein binding in the medium and non-specific binding should be considered and unbound concentrations used for the evaluation.)
Controls	Vehicle control. Positive control (known strong inducer) Negative control (known non-inducer)	Vehicle control. Positive control (known strong inducer)
Positive controls	Omeprazole and Lansoprazole for CYP1A2; phenobarbital for 2B6; rifampicin for CYP2C8, 2C9, 2C19 and 3A4; range of concentrations is provided	Rifampicin (20µM) for PXR, CITCO (≤100 nM) for CAR, omeprazole (50µM) for the Ah-receptor and dexamethasone (50µM) for GR
Vehicle control	Required	Required
Negative control (no-inducer)	Required	Not-required
Hepatocyte quality control	n.s.	If cells from a donor do not respond satisfactorily to the positive controls, if the viability of the cells is <80% at the start of the incubation, or if the viability at the end of the incubation deviates markedly from the other donors, the cells should be replaced by hepatocytes from a new donor.
Data analysis	The increase in mRNA is compared to the vehicle control	The levels of mRNA are compared to the control (vehicle) incubations.

Positive enzyme induction	At least one donor exceeds the predefined threshold (e.g. R<0.9)	Response gives rise to a more than 100% increase in mRNA and the increase is concentration dependent
Negative enzyme induction (no inducer)	All donors exceed the predefined threshold (e.g. R≥0.9)	Response is <100% in mRNA are compared to the control (vehicle) and is less than 20% of the response of the positive control (rifampicin 20 µM or, for Ah-receptor activation, omeprazole 50 µM).
Positive control inducer	Not used in the quantitative evaluation	Used for assessing reliability of the response and to interpret negative response
In vivo	In case of a positive or inconclusive in vitro result, conduct in vivo studies with strong inhibitor(s)/inducer(s) or if appropriate, compare PK in different genotypes	A positive or inconclusive in vitro result should be confirmed in vivo or lack of induction potential needs to be shown in another in vitro study

CYP isoforms 1A2, 2B6, 2C9 and 3A4

The four P450 iso-enzymes 1A2, 2B6, 2C9 and 3A4 were were selected as they are inducible in humans, are involved in most of the Phase I detoxifying processes in human liver and are recommended by EMA and FDA drug-drug interaction Guidelines. CYP3A4 is the most abundant isoform, constituting 30% of all the P450 liver enzymes in humans. The CYP2C family accounts of 30-40% of human hepatic P450, with CYP2C9 being the most highly expressed (Fahmi *et al*, 2010).

Table 08 Characteristics of the CYP enzymes subject for this investigation (see also Pelkonen et al, 2008)

1A2	Metabolic activation and deactivation of chemicals and environmental pollutants. CYP1A2 oxygenates heterocyclic aromatic amines/amides to reactive intermediates that subsequently lead to DNA and protein adducts formation. It also dealkylates phenacetin to ultimate metabolites that produce liver necrosis. However, metabolism by CYP1A2 can also decrease the carcinogenic effects of aflatoxin and other xenobiotics.	Ma and Lu, 2007
CYP2B6	Metabolises several drugs as bupropion (model substrate), cyclophosphamide, artemesinin, nevirapine and efavirenz.	Turpeinen et al 2006
CYP2C9	Has a broad range of drug substrates such as anti-inflammatory drugs (e.g. diclofenac, a model substrate), oral hypoglycemics (e.g. tolbutamide), anticoagulants (e.g. warfarin) and angiotensin receptor blockers (e.g. losartan)	
CYP3A4	Is the most abundant hepatic enzyme metabolising about 50% of metabolic cleared drugs includes benzodiazepines such as midazolam (model substrate), antihistamines such terfenadine, antifungals such ketoconazole, anesthesia sich as eflentanil and antihypertensives such felodipine and antiarrythimics such as verapamil. The enzyme is also metabolising paracetamol into the reactive metabolite NAPQI and aslo xenobiotics such as aflatoxin and benzphetamin into reactive metabolites	Luo et al. 2002

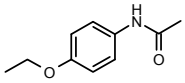
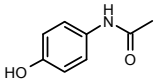
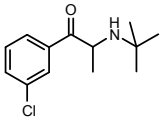
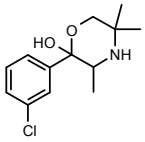
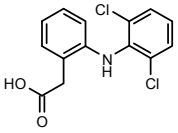
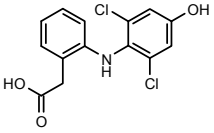
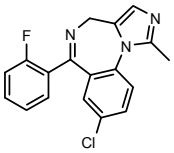
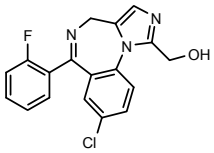
4.7 Standard Operating Procedure (SOP) of the *in vitro* methods

The final version of the SOPs "Cytochrome P450 induction in human cryopreserved hepatocytes (n-in-one incubations on 48-well plates)"and "Cytochrome P450 induction in CryoHepaRG® cells (n-in-one incubations on 96-well plates)" are provided in Appendix 06 and Appendix 07.

The CYP induction *in vitro* method is an *in vitro* cell based method in which the potential of four CYP isoforms (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) to be induced by selected test items is assessed in Cryoheps and CryoHepaRG® cells by measuring CYP-selective probe activities with an analytical method (LC/MS-MS).

The CYP induction *in vitro* method is performed with one 96-well-plate (CryoHepaRG®) or 48-well-plate (Cryoheps) for one cell batch (Figure 03). Cells are thawed on a Friday morning and allowed to attach for 4-6 hours. The medium is refreshed and the cells are allowed to recover for 72 hours. On Monday morning, medium is replaced by the test items and reference compounds dissolved in serum-free induction medium. The lead laboratories declared that there are no differences in terms of serum and protein content between the media used for the two test system. The induction solutions are renewed after 24 ± 0.3 h (multi - challenge exposure). After a total induction time of 48 ± 0.3 h (CryoHepaRG®) or 72 ± 0.5 h (Cryoheps), the probe substrate reaction is carried out. A cocktail of four P450 substrates is added to each well and incubated for 60 ± 3 min (CryoHepaRG®) or 30 min (Cryoheps), at 37 ± 1°C. At the end of the incubation time, the reaction is quenched by the addition of stop solution (acetonitrile + internal standards) and the samples are analysed for the specific products shown in Table 09 by means of LC/MS-MS.

Table 09 Specific P450 reactions (*cryoHepaRG®; ** cryoheps)

Isoenzyme	Probe Substrate	Product	Final test concentration [µM]	Incubation time [min]
CYP1A2	 Phenacetin	 Acetaminophen	26*; 10**	60* 30**
CYP2B6	 Bupropion	 Hydroxybupropion	100	
CYP2C9	 Diclofenac	 4-Hydroxydiclofenac	9*; 10**	
CYP3A4	 Midazolam	 1-Hydroxymidazolam	3	

Analytical quantification of products in incubation supernatants is performed by application of LC/MS-MS by coupling of analytical HPLC (High Performance Liquid Chromatography) and mass spectrometry (MS). HPLC is applied for concentration and purification of the product to be detected, whereas MS is applied for its specific quantification. For quantification the internal standard method is applied. An internal standard (INST) is a chemical that is added in a constant amount to the samples, the blank and the calibration standards in a quantitative analysis for correction for the loss of analyte during sample preparation or sample inlet. Griseofulvin (CryoHepaRG®) and DDIBA (5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid) (Cryoheps) are the internal standards. The selection of the INST was based on lead laboratory experience and historical data. The use of different compounds as INST has no impact on the quantitative results by LC-MS, as the INST is only volume marker, guaranteeing that the sample volume injected is constant.

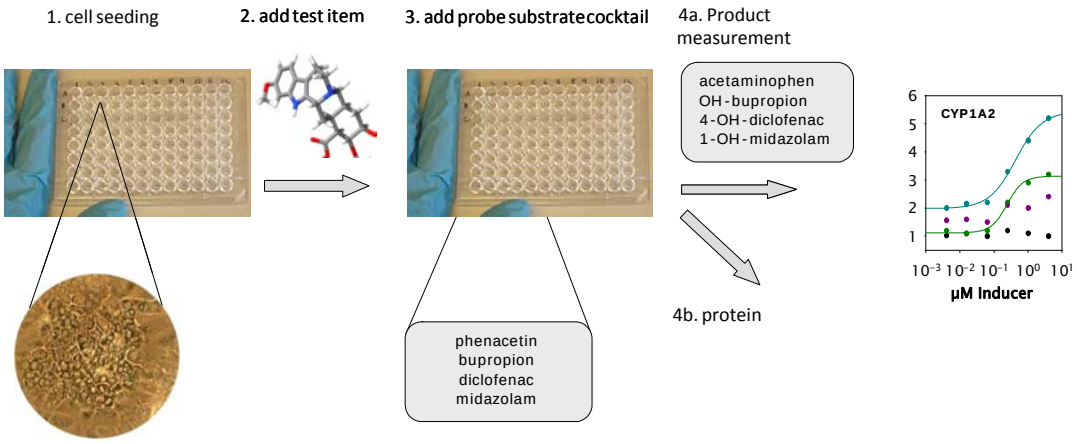


Figure 03 Experimental designs for CYP induction. Cells are seeded (1). Following 72 hours recovery, they are exposed to the test item (2). Fresh preparation of test item solution in medium is replaced every 24 hours. When exposure time is finished the test item solution is removed and the CYP selective substrates cocktail is added (3) to each well. Formation of the specific products acetaminophen (CYP1A2), hydroxybuproprion (CYP2B6), 4-hydroxydiclofenac (CYP2C9) and 1-hydroxymidazolam (CYP3A4) is analysed in the incubation supernatants following acetonitrile precipitation (4a). Data are normalised to protein content of each well (4b).

One complete experiment requires three experimental steps:

(I) solubility
(II) cytotoxicity
(III) induction

I. Solubility

Test items were investigated for their solubility in DMSO (or DMSO:water 1:1 blend) and for their solubility in the experimental conditions (cell culture medium, 37°C, 5%CO2). Based on the results, the highest soluble concentration to be used as starting concentration in cytotoxicity experiments was defined. When dealing with *in vitro* methods, it is very important to know the actual test item concentration that reaches the cells. For this reason, the solubility part was run in parallel at EURL ECVAM by the independent TIM team using not visual inspection (as specified in the SOPs) but nephelometer analysis.

II. Cytotoxicity

The cytotoxic potential of test items was assessed. Based on the results, the highest non-cytotoxic test item concentration to be used as starting concentration in induction experiments was defined.

For cryoheps, seven different concentrations per chemical were tested (n=3) on three batches of human cryopreserved hepatocytes (S240408, B270808, S2406A). The positive cytotoxicity control was 25µM chlorpromazine.
For cryoHepaRG®, eight different concentrations per chemical were tested (n=3) on one batch of cryoHepaRG® (HPR116035). The positive cytotoxicity control was 8µM doxorubicin.
The prototypical inducers[2] (25 µM β-naphthoflavone, 500 µM phenobarbital and 10 µM rifampicin) (Pelkonen et al, 2008) and the negative solvent control (0.1 %DMSO) were always run in parallel.

III. Induction

The potential of test items to induce one or more of the four selected CYPs is assessed. After a recovery period, cells were exposed to the test items. Subsequently, the conversion of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 probe substrates is measured using the "cocktail approach". A cocktail of the four CYPs' substrates is simultaneously applied and the corresponding products formation is simultaneously by LC/MS-MS analysis ("n-in one" reaction).
Six different concentrations per chemical were tested (n=3) on three cell batches (Cryoheps: S240408, B270808, S2406A; CryoHepaRG®: HPR116035, HPR116020, HPR116036). The prototypical inducers (25 µM beta-naphthoflavone, 500 µM phenobarbital and 10 µM rifampicin) and the negative solvent control (0.1% DMSO) were run in parallel.

Selection of positive cytotoxicity control

The aim of the cytotoxicity assay was to determine if the test items possess cytotoxic potential for cryohepsatocytes and cryoHepaRG® cells. The resulting data were used to determine the test item concentrations for the subsequent CYP induction studies.
Doxorubicin and chlorpromazine served as reference compounds with well-known cytotoxic properties. Based on the acceptance criteria stated in the final SOPs *doxorubicin has to lead to a reduction of cellular viability for 50-70% while chlorpromazine to produce a fractional survival of cells equal to or less than 70%*.

Prototypical CYP inducers

The reference prototypical inducers for the four selected CYP isoforms beta-naphthoflavone (CYP1A2), phenobarbital (CYP2B6) and rifampicin (CYP2C9, CYP3A4) were tested at single concentrations (25 µM, 500 µM, 10 µM) in accordance with the FDA Guidelines for drug-drug interaction (FDA, 2012) in all the cytotoxicity and induction experiments.
The experiments for the selection of the appropriate positive controls were performed beforehand to define the concentration and acceptance criteria for the experiments using cryoHepaRG® (see "Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 by Phenobarbital and Omeprazole in HepaRG® Cells" Short Report corresponding to Pharmacelsus Project No. HepaRG® Prevalidation Study of May 12, 2009).

[2]FDA and EMA Guidelines recommend that strong inducers should be included as positive controls to verify functioning regulation pathways via PXR, CAR and the Ah-receptor

None of the prototypical inducer was cytotoxic for cryoheps or cryoHepaRG® at the tested concentrations.

Optimisation of the SOPs

Following the original CYP induction SOPs submitted to EURL ECVAM, revisions of the SOPs were made in collaboration with Kaly Cell and Pharmacelsus GmbH. Both SOPs needed further test definition and test description additions to continue with the validation project and initiate the ring trial.

SOP Cytochrome P450 induction in CryoHepaRG® cells (n-in-one incubations on 96-well plates) - version 02 was prepared and released by Pharmacelsus GmbH on May the 20th 2012 and approved by the trial coordinator on June the 5th 2012. This version of the SOP is the one suggested for future use of CYP induction on CryoHepaRG cell line (**Appendix 06**).

SOP Cytochrome P450 induction in human cryopreserved hepatocytes (n-in-one incubations on 48-well plates) version 08 was prepared and released by Kaly Cell on November the 5th 2012 and approved by the trial coordinator on November the 5th 2012. This version of the SOP is the one suggested for future use of CYP induction on human cryopreserved hepatocytes (**Appendix 07**).

A detailed description of the modifications produced to improve the SOPs during the project is described in **Appendix 08** and **Appendix 09**.

More complex changes or improvements of the experimental design which took place during the validation project are the following:

I. Cryoheps: From 24 to 48 hours recovery period after seeding

An important difference between the two SOPs was the recovery period after thawing and before proceeding with the 72h test item treatment. For Cryoheps it was 24h instead of 48h, as recommended by FDA Guidelines.
During Module 2, the cryoheps' SOP was better defined in terms of cell recovery time after seeding and it was agreed to move from a 24 h to a 48 h. Based on the results performed on cryoheps batch HHC170407:

(I) the basal activities of all four CYPs after the 72h treatment with 0.1% DMSO (negative control) were about half after 48h recovery compared to a 24h recovery period;
(II) following 48h recovery, omeprazole induced CYP1A2 in a concentration-dependent way, as observed following the 24h recovery period. However the induction was higher in cells which were recovered for 48h.

Astra Zeneca performed the same protocol on the same cell batch.

Conclusions: by extending the pre-culturing period from 24 hours to 48 hours recovery before starting the induction experiments, the acceptance criteria for the prototypical inducers were met, due to lower CYP activities in untreated cells (0.1% DMSO).

II. Cryoheps: avoid working in the week-end

The lead laboratory was asked to further optimise the SOP in order to make it more workable and perform the SOP in 5 instead of 6 working days.

Cryopheps cell batch B270808 (as not enough cells were available from Cryoheps batch HHC170407) was thawed and seeded. Cells underwent medium change at different times after plating (24 and 72h) and the induction assay with reference prototypical inducers was performed after 24, 48 and 72h from cell seeing. For comparison, treatment with prototypical inducers started 24h, 48h and 72h after seeding.

Based on the results, confirmed also with Cryoheps cell batch N2309VT:

- 24h recovery after plating: (I) the response of CYP1A2 and CYP3A4 to their prototypical inducers met the acceptance criteria. The response of CYP2B6 and CYP2C9 did not meet the criteria due to the high response of the negative solvent control (0.1% DMSO); (II) with a medium exchange 7h after seeding, the acceptance criteria were met for CYP1A2, CYP2B6 and CYP3A4, but not for CYP2C9.
- 48h recovery after plating: (I) the basal activity for all four CYPs of the negative solvent control (0.1% DMSO) was less than after a 24h recovery period (II) the response of all four CYPs to their prototypical inducers met the acceptance criteria.
- 72h recovery after plating: (I) except for CYP1A2, the basal activities of all CYPs of the negative solvent control (0.1% DMSO) was less than after a 24h recovery and slightly higher than after 48h recovery. However the responses to the prototypical inducers were higher compared to those obtained following 24 and 48h recovery. With medium exchange 7h after plating, the acceptance criteria were met for all four CYPs.

Conclusions: the recommended SOP foresees:

(I) thawing and seeding cells on Friday,
(II) 7h after seeding medium exchange with fresh medium plus additive
(III) 72h after seeding (Monday) start induction experiments

The optimised SOP avoids working during the week-end.

III. Cryoheps: positive control for cytotoxicity: from 0.1 μM doxorubicin to 25 μM chlorpromazine (version 05):

Doxorubicin 0.1 μM, used as cytotoxicity positive control in cryoHepaRG® test method, was initially used also for Cryoheps. The lead laboratory changed the acceptance criteria from *doxorubicin 0.1 μM has to lead to a reduction of cellular viability for 50-70%* (as stated in the HepaRG®' SOP) to *doxorubicin 0.1 μM has to lead to a reduction of cellular viability for 30-70%*.

However, the high reproducible results obtained for CryoHepaRG® with doxorubixcin were not observed with Cryoheps.
Although, during the meeting of 16-17 September 2010 (**Appendix 03**), the poor reproducibility with Cryoheps was ascribed to technical issues (i.e. weighing of less than 1 mg of chemical), the lead laboratory proposed to use another chemical as cytotoxicity positive control. Chlorpromazine was selected due to the availability of historical data from the previous European project Predict IV.
Following evaluation of the KaLy-Cell report "Assessment of chlorpromazine as positive control for cytotoxicity: Additional results to module 1 test definition" in which it was concluded that *a concentration of 20μM chlorpromazine is likely to reduce Cryoheps cell viability by 50% ± 20% within 72 h of incubation,* the biostatistician recommended that the use of a 20μM concentration was not suitable (**Appendix 10**) to meet the acceptance criterion. During Module 4, the new acceptance criterion "*the positive control chlorpromazine at 20 μM had to induce between 30% and 70% of cell viability reduction (arithmetic mean) compared to the negative control*…"was not met. VMG asked the lead laboratory to perform extra experiments to identify the best chlorpromazine concentration and to evaluate intra - and between batches variability.
This step is crucial as chlorpromazine is used in the validation project as positive control for assessing the sensitivity of the human primary cryohepatocytes cell batches to its cytotoxicity. Cytotoxicity, based on these data, proved to be highly valuable to control the aspects of donor variability.
Between 2011 and 2012 extra experiments (amendment 01 - 3 October 2011; amendment 02 - 20 October 2011; and amendment 03 - 11 January 2012) were performed by the lead laboratory to identify the experimental conditions for the cytotoxicity positive control and to define the new acceptance criterion. The new experiments were performed on different Cryoheps batches and using different concentrations of chlorpromazine (**Appendix 11 and 12**). The VMG analysed the data and considered the variability between batches of Cryoheps reflecting the *in vivo* situation. This variability made it difficult to give a strict single concentration that would always be fulfilled as an acceptance criterion for cytotoxicity. There will always be outliers if a sufficient large number of Cryoheps batches are studied. Based on data provided, the VMG proposed 25 μM chlorpromazine to comply with requests from FDA and other regulatory entities and the acceptance criterion was modified in SOP version 06: "*assay meets the criteria if the positive control chlorpromazine at the concentration of 25 μM produces equal to or less than 70% fractional survival (FS) of the cell (calculated based on an arithmetic mean of replicates)*".

I. CryoHepaRG®: from fresh human HepaRG® cells to cryopreserved HepaRG® cell line

During Module 1 test definition, Biopredic, the supplier of the HepaRG® test system, announced that they would change from supplying fresh human HepaRG® cells to a cryopreserved HepaRG® cell line, the product that, from that time, would have been on the market and would guarantee a better controlled shipment. The difference between the CryoHepaRG® cell batches is referred to differences in time of preparation (differentiation); the source is always the same. Based on reliability issues encountered using freshly shipped HepaRG® cells, VMG agreed to continue the validation project with CryoHepaRG® and asked the lead laboratory to repeat Module 2 – within-

laboratory reproducibility - already performed with fresh HepaRG® cells, to assess if the cryopreserved product was performing as the fresh one.
Conclusion: CryoHepaRG® cells performed as good as fresh human HepaRG® cells. Between-batch variability was lower for CryoHepaRG® cells than for fresh HepaRG® cells. CryoHepaRG® cells were used for the whole validation project.

4.7.1 Acceptance criteria

The CYP induction SOPs, as submitted to EURL ECVAM by the lead laboratories, contained a set of acceptance criteria for the evaluation of runs to determine whether the obtained results are valid. The main change was in the cryoheps 'SOP with respect to the new acceptance criterion for the cytotoxicity positive control chlorpromazine (as refereed above). The laboratories raised no issues with respect to meeting these criteria during the project.

CYP enzymatic activity was normalized to protein content not to cell number (e.g. DAPI staining).

The following assessment criteria were discussed and agreed by the VMG
• Acceptance criteria for CYP induction:
- Exposure to reference items (positive controls) has to lead to a ≥ 2-fold increase of enzymatic activity (of statistical significance) at the defined fixed concentrations (Kanebratt, 2008).

- A test item is considered a potent inducer if a **≥2-fold** increase of enzymatic activity (of statistical significance) is measured. The criterion was a VMG decision, based on its experience with CYP activity. As a ≥2-fold increase is just point information, the VMG considered also important to observe a **dose response induction curve**. The VMG suggested that at least 2 out of the 6 concentrations should be above the background, to be sure data are relevant.

Cryoheps:
• Acceptance criteria with regards to the cells:
- After thawing, cell viability will need to be in the range +/- 10% of that given by KaLy-Cell, and attachment rate, measured by morphological observation of the cell monolayer, needs to be in the range +/- 10% of that given by KaLy-Cell.
- 70% confluent hepatocyte monolayer minimum after the 24h attachment period (morphological observations, see Figure 8–3).
- Less than 50% protein lost at the end of the 72-h induction period (T72 versus T0).
- Known chemical inducers (e.g. β-naphthoflavone, phenobarbital, and rifampicin) are included in every study. The cells are exposed to the reference items at a defined concentration for 72 hours in parallel to the exposure of the test items.

• Acceptance criteria for cytotoxicity assay:

- For the negative control, RFU > 100,000 have to be detected after 3 h of reagent incubation (specification for KaLy-Cell Multiplate Reader BioTek Synergy HT fluorimeter). If the optical density of the negative control wells is found < 100,000 the metabolic activity of the cell batch cannot be guaranteed and the assay needs to be repeated using a new cell batch.
- The positive control chlorpromazine at 25 µM has to produce equal to or less than 70% fractional survival (FS) of the cells (calculated based on an arithmetic mean of replicates).
- At least, two non-toxic concentrations should be found or the cytotoxic assay should be repeated with lower test item concentrations.
- Negative control and reference inducers should be ≥80%FS.

- Acceptance criteria for protein standard curve
  - The standard curve should have a correlation coefficient (r2) equal or greater than 0.95.
- Acceptance criteria for selection of appropriate test concentrations:
  - Test item has to be dissolved at all concentrations chosen for induction in induction medium (see chapter 8.1).
  - The highest concentration chosen for induction must not decrease cellular viability below 80% after 72 hours of incubation (see chapter 8.2).
  - In order to cover a full-dose response range, the highest concentration is serially diluted at 6 levels.

• Acceptance criteria for sequence analysis:
  - No more than 33.3% (2 of 6, 3 of 9, 4 of 12) of QC should be excluded (for all the reasons e.g.: loss of sample QC, poor injection, a value greater than ± 15 % of the nominal value ...).
  - At least 50% of a level of QC (QC1, QC2 and QC3) must be accepted within a sample list.
  - All blocks of QC must have at least 1 QC accepted.

CryoHepaRG®:

- Acceptance criteria after thawing and seeding:
  - minimum cell viability: 80 % after thawing
  - minimum recovery per vial: 4.5 x 10^6 cells/vial
  - About 80% confluent HepaRG® monolayer after the 72 h attachment period (morphological observation, see Figure 6 1).

- Acceptance criteria for cytotoxicity assays
  - For the negative control, RFU (relative fluorescence units) > 100,000 have to be detected after 3 h of reagent incubation (specification for Pharmacelsus Perkin Elmer Wallac Victor multiwell-plate fluorimeter). If the optical density of the negative control wells is found < 100,000 RFU, the metabolic activity of the cell batch cannot be guaranteed and the assay needs to be repeated using a new cell batch. The resulting RFU has to demonstrate the metabolic activity of the cells in the experiment. The negative control acceptance criterion should be established based on the analysis of historical data set for the equipment used.

- The positive control doxorubicin at 8 µM has to induce at least 30-70% of cell viability reduction (arithmetic mean) compared to the negative control.

4.8 Technical limitation and drawbacks of the test method

Test items that are not soluble or stable in the solvents recommended by the SOP cannot be tested. Some of the test items could not be tested because they were insoluble or unstable in solution.

For xenobiotics that interfere with the cytotoxicity assay reagent chemistry or the analytical determinations of metabolites cannot be tested an alternative approach has to be established e.g. xenobiotics that interfere with the cytotoxicity fluorescence measurement should be using an absorbance based cytotoxicity assay.

4.8.1 Limitation in applicability

The human *in vitro* CYP induction methods under investigation in this validation project measures not mRNA, but the functional endpoint, enzyme activity which is of major importance for the toxicity endpoints e.g. because of its capacity to produce reactive metabolites and effects on xenobiotic cellular concentrations. As a consequence, one of the limitations of this project is that the validated in vitro method does not include measurement of mRNA, which is required by FDA/EMA guidance on drug induction studies. However, as indicated before, it is well documented by solid scientific evidence that there is discrepancy between mRNA induction and catalytic activity and the lack of positive correlation between CYP activity and the specific CYP mRNA level ascribed to several different kinds of post-transcriptional control mechanisms including, factors controlling translation and post-translational insertion in the membranes and phosphorylation (see section: CYP induction: mRNA versus enzymatic activity). Although the understanding of the mechanism of transcriptional regulation of CYPs has progressed, the post-transcriptional regulation is still largely unclear.
Despite a variety of scientific evidence that mRNA is not always the adequate endpoint for evaluating CYP induction (see section: CYP induction: mRNA versus enzymatic activity for details), it is also of importance to note that the activity may be inhibited by metabolites of the putative inducer. Nowadays many high throughput in vitro methods are available for measuring the activation of nuclear receptors by xenobiotics. However, the observed activation of a nuclear receptor in an *in vitro* method does not necessarily indicate induction of the CYP enzyme activities (Abass et al, 2012).

Since the two test systems have only been tested with drugs, the applicability to other xenobiotics such as pesticides or industrial chemicals is strictly speaking unknown. However, it is probable that any chemical substance capable of binding to appropriate nuclear receptor

("xeno-sensor") and triggering the induction process should be a candidate inducer, whatever its chemical domain and use class. There is currently available ample published literature suggesting a more general applicability of the CYP induction test than pharmaceuticals (Pelkonen et al 2008; Hukkanen 2012; Abass et al 2012).

4.9 Conclusion of the Validation Management Group on Module 1

The CYP induction protocol proved to be generally robust for the purposes of this study, only minor clarifications were made to the SOP during the course of the study in relation to specific elements of the procedure and the data interpretation to minimise the sources of variability.

The additions in the sections describing the analysis sequence, data recording and analysis and calculation of results were introduced largely to resolve ambiguities and minor omissions in the original SOP in order to improve clarity and consistency of data generation and interpretation.

The cryoheps SOP was optimised to ensure completion of test runs within a working week and avoid commitment during the week-end.

Acknowledging that there will always be between human hepatocytes donor variability (which can be considered as an added value of this test system), chlorpromazine was used in the validation trial as positive control for assessing the sensitivity of the human cryohepatocytes cell batches to its cytotoxicity. Cytotoxicity, based on these data, proved to be highly valuable to control the aspects of donor variability.

The selection of a set of test items to be assayed blindly by test facilities was a major task of the VMG and proved to be difficult due to the restricted set of compounds with human (clinical) *in vivo* data on induction.

Following these procedural clarifications to the SOP, the VMG believes the supporting documents (including the original submission to ECVAM and associated scientific publications) and the current study findings adequately demonstrate the intended purpose, the need for, the status of development, and the scientific and mechanistic basis and relevance of the CYP induction test method.

In conclusion, the VMG believes that Module 1, Test Method Definition, is satisfied

5 WITHIN-LABORATORY REPRODUCIBILITY (MODULE 2)

Reference documents:

- *Statistical Report (**Appendix 13**)*

In this module information was gathered to assess the within-batch, within-laboratory and between-batch reproducibility of selected CYP enzymes in CryoHepaRG® cells and cryopreserved human hepatocytes. The within-laboratory reproducibility was based on preliminary pilot experiments performed in the lead laboratories but mainly on the data generated during the between-laboratory study when 13 blind coded test items were tested.

5.1 Preliminary experiments performed in the lead laboratories

5.1.1 CryoHepaRG

The study design for Module 2 was the following: (see Study Plan Module 2; Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 by troglitazone and omeprazole in CryoHepaRG® cells; approved by the trial manager on June 8th, 2010).

Within-batch reproducibility was tested by:

- One batch cryoHepaRG® (HPR116036)
- Three consecutive assays in independent experiments
- Two compounds (omeprazole, troglitazone)
- Compounds not blinded, test concentrations given
- First operator
- Only in the lead laboratory

Between-batch and **within-lab**oratory reproducibility was evaluated by:

- Three batches cryoHepaRG® (HPR116036, HPR116035, HPR116020)
- Two compounds (omeprazole, troglitazone)
- Compounds not blinded, test concentrations given
- First operator
- Second operator performs additional assay on the second and third batch

Experiment	Operator	batch	Batch #
1	1	1	HPR116035
2	2	1	HPR116035
3	1	2	HPR116020
4	2	2	HPR116020
5	1	3	HPR116036
6	1	3	HPR116036
7	1	3	HPR116036

Within-batch reproducibility was assessed in experiment 5, 6 and 7
Between-batch reproducibility was assessed in experiment 1, 3 and 5
Between-operator reproducibility was assessed in experiment 1, 2 and 3, 4
Within laboratory reproducibility was assessed in experiments 1-7.

Table 10 Positive control inducers

CYP450	Reference inducer	Substrate	Product
1A2	25 µM BNF	Phenacetin	Acetaminophen
2B6	500 µM Phenobarbital	Bupropion	Hydroxybupropion
2C9	10 µM Rifampicin, 500 µM Phenobarbital	Diclofenac	4-Hydroxydiclofenac
3A4	10 µM Rifampicin, 500 µM Phenobarbital	Midazolam	1-OH-Midazolam

25 µM β-Naphthoflavone, 10 µM rifampicin and 500 µM phenobarbital served as positive controls for induction based on experiments performed beforehand to define the concentration and acceptance criteria for the experiments using HepaRG® cells as well as CryoHepaRG® (see reports: "Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 by phenobarbital and omeprazole in HepaRG Cells" Pharmacelsus - HepaRG Prevalidation Study of May 12, 2009; "Test item concentration finding by evaluation of cytotoxicity of test items omeprazole and troglitazone towards HepaRG® cells / ECVAM validation study module 1: test definition" Pharmacelsus - HepaRG® / CYP Induction Validation Trial of February 24, 2010; "Follow up study of test item concentration finding by evaluation of cytotoxicity of test items omeprazole and troglitazone towards HepaRG® cells: Transfer of cytotoxicity determination and induction testing on CryoHepaRG® cells / ECVAM validation study module 1: test definition" Pharmacelsus - 2010ECV001: CryoHepaRG® / CYP Induction Validation Trial of August 17, 2010).
Based on data provided by the lead laboratory (Table 11), the applicability of 10 µM rifampicin as positive control inducer for CYP3A4 and 25 µM beta-naphthoflavone as positive control inducer for CYP1A2, was proven with CryoHepaRG® of three different batches because all met the criteria for CYP induction of ≥ 2-fold increase of enzymatic activity, which was also statistically greater than control values.

Table 11: n-fold induction by prototypical inducers. Mean is the mean value of all 7 runs.

CYP	Reference Items	Mean	SD	CV [%]
1A2	25 µM BNF	21.2	8.6	40.4
2B6	500 µM Phenobarbital	6.6	2.3	35.0
2C9	10 µM Rifampicin	1.3	0.3	22.8
3A4	10 µM Rifampicin	5.8	2.2	37.9

All assays met the acceptance criteria for CYP2B6 induction by 500 µM phenobarbital. The criterion for induction of 2C9 by 10 uM rifampicin was not met.

Test items

Omeprazole (100 µM, 66.6 µM, 44.4 µM, 29.6 µM, 19.7 µM, and 13.1 µM) and Troglitazone (10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM and 0.3125 µM) were used as test items in induction assay.

Table 12: n-fold induction rates for the highest test concentration of omeprazole and troglitazone (N=7, n=3) N=number of assays; n=number of plate replicates.

assay	batch	Test item concentration	operator	CYP1A2	CYP2B6	CYP2C9	CYP3A4
1	HPR116035	Omeprazole 100µM	1	16.6±1.9	1.2±0.1	0.7±0.1	0.7±0.1
2			2	12.5±2.5	0.9±0.2	0.5±0.01	0.9±0.1
3	HPR116020		1	21.4±4.8	1.9±0.3	0.9±0.2	0.7±0.1
4			2	17.1±1.8	1.9±0.2	0.8±0.1	0.9±0.1
5	HPR116036		1	10.8±1.5	1.3±0.1	0.8±0.1	0.7±0.1
6			1	11.3±1.7	0.8±0.1	0.5±0.1	0.4±0.1
7			1	16.1±1.2	1.1±0.1	0.9±0.1	0.4±0.03
1	HPR116035	Troglitazone 10µM	1	5.9±0.7	2.8±0.24	1.3±0.1	2.7±0.4
2			2	3.0±0.4	2.4±0.19	1.2±0.1	3.0±0.2
3	HPR116020		1	1.7±0.5	2.2±0.34	1.1±0.2	1.6±0.3
4			2	2.7±0.2	2.8±0.10	1.4±0.1	2.7±0.1
5	HPR116036		1	1.6±0.2	2.2±1.60	1.9±0.2	2.4±0.4
6			1	2.2±0.2	2.3±0.17	1.2±0.1	2.1±0.1
7			1	2.0±0.3	2.3±0.20	1.4±0.03	2.3±0.1

All assays demonstrated that CYP1A2 was inducible in CryoHepaRG® cells by omeprazole (mean fold induction 15.1, SD 3.8, CV=25.1%) at the highest test concentration (100 µM). Troglitazone, at the highest concentration of 10 µM weakly induced CYP1A2 (mean fold induction 2.7, SD 1.5, CV=54.4%).

Troglitazone at the highest concentrations was a weak inducer of CYP2B6 (mean 2.5, SD 0.3, CV=12.7%).

Neither CYP2C9 nor CYP3A4 were induced by omeprazole.

CYP3A4 was inducible by 10 µM troglitazone (mean 2.4, SD 0.5, CV=19.0%) whereas CYP2C9 was not induced by troglitazone.

The within-laboratory reproducibility was evaluated based on the induction values (Table 12) using the ≥2 fold induction classification by calculating the proportion of tests that yielded ≥2-fold induction. Date for omeprazole showed 100% within-batch (3/3), between-batch (3/3) and within-laboratory (7/7) reproducibility for the induction of all four CYPs. Based on data for troglitazone, the within-batch, between-batch, and within-laboratory reproducibility was 100% for CYP2B6 and CYP2C9. However, the within-batch and between-batch reproducibility for CYP1A2 was 67% (2/3) and the within laboratory reproducibility was 71% (5/7). For CYP3A4, the within-batch reproducibility was 100 % (3/3), the between-batch reproducibility was 67% (2/3) and the within laboratory reproducibility was 71% (5/7). This was not considered as an issue as troglitazone induction values were close the threshold of 2. Troglitazone might be a weak inducer and thus borderline compound for the established classification rule.

Acknowledging the limited number of tests item (omeprazole and troglitazone) and the limited data sets, overall, the VMG agreed that the in vitro method was reproducible.

5.1.2 Cryoheps

The aim of the study was to show the within-batch, within-laboratory and between-batch reproducibility of selected CYP enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) induction in cryopreserved human hepatocytes (see Study Plan Module 2; Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 by troglitazone and omeprazole in cryoheps; approved by the trial manager on May 12th, 2010).
The within-batch reproducibility was initially performed on three cryoheps cell batches (HHC170407; B270808; S270407). However, the high basal activity for the four CYP required the optimisation of the SOP in order to start the 72h treatment period 48h post seeding instead of 24h post seeding.
On December 2010 it was agreed by VMG and lead laboroatory to optoimise the cryoheps SOP by extending the pre-culturing period from 24 hours to. 48 hours. Also it was mentioned that literature describes that a longer recovery is necessary when hepatocytes are cryopreserved. Nevertheless, the protocol with 48h recovery period which consists of 6 working days performed by both KaLy-Cell and AstraZeneca, was not perceived by AstraZeneca as very workable, due to the necessary work on the Saturday. Therefore Kaly-Cell optimised the protocol, in order to avoid working in the week end.:
Following SOP optimisation, the within-batch reproducibility study was then repeated on the cryopreserved hepatocyte batch HHC170407. Three consecutive induction assays in independent experiments were performed using omeprazole as test item, tested at six given concentrations (50 µM, 20 µM, 8 µM, 3.2 µM, 1.28 µM, and 0.512 µM).

Table 13: Positive control inducers

CYP450	Reference inducer	Substrate	Product
1A2	25 µM BNF	Phenacetin	Acetaminophen
2B6	500 µM Phenobarbital	Bupropion	Hydroxybupropion
2C9	10 µM Rifampicin, 500 µM Phenobarbital	Diclofenac	4-Hydroxydiclofenac
3A4	10 µM Rifampicin, 500 µM Phenobarbital	Midazolam	1-OH-Midazolam

25 µM β-Naphthoflavone, 10 µM rifampicin and 500 µM phenobarbital served as positive controls for induction based on experiments performed beforehand to define the concentration and acceptance criteria for the experiments using cryopreserved human hepatocytes. Table 14 summarizes the results on induction by the prototypical inducers (positive control)

Table 14: n-fold induction by prototypical inducers. Mean is the mean value of all 3 runs.

		48h			24h		
CYP	**Reference Items**	**Mean**	**SD**	**CV [%]**	**Mean**	**SD**	**CV [%]**
1A2	25 µM BNF	14.2	6.3	44.7	3.2	0.8	24.2
2B6	500 µM Phenobarbital	2.7	0.3	9.3	1.7	0.9	23.9
2C9	10 µM Rifampicin	2.0	0.3	15.4	1.1	0.2	13.9
3A4	10 µM Rifampicin	6.3	1.0	15.7	2.6	0.2	9.7

With a 24h recovery after plating, the response of CYP1A2 and CYP3A4 to their respective prototypical inducer met the criteria while the response of CYP2B6 and CYP2C9 did not.

With a 48h recovery after plating, the basal activities of all CYPs after the 72h of 0.1% DMSO exposure were about half compared to those obtained with a 24h recovery period. The response of CYP1A2, CYP2B6, CYP3A4 and CYP2C9 to their respective prototypical positive inducer were ≥2-fold induction, thus, meeting the acceptance criteria.

Test items

Table 15: n-fold induction rates for the highest test concentration (50µM) of omeprazole (3 plate replicates) in Cryohep cells batch HHC170407 based on SOP 24 h (assay 1) and a 48h (assays 2-4) recovery after plating.

assay	CYP1A2	CYP2B6	CYP2C9	CYP3A4
1 24h	2.6±0.2	0.9±0.1	0.8±0.1	0.2±0.1
2 48h	13.5±1.0	1.7±0.2	2.1±0.4	0.6±0.1
3 48h	23.7±4.2	3.5±0.8	2.2±0.1	0.8±0.1
4 48h	11.0±1.3	2.4±0.5	1.9±0.2	0.6±0.1

As shown in table 15, all assays demonstrated that CYP1A2 was inducible in Cryoheps cells by omeprazole (mean 16.1, SD 6.3, CV=39.0%) at the highest test concentration (50 µM). Omeprazole at the highest concentrations was weak inducer of CYP2B6 (mean 2.6, SD 0.9, CV=35.9%). CYP2C9 (mean 2.1, SD 0.3, CV=12.9%) and CYP3A4 (mean 0.7, SD 0.1, CV=18.9%) were considered notinduced by omeprazole.
When treatment started 24h after plating, omeprazole, induced CYP1A2 (N=1, n=3; mean 2.6, SD 0.2, CV=8.4%) at the highest test concentration (50 µM) and did not induce CYP2C9, CYP3A4 and CYP2B6 (Table 15).
For CYP2C9, the prototypical inducer (RIF) did not met the acceptance criteria in 1/3 48h assays (assay 2) and in the 24h assay (assay 1). As shown in Table 15, omeprazole n-fold induction value was always above threshold 2 for CYP1A2. CYP2B6 and CYP2C9 n-fold induction rates were around the threshold 2. CYP3A4 was not induced by omeprazole, all values were <1.

The VMG concluded that omeprazole showed good within-batch reproducibility for CYP1A2 and CYP3A4 enzymes. The induction values for CYP2B6 and CYP2C9 were around the threshold 2 showing that omeprazole is weak inducer for these CYP isoforms in this specific cell batch.

5.2 Experiments performed in all the laboratories

During Module 4 (between laboratory reproducibility) each laboratory tested 13 blind coded test items on three different cell batches of cryoHepaRG and cryopreserved human hepatocytes for their potential induction of the four CYP isoforms.
Although the main purpose was to evaluate the between-laboratory reproducibility, the data generated during this Module can be used also for evaluating the between-batch and within-laboratory reproducibility.
The within-batch reproducibility cannot be evaluated on data generated during Module 4 as the experimental design was aimed primarily to assess the between-laboratory reproducibility and therefore each cell batch was tested only once (in triplicates per test item).
Table 16 and 17 summarise the between-batch reproducibility. Values are generated based on Table 3 and table 6 of the statistical report, to which you should refer for a detailed analysis.

Relative frequencies of 100% reproducibility of classification[3] across three cell batches are reported and the frequencies are aggregated over all test items and concentrations. Please note that this measure is underestimating the ideal reproducibility values (e.g. for test item that shows dose-response, a concentration at which induction changes from a value <2 to a value >2 might not be a good candidate to measure the reproducibility).

Table 16: Between-batch reproducibility based on 2-fold induction threshold classification in cryoHepaRG cells

Laboratory	CYP1A2	CYP2B6	CYP2C9	CYP3A4
Janssen	82% (46/60)	75% (45/60)	93% (56/60)	87% (52/60)
Pharmacelsus	72% (43/60)	75% (45/60)	80% (48/60)	92% (55/60)
EURL ECVAM	85% (51/60)	78% (47/60)	90% (54/60)	88% (53/60)

Table 17: Between-batch reproducibility based on 2-fold induction threshold classification in cryohep cells

Laboratory	CYP1A2	CYP2B6	CYP2C9	CYP3A4
AstraZeneca	66% (39/59)	78% (46/59)	83% (49/59)	78% (46/59)
KaLy-Cell	82% (42/51)	60% (30/50)	82% (41/50)	82% (41/50)
EURL ECVAM	77% (54/70)	60% (42/70)	83% (58/70)	74% (52/70)

For all the CYP enzymes a higher reproducibility was obtained for the cryopreserved HepaRG than the cryoheps. However, this result can be expected because cryoHepaRG cell batches were generated from one donor, while cryopreserved hepatocytes originated from three different donors. The between-donor variability in the cryoheps provides added value because it is closer to what actually happens in the human population.
Although high reproducibility was obtained for CYP2C9 in both test systems, it should be noted that the acceptance criteria for the inducer positive control (10μM rifampicin) were not met.

[3] The easiest implementation is to calculate (estimate) the n-fold induction by dividing the averaged measured enzyme activity of treated cells by the averaged measured enzyme activity of control cells. (The average is taken over the values related to wells on plate assigned for cell exposure). The resulting value is then compared with a threshold 2.

5.3 Conclusion of the Validation Management Team on Module 2

On the basis of the experiments conducted specifically for Module 2 and of those produced for Module 4 (see Module 4) the VMG concluded that within-laboratory reproducibility was at a sufficient level.

6 TRANSFERABILITY (MODULE 3)

Reference documents:

- *Training plan cryoHepaRG® (**Appendix 14**)*
- *Study plan "Module 3" of 13/07/2010 (**Appendix 15**)*
- *Study report "Transfer of assays for cytotoxicity and induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 by Troglitazone and Omeprazole in CryoHepaRG® cells according to STUDY PLAN Module 3 of 13/07/2010"of 07/10/2011 (**Appendix 16**)*
- *Statistical Report (**Appendix 13**)*
- *Agenda cryoHepaRG transfer Workshop (**Appendix 17**)*
- *Agenda cryoheps transfer Workshop (**Appendix 18**)*

6.1 General aspects

The CYP induction test method can be performed in any laboratory working with minimum standards in cell culture (Good Cell Culture Practice) and equipped with analytical instruments (e.g LC-MS/MS). All apparatus and reagents needed for the performance of the method are readily available commercially. The cryoHepaRG® cells are available from different suppliers in Europe, USA, Japan and Brazil.

Kaly Cell and Pharmacelsus, being the lead laboratories, were responsible for the training of the personnel of the other laboratories participating in the project and for providing advice during the test method transfer in order to ensure that the procedure described in the respective SOPs were clearly understood and promptly implemented.

The trained personnel were then responsible for the transfer of the test method to their own laboratories under the supervision of the lead laboratories (Module 3).

The schedule for the training of these laboratories as well as the details for the transfer experiments, were drafted by the lead laboratories on the basis of their experience with the test method.

To demonstrate successful method transfer the laboratories had to perform the test method procedure by testing in-house two chemicals: omeprazole and troglitazone and met the transfer acceptance criteria as defined in the study plan Module 3.

The two compounds used in this module were proposed by the lead laboratories and approved by VMG prior of initiating the transfer module.

The two chemicals were not supplied by the TIM group at EURL ECVAM, but purchased by the laboratories. The results were sent directly to the lead laboratories for evaluation.

6.2 Preliminary experiments performed in the lead laboratories

6.2.1 CryoHepaRG

Training

Pharmacelsus provided training to study personnel of IBET and EURL ECVAM on solubility, cytotoxicity and induction on the 7-9 October 2008 on the protocol for the fresh HepaRG® cells. Due to the announcement that Biopredic would no longer provide fresh cells, the training session was repeated with the new cryopreserved HepaRG® test system on the 1st and 2nd of July 2010. Janssen Pharmaceutica took part to this training session.

Transfer of the test method to the laboratories

During Module 3, which is defined as the assessment of transferability, in parallel to the experiments of module 2 (within-laboratory reproducibility) performed at the lead laboratory, Janssen Pharmaceutica and EURL ECVAM performed two independent runs of the cytotoxicity assay and two independent induction assays to verify transferability of the method. The experiments were performed on cryoHepaRG® cell batch HPR116036 according to the following scheme:

I. Transferability of cytotoxicity assessment
   a. One batch HepaRG®
   b. Two assays
   c. Two compounds (omeprazole, troglitazone)
   d. Compounds not blinded, test concentrations given (SOP)
   e. One operator

II. Transferability of induction assay
   a. One batch HepaRG®
   b. Two assays
   c. Two compounds (omeprazole, troglitazone)
   d. Compounds not blinded, test concentrations given (SOP)
   e. One operator

For module 2, the lead laboratory performed a within-laboratory and within-batch reproducibility study for the induction assay (one operator, three independent experiments) using batch HPR116036 in parallel in order to directly compare to the module 3 results. The results of the cytotoxicity studies from Janssen Pharmaceutica and EURL ECVAM were compared to the data generated by the lead laboratory during module 1 (test definition).
The two test items were used at the following concentrations, based on solubility and cytotoxicity data of Module 1, test definition.

Cytotoxicity assay:

100 μM for Troglitazone and 100 μM for Omeprazole, followed by a serial 1:3 dilution.
   a. Omeprazole was tested at 100.00-33.33-11.11-3.70-1.23-0.412-0.137-0.045μM
   b. Troglitzone was tested at 100.00-33.33-11.11-3.70-1.23-0.412-0.137-0.045μM
   c. Doxorubicin, the positive control for cytotoxicity was tested at 8μM

Induction assay:
   a. Omeprazole, starting concentration 100μM, followed by 1:1.5 dilutions:
      100 μM – 66.6 μM – 44.4 μM – 29.6 μM – 19.8 μM – 13.2 μM
   b. Troglitazone, starting concentration 10μM, followed by 1:2 dilution:
      10 μM – 5 μM – 2.5 μM – 1.25 μM – 0.625 μM – 0.313 μM
   c. 25 μM b-Naphthoflavone, 10 μM rifampicin and 500 μM phenobarbital were the positive controls for induction

The laboratories submitted the results directly to the lead laboratory, which on the 23rd of August 2010, produced an interim transfer report. The results submitted by Janssen Phharmaceutica were accepted by the lead laboratory and thus, the CYP induction assay was successfully transferred to this laboratory. The results submitted by EURL ECVAM were not accepted, as they did not meet the bioanalytical acceptance criteria defined in the SOP. The calibration curve acceptance criterion that at least 75% of the calibration standards have to be within 15% (20% for lower limit of quantification -LLOQ) of the nominal concentration was neither met for hydroxybupropion nor for 4-hydroxydiclofenac. For acetaminophen the sensitivity of the method was too low. The LLOQ was established at 62.5 nM. As a result, the values for the solvent treated control could not have been reported quantitatively because they were below LLOQ. Therefore, it was not possible to evaluate the n-fold induction of the other compounds.

The lead laboratory recommended that EURL ECVAM optimise the analytical method and then validate the method.

In June 2011, after optimisation and validation of the LC-MS method, two induction runs were repeated as recommended by the lead laboratory. The *in vitro* method acceptance criteria were met for protein analysis and LC-MS analysis.
The standard curves used to evaluate protein content in the wells met the acceptance criterion of correlation coefficient (r2) >0.9. Regarding LC-MS analysis, 5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid (DDIBA) was used as internal standard instead of griseofulvin and for both runs the acceptance criteria were met as:

- at least 75% of the calibration standards were within 15% (20% for LLOQ) of the nominal concentration and the number of valid calibration standards was then above 6;
- LLOQ and ULOQ for all compounds were established at the levels of 7.8 nM and 2000 nM, respectively and were part of the calibration curve;
- at least 50% of samples within each QC level were within 15% of the nominal concentration;
- CV of DDIBA was below 20 %;
- The peak shape, resolution and retention time of the peaks of interest were adequate and consistent.

Experimental data were submitted to the lead laboratory for evaluation and a study report "Transfer of assays for cytotoxicity and induction of CYP1A2, CYP2B6, CYP2C9,and CYP3A4 by Troglitazone and Omeprazole in CryoHepaRG® cells according to Study Plan Module 3 of 13/07/2010" was produced for VMG submission.

Janssen Pharmaceutica and EURL ECVAM performed the cytotoxicity assay with omeprazole and troglitazone in two independent runs. EURL ECVAM met the acceptance criteria as defined in the SOP. The IC50 in in run 1 was 2-3fold higher than reported previously. In addition, in the concentration-response curves differed slightly from the experiments performed by the lead laboratory and Janssen Pharmaceutical. One cytotoxicity assay (run 2) performed at Janssen did not met the acceptance criteria (see Table 18) since the fractional survival of the positive control (8 µM doxorubicin) was found below 30%-70%. However, as the IC50 value of troglitazone and the shape of the concentration-response curve of omeprazole were comparable to the data reported by the lead laboratory in previous experiments, the assay was considered as accepted.

Table 18: IC50 values of test items and corresponding positive control (8µM doxorubicin)

Test item	Laboratory	Cell batch	IC50[4] [mM]	% fractional survival positive control[5]
omeprazole	Pharmacelsus[6]	HPR116020	n/a[7]	46.2±0.8
troglitazone			41.6	45.6±1.4
omeprazole	Janssen Pharmaceutica (run1)	HPR116036	n/a	55.9±0.9
troglitazone			32.2	54.2±1.6
omeprazole	Janssen Pharmaceutica (run2)		n/a	22.6±0.5
troglitazone			29.7	22.5±0.8
omeprazole	EURL ECVAM (run1)		n/a	61.2±3.0
troglitazone			97.5	55.9±4.8
omeprazole	EURL ECVAM (run2)		n/a	58.8±5.0
troglitazone			29.9	51.0±0.4

[4] IC50 were calculated by plotting log(test item concentration) against %fractional survival using the equation: Y=Bottom+(Top-Bottom)/(1+10^((LogIC50-X)*HillSlope)), if applicable

[5] Acceptance criterion for cytotoxicity assay positive control is 30-70% fractional survival in presence of 8µM doxorubicin. Results are expressed as arithmetic mean ± SD

[6] Test definition assay reported by Pharmacelsus (August 17,2010)

[7] n/a not applicable (% fractional survival>50%)

Table 19: Induction of CYP enzyme activity in cryoHepaRG® cell batch HPR 116036, following exposure to 100 μM omeprazole in two or three independently performed assays per laboratory

Test item	Laboratory	CYP1A2	CYP2B6	CYP2C9	CYP3A4
run 1	Pharmacelsus	10.8±1.5	1.3±0.1	0.8±0.1	0.7±0.1
run 2		11.3±1.7	0.8±0.1	0.5±0.1	0.4±0.1
run 3		16.1±1.2	1.1±0.1	0.9±0.1	0.4±0.03
run 1	Janssen Pharmaceutica	16.9±3.0	1.6±0.3	0.8±0.1	0.9±0.1
run 2		8.6±1.0	0.5±0.1	0.4±0.1	0.4±0.04
run 1	EURL ECVAM	21.6±8.3	1.7±0.4	1.3±0.5	0.6±0.02
run 2		95.3±10.3	4.8±0.8	1.7±0.3	0.7±0.1

Table 20 Induction of CYP enzyme activity in cryoHepaRG® cell batch HPR 116036, following exposure to 10 μM troglitazone in two or three independently performed assays per laboratory

Test item	Laboratory	CYP1A2	CYP2B6	CYP2C9	CYP3A4
run 1	Pharmacelsus	1.6±0.2	2.2±1.6	1.9±0.2	2.4±0.4
run 2		2.2±0.2	2.3±0.2	1.2±0.1	2.1±0.1
run 3		2.0±0.3	2.3±0.2	1.4±0.03	2.3±0.1
run 1	Janssen Pharmaceutica	1.7±0.2	2.5±0.2	1.5±0.3	2.3±0.3
run 2		2.0±0.4	3.1±0.4	1.4±0.1	2.5±0.3
run 1	EURL ECVAM	3.0±2.0	2.1±1.3	1.2±0.8	2.4±1.3
run 2		3.7±1.13	3.0±1.0	1.9±0.5	2.7±0.9

Transfer experiments at Janssen were performed in June-July 2010. Transfer experiments at EURL ECVAM were performed between July 2010 and June 2011, as extensive work was done to optimise and validate the LC-MS analysis.

Following the teleconference in September 2011, VMG approved the successful method transfer of cytotoxicity and induction satisfied that all the acceptance criteria as stated in the SOP were met.

6.2.2 Cryoheps

Training

Kaly Cell provided training to study personnel of Astar Zeneca and EURL ECVAM on seeding and cultivating cryoheps and on the induction assay from the 19th to the 21st of April 2010 following the cryoheps SOP.

Transfer of the test method to the laboratories

During Module 3, which is defined as the assessment of transferability, in parallel to the experiments of module 2 (within-laboratory reproducibility) performed at the lead laboratory, Astra Zeneca and EURL ECVAM performed two independent runs of the cytotoxicity assay and two independent induction assays to verify transferability of the method. The experiments were performed on cryohep cell batch B240608 according to the following scheme:

I. Transferability of cytotoxicity assessment
   a. One batch cryoheps
   b. Two assays
   c. Two compounds (omeprazole, troglitazone)
   d. Compounds not blinded, test concentrations given (SOP)
   e. One operator

II. Transferability of induction assay
   a. One batch cryoheps
   b. Two assays
   c. Two compounds (omeprazole, troglitazone)
   d. Compounds not blinded, test concentrations given (SOP)
   e. One operator

For module 2, the lead laboratory performed a within-laboratory and within-batch reproducibility study for the induction assay (one operator, three independent experiments) using batch B240608 in parallel in order to directly compare to the module 3 results. The results of the cytotoxicity studies from AstraZeneca and EURL ECVAM were compared to the data generated by the lead laboratory.
The two test items were used at the following concentrations, based on solubility and cytotoxicity data of Module 1, test definition.

Cytotoxicity assay:

50 μM for Troglitazone and 33.65 (Astra Zeneca) μM / 50 (EURL ECVAM) μM for Omeprazole, followed by a serial 1:3 dilution.

a. Omeprazole was tested at: 50 μM – 25 μM – 12.5 μM – 6.25 μM – 3.13 μM – 1.56 μM
   33.65μM – 16.8 μM – 8.4 μM – 4.2 μM – 2.1 μM – 1.05 μM
b. Troglitzone was tested at 50 μM – 25 μM – 12.5 μM – 6.25 μM – 3.13 μM – 1.56 μM c. Doxorubicin, the positive control for cytotoxicity was tested at 0.1μM

Induction assay:

a. Omeprazole, starting concentration 100μM, followed by 1:1.5 dilutions:
   50 μM – 25 μM – 12.5 μM – 6.25 μM – 3.13 μM – 1.56 μM
b. Troglitazone, starting concentration 10μM, followed by 1:2 dilution:
   3 μM – 1.5 μM – 0.75 μM – 0.375 μM – 0.187 μM – 0.093 μM

c. 25 µM beta-naphthoflavone, 10 µM rifampicin and 500 µM phenobarbital were the positive controls for induction

The laboratories submitted the results directly to the lead laboratory, which in June 2011, produced a transfer report. The results submitted by Astra Zeneca and EURL ECVAM were accepted by the lead laboratory and thus, the CYP induction assay was successfully transferred to this laboratory.

Table 21 Induction of CYP enzyme activity in cryohep cell batch HHC170407, following exposure to Omeprazole in Kaly-Cell and AstraZeneca laboratories

Omeprazole [µM]	CYP1A2		CYP2B6		CYP3A4		CYP2C9	
	Kaly-Cell	AstraZeneca	Kaly-Cell	AstraZeneca	Kaly-Cell	AstraZeneca	Kaly-Cell	AstraZeneca
0.512	2.1	1.8	1.0	1.0	1.0	0.9	1.5	1.1
1.28	3.5	1.5	1.0	0.8	0.9	0.6	1.4	0.9
3.2	7.7	4.3	1.2	1.1	1.0	0.7	1.6	1.1
8	14.2	9.8	1.2	1.6	0.9	1.0	1.8	1.3
20	15.6	15.7	1.6	2.6	0.8	1.5	1.9	1.5
50	14.4	17.0	2.5	2.6	0.7	1.3	2.1	1.4

The assays performed in Kaly Cell and AstraZeneca with batch HHC170407 met the positive control criteria for all CYPs (except AstraZeneca for CYP2C9). The results on n-fold induction of omeprazole are shown in Table 21. The values of n-fold CYP induction obtained by AstraZeneca were similar to those obtained by KaLy-Cell. Omeprazole was found strong inducer for CYP1A2 with concentration dependent response. The CYP2B6 was induced at highest concentration (50µM) tested at both laboratories.

Table 22 Induction of CYP enzyme activity in cryohep cell batch B240608, following exposure to Omeprazole in EURL ECVAM and AstraZeneca laboratories

Omeprazole [µM]	CYP1A2		CYP2B6		CYP3A4		CYP2C9	
	EURL ECVAM	Astra Zeneca	EURL ECVAM	Astra Zeneca	EURL ECVAM	Astra Zeneca	EURL ECVAM	Astra Zeneca
0.512	1.2	1.0	1.1	0.8	0.9	0.7	1.1	0.9
1.28	1.6	1.6	1.1	1.0	0.8	0.8	1.2	1.3
3.2	2.2	2.2	1.0	0.9	0.5	0.5	1.2	1.3
8	3.4	3.3	0.9	0.8	0.3	0.3	1.1	1.3
20	4.9	4.7	0.8	0.6	0.2	0.2	1.3	1.3
50	6.1	4.1	0.8	0.4	0.1	0.1	1.2	1.3

The assays performed in EURL ECVAM and AstraZeneca with batch B240608 did not meet the positive control criteria for all CYPs but CYP1A2. The VMG decided to take these results into account for transferability evaluation as the profile for the 4 CYP enzyme activities was as expected. The results on n-fold induction of omeprazole are shown in Table 22. As seen from Table 22, the pairs of values at given concentration for both laboratories are either both below or above the threshold 2 resulting to 100% reproducibility. Omeprazole was found strong inducer for CYP1A2 with concentration dependent response. None of the remaining 3 CYPs was induced by omeprazole in batch B240608.

6.3 Conclusion of the Validation Management Team on Module 3

Based on scientific considerations and the data sets received both the lead laboratories consider the two additional test facilities fully competent and ready to move to Module 4 (teleconference minutes 13 September 2011). The VMG had no additional concerns to proceed to Module 4 (between laboratory variability). At that stage of the validation project, acknowledging the limited data sets based only on two test items, the two test methods seemed reliable to VMG, but only additional full data sets from Modules 4a and 4b would allow the VMG to draw conclusion on reliability.

The VMG concluded that the CYP induction test method was successfully transferred from the lead Laboratories, one for HepaRG cells and the other for human primary hepatocytes, to the other laboratories. All the problems experienced by the participating laboratories during the transfer phase proved to be due either to reagents or instrument configuration which were resolved and addressed in the revised SOP where appropriate.

Concerning the acceptance criteria that were set prior to the initiation of the study both laboratories experienced problems in fully meeting some of them.

Being LC-MS based, the CYP induction test method requires strict adherence to the stringent and demanding criteria required for the implementation of bioanalytical methods for quantitative determination of parent compounds and metabolites. As demonstrated by some of the issues encountered during this transfer phase, performance of the CYP induction assay requires a sufficiently powerful MS machine and detailed understanding of the techniques and strict adherence to the specified equipment and procedural details.

In conclusion the VMG considers that the CYP induction test method can be readily transferred among properly equipped and staffed laboratories. The biochemical techniques involved are commonly used in modern analytical laboratories and human cell cultures do not require any extraordinary machinery or environment. Experienced personnel can readily be trained in the test method, and the necessary equipment and supplies can be readily obtained. The CYP induction SOPs are clearly written and the analysis can be performed without difficulties.

6.4 Experiments performed in all the laboratories

During Module 4 (between laboratory variability) each laboratory tested 13 blind coded test items for solubility and cytotoxicity. As a result, in the induction experimental phase, 10 test items were evaluated on three different cell batches of cryoHepaRG cells and 12 test items on three different cell batches of cryoheps.
The main purpose of Module 4 was to evaluate the between-laboratory reproducibility.

Table 23 Between laboratory reproducibility based on 2-fold induction threshold classification in cryohepaRG cells

Cell batch	CYP1A2	CYP2B6	CYP2C9	CYP3A4
HPR116020	95% (57/60)	82% (49/60)	93% (56/60)	90% (54/60)
HPR116035	83% (50/60)	75% (45/60)	90% (54/60)	95% (57/60)
HPR116036	68% (41/60)	70% (42/60)	83% (50/60)	90% (54/60)

Table 24 Between laboratory reproducibility based on 2-fold induction threshold classification in cryoheps cells

Cell batch	CYP1A2	CYP2B6	CYP2C9	CYP3A4
B270808	80% (45/56)	67% (37/55)	84% (46/55)	71% (39/55)
S240408	58% (35/60)	37% (22/60)	77% (46/60)	55% (33/60)
S2406A	74% (52/70)	63% (44/70)	90% (63/70)	61% (43/70)

Table 23 and 24 summarise the between laboratory reproducibility. Values are generated based on Table 2 and table 5 of the statistical report, to which you should refer for a detailed analysis. Relative frequencies of reproducibility of classification across three laboratories are reported and the frequencies are aggregated over all test items and concentrations. Please note that this measure is underestimating the ideal reproducibility values (e.g. for test item that shows dose-response, a concentration at which induction changes from a value <2 to a value >2 might not be a good candidate to measure the reproducibility).

Due to cryohep cell recovery issue after thowing not all test item concentrations were always assessed and therefore the denominator in the frequency ratio is varying in Table 24.

Analysis of activity and induction results produced by different laboratories with the same batches indicated that concordance was dependent on test system used used.

CryoHepaRG showed higher reproducibility for the 4 CYPS under investigation compared to cryoheps. The VMG concluded that the BLR is satisfactory for all CYPs. The highest reproducibility value was observed for CYP3A4 (all ≥ 90%).

CryoHeps showed lower reproducibility for the 4 CYPS under investigation compared to cryoHepaRGs. Based on the information generated, and not having the availability of such historical data for other similar ring trials (since this validation project was the first in its kind) , the VMG concluded that the BLR is not satisfactory for one (Batch S240408) out of the three batches for the four CYPs. For this batch the lowest reproducibility value was observed for CYP2B6 (all 37%). The other two batches showed BLR values between 61% and 80% (CYP2C9 excluded).

7 BETWEEN-LABORATORY REPRODUCIBILITY (MODULE 4)

Reference documents:

- *Statistical Report (**Appendix 13**)*
- *Hill curve fit to HepaRG and cryoheps data (**Appendix 17**)*

The between laboratory reproducibility was assessed on the basis of the potential of the thirteen selected test items to induce CYP1A2, CYP2B6, CYP2C9 and CYP3A4. However, all data sets even form the previous Modules contributed to the overall evaluation of the reliability of the test methods.

The experimental design of Module 4 foresees three phases (as described in chapter 5. Standard Operating Procedure (SOP) of the in vitro methods):

(1) Solubility
(2) Cytotoxicity
(3) Induction

It was decided by VMG prior to the initiation of Module 4 to split it into Module 4a, where the first four test items were assessed and Module 4b where the remaining nine test items were tested. At the end of Module 4a, the laboratories were asked to provide study reports and data for VMG. Only after the evaluation of the laboratory's reports, the VMG gave the green light to the laboratories to proceed with Module 4b.

In this validation report data on solubility and cytotoxicity are summarized (Table 25 and 26). The primary analysis is performed on induction as it is the data on which the between laboratory, between batches and within-batch evaluation is evaluated. All the data from Module 4a and 4b are analysed together.

7.1 Solubility

For *in vitro* methods the concentration of the test item in the exposure medium is important in order to adequately estimate the exposure that affectst with cellular receptors and cause the biological effect measured (endpoint) on the test system. The aim of solubility testing was to identify the highest soluble concentration (Table 25 and 26) of the test item to be used as a starting concentration in cytotoxicity assay.

Table 25 Results on the thirteen test items solubility (mg/mL) and solubility in exposure medium (µg/mL): *cryoHepaRG* method SOP visual inspection (Detailed description in Appendix 06)

Chemical Name	**Pharmacelsus**	**Janssen Ph.**	**EURL ECVAM**	TIM[8]
	solubility (DMSO)[9]	solubility (DMSO)[8]	solubility (DMSO)[8]	solubility (DMSO)[8]
	solubility (medium)[10]	solubility (medium)[9]	solubility (medium)[9]	solubility (medium)[9]
Omeprazole	40	40	40	40
	40	40	40	40
Carbamazepine	40	40	40	40
	40	40	40	40
Phenytoin sodium	40[11]	20	40	40 [10]
	40	20	40	40
Penicillin G sodium	40	40	40	40
	40	40	40	40
Indole carbinol	40	40	40	40
	5	***10***	***20*** [12]	***10***
Rifabutin	40	40	40	40
	40	***20***	40	40
Sulfinpyrazone	40	40	40	40
	40	40	40	40
Bosentan hydrate	40	40	40	40
	40	40	40	40
Artemisinin	40	40	40	40
	40	40	40	40
Efavirenz	40	40	40	40
	20	40	40	**20**
Rifampicin	40	40	40	40
	40	40	40	40
Metoprolol	40	40	40	40
	40	40	40	40
Sotalol hydrochloride	40	40	40	40
	40	40	40	40

[8] Test item Management group
[9] stock solutions (DMSO solvent): 40 mg/mL limit concentration
[10] GlutaMAX medium dilutions (x1000, with incubation): 24 hours at 37°C
[11] DMSO+water (1:1) blend
[12] precipitation observed

Table 26 Results on the thirteen test items solubility (mg/mL) and solubility in exposure medium (µg/mL): cryoheps method SOP visual inspection (Detailed description in Appendix 06)

Chemical Name	**AstraZeneca**	**KalyCell**	**EURL ECVAM**	TIM
	solubility (DMSO)	solubility (DMSO)	solubility (DMSO)	solubility (DMSO)
	solubility (medium)	solubility (medium)	solubility (medium)	solubility (medium)
Omeprazole	40	40	40	40
	40	40	40	40
Carbamazepine	40	40	40	40
	40	40	40	40
Phenytoin sodium	20	40	10	40
	20	40	10	40
Penicillin G sodium	40	40	40	40
	40	40	20	40
Indole carbinol	40	40	40	40
	20	***20***	***20***	40
Rifabutin	40	40	40	40
	40	40	40	40
Sulfinpyrazone	40	40	40	40
	40	40	40	40
Bosentan hydrate	40	40	40	40
	40	40	40	40
Artemisinin	40	40	40	40
	40	40	40	40
Efavirenz	40	40	40	40
	40	40	40	40
Rifampicin	40	40	40	40
	40	40	40	40
Metoprolol	40	40	40	40
	40	40	40	40
Sotalol hydrochloride	40	40	40	40
	40	40	40	40

Based on the reported results, VMG agreed that all test items, exept from indole carbinol, had to be tested in cytotoxicity assay at the starting concentration of 40µg/ml (working concentration) to determine if they possess cytotoxic potential. Because indole carbinol precipitated after the 24 hour incubation, it was not tested for cytotoxicity. All chemicals were dissolved in DMSO apart from phenytoin which had to be prepared in a blend DMSO:water 1:1.

7.2 Cytotoxicity

Based on the SOPS, only test item concentrations which lead to less than 10% reduction of cellular viability within 48 hours in the HepaRG® test system can be employed in induction assays. For the cryopreserved human hepatocytes the test item concentrations that lead to less than 20% reduction of cellular viability in vitro within 72 hours can be further used for the induction assay.
For cryoheps all test items were tested at the concentration starting from 40 µg/ml followed by a 1:1 dilution ratio (40 µg/ml – 20 µg/ml – 10 µg/ml – 5 µg/ml – 2.5 µg/ml – 1.25 µg/ml – 0.63 µg/ml). Each concentration was tested in triplicate.
Positive inducers (β-naphtoflavone, phenobarbital and rifampicin) were tested at the concentrations used for the subsequent induction experiments, i.e. 25 µM, 500 µM and 10 µM, respectively (n = 3). Chlorpromazine at 25 µM was included as the positive control (n = 3).
For cryoHepaRG, the highest applicable test concentration was 40 µg/ml followed by a serial 1:3 dilution (i.e. 40.00 – 13.33 – 4.44 – 1.48 – 0.49 – 0.16 – 0.055 – 0.018 µg/ml). Each concentration was tested in triplicate and doxorubicin served as positive control.

Based on cytotoxicity results provided by the laboratories, rifabutin and efavirez were considered cytotoxic in cryoHepaRG cells and excluded for further induction assays. Rifabutin, bosentan and efavirez were tested in cryoheps, although at a starting concentration < 40 µg/ml (20; 10; 2.5 µg/ml). In November 2011 (Module 4a) and in March 2013 (Module 4b), the VMG agreed to perform the Module 4a and 4b-induction with the highest test concentrations as follows, applying a 1:3 serial dilution:

Test item	**cryoHepaRG** µg/ml	**Cryoheps** µg/ml
Omeprazole	40	40
Carbamazepine	40	40
Phenytoin sodium	30[13]	40[12]
Penicillin G sodium	40	40
Indole carbinol	Excluded for solubility issues	
Rifabutin	cytotoxic	20
Sulfinpyrazone	40	40
Bosentan hydrate	40	10
Artemisinin	40	40
Efavirenz	cytotoxic	2.5
Rifampicin	40	40
Metoprolol	40	40
Sotalol hydrochloride	40	40

[13] The solvent to be used was a 1:1 blend DMSO:water

7.3 Induction: Assessment of reproducibility in different laboratories

Between laboratory reproducibility based on the potential of blinded compounds to induce CYP1A2, CYP2B6, CYP2C9 and CYP3A4 enzyme activity was examined in Module 4. Tested compounds not excluded because of solubility or/and cytotoxicity issues were examined in the induction phase of Module 4. Thus, 10 test items were evaluated in the cryoHepaRG system and 12 substances were evaluated in the cryoheps system.

In this section between-batch and between-laboratory reproducibility has been evaluated. Three laboratories participating in the cryoHepaRG validation study are Pharmacelsus GmbH (lead laboratory), Janssen Pharmaceuticals and EURL ECVAM, whereas validation assay on cryopreserved primary human hepatocytes (cryoheps) was performed in Kaly-Cell (lead laboratory), AstraZeneca and EURL ECVAM laboratories.

7.3.1 Statistical analysis of induction potential of test items

The CYP induction results are expressed as P450 activities in pmol/min/mg protein.
The induction potential of a test item is calculated in two ways:
• n-fold induction of solvent-treated control (0.1% DMSO), i.e. calculated by normalizing the enzymatic activity in presence of the test item to the enzymatic activity in absence of the test item,
n-fold induction = activity of treated cells/activity of control cells,
• the percentage response is calculated as a percentage of the positive control (PC) as follows:
Percentage of PC = (activity of treated cells) - (activity of control cells)/[(activity of positive control cells) - (activity of control cells)],
where ”Treated cells” are cells treated with the test compound, ”Control cells” are cells treated with 0.1% DMSO and ”Positive control cells” are cells treated with the reference inducer.

7.3.2 Statistical definition of potent inducer

It was decided earlier by VMG (module 1) that a compound shall be a potent inducer in the *in vitro* system when observed ≥2-fold increase in enzyme activity of probe substrate at inducer concentrations < 500 µM. The implementation of the above mentioned threshold of 2-fold increase in enzyme activity is not straightforward.

Approach 1: The easiest implementation is to calculate (estimate) the n-fold induction by dividing the averaged measured enzyme activity of treated cells by the averaged measured enzyme activity of control cells. (The average is taken over the values related to wells on plate assigned for cell exposure). The resulting value is then compared with a threshold 2.

Approach 2: The second implementation is based on the assumption that the threshold is related to underlying population induction and not sample induction. This implementation tests the hypothesis that enzyme activity in controls cells (treated by solvent only) is less than twice the activity of treated cells (by specific compound at given concentration). The implementation requires not only estimated averaged n-fold induction as in Approach 1 but estimated variance of the estimated n-fold induction.

Approach 3: The last possible understanding of the rule above is to provide user-friendly implementation of testing the hypothesis of equal enzyme activity in controls cells (treated by solvent only) and treated cells (by specific compound at given concentration), i.e. twice higher activity shall sufficiently guarantee that the underlying ”population” enzyme activity of treated cells is well above activity of control cells. In this case, it would be more practical to test the hypothesis of equal enzyme activity in controls cells and treated cells.

In the statistical report, all 3 approaches have been used to present the results. However, in further analysis and interpretation of induction results additional criteria have been employed (see below).

7.3.3 Initial evaluation of reproducibility between batches and laboratories

Based on the above mentioned statistical analysis, a measure of between-batch reproducibility for given laboratory (BBR-lab) and a measure of between-laboratory reproducibility for a given batch (BLR-b) are constructed and summarized in Tables 1-6 of the Statistical Report.

• BBR-lab is represented by frequency of n-fold induction being ≥2. Frequency is taken over three batches (for a given laboratory, concentration and enzyme). Max Frequency is 3, i.e. for all three batches n-fold induction > 2. Min Frequency is 0, i.e. for all three batches is n-fold induction < 2. (see Table 3 and 6 in Stat Report)

• BLR-b is represented by frequency of n-fold induction being ≥2. Frequency is taken over three labs(for a given batch, concentration and enzyme). Max Frequency is 3, i.e. in all three labs n-fold induction ≥2. Min Frequency is 0, i.e. in all three labs is n-fold induction < 2. (see Table 2 and 5 in Stat Report)

An overall measure of between laboratory reproducibility (BLR) is defined as a frequency of n-fold induction being > 2. Frequency is taken over three batches and three laboratories (for a given chemical, concentration and enzyme). Max Frequency is 9 (in red), i.e. in all batches and laboratories n-fold induction > 2. Min Frequency is 0(in green), i.e. in all laboratories for all batches is n-fold induction < 2. (See Table 1 and Table 4 in Stat Report).

As can be seen from Tables 1 – 6 of the Statistical Report, the between batch and between laboratory reproducibility is quite good for cryoHepaRG cells. In case of cryoheps the between batch reproducibility is not as good when seen from n-fold induction indicator. However, in this analysis the principal indicator is the statistically significant ≥2-fold increase in enzyme activity. Later, a more in-depth analysis of induction status will be performed and assessed.

7.3.4 Assessment on the basis of basal activities and positive model inducers

This analysis is based on sections 2.3 of the statistical report, especially tables 7-14 and figures 1-12.

Each plate in the induction experiments included wells for the measurement of both basal activities and those induced by positive control inducers beta-naphthoflavone (BNF), phenobarbital (PB) and rifampicin (RIF). These activities should be very illustrative about the variability of four CYP-selective acitivies in both cell lines with three batches in each in five laboratories and these over the time period of induction experiments.

Table 27 contains the average CYP activities in basal and induced cells of different batches. The variability of CYP activities is markedly higher in cryoheps compared to cryoHepaRG cells, reflecting the heterogeneity of CYP expression in human liver and/or quality of the cells (Hewitt et al 2007). This is in agreement with the characterization CYP activity of HepaRG cells and primary human hepatocytes as untreated cells and in response to several prototypic inducers (Gerets et al 2012). However, the variability could also originate from isolation, freezing and thawing procedures.

Table 27. Basal and induced CYP enzyme activities in different batches of cryoHepaRG cells and cryoheps.

CryoHepaRG

	Basal Activity			BNF			RIF			PB		
	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036
CYP1A2	0.8 *	0.7	0.8	36.9	28.5	22.0	4.7	4.6	3.8	5.8	5.6	4.4
CYP2B6	0.9	1.1	0.9	2.0	2.2	1.6	2.2	2.3	1.9	9.4	10.7	7.6
CYP2C9	25.2	16.4	14.8	23.0	15.2	11.5	30.4	20.1	16.9	55.9	37.0	30.7
CYP3A4	4.2	2.8	4.4	1.3	0.9	1.1	32.2	33.3	30.4	31.5	31.8	30.1

*CYP enzyme activity average values over plates and laboratory are expressed as pmol specific probe substrate metabolite /mg protein/min

Cryoheps

	Basal Activity			BNF			RIF			PB		
	B270808	S240408	S2406A	B270808	S240408	S2406A	B270808	S240408	S2406A	B270808	S240408	S2406A
CYP1A2	7.6*	14.7	6.1	128.0	186.1	138.2	9.1	11.7	10.8	17.1	23.0	17.6
CYP2B6	1.3	3.1	2.0	1.8	4.7	3.6	7.8	12.5	13.4	10.9	25.5	27.9
CYP2C9	44.5	32.8	27.5	38.3	31.6	23.0	80.7	64.8	51.8	72.6	53.0	45.1
CYP3A4	5.9	20.5	3.8	2.8	14.6	2.3	52.8	61.2	44.0	52.0	55.4	40.7

*CYP enzyme activity average values over plates and laboratory are expressed as pmol specific probe substrate metabolite /mg protein/min

As an example, Tables 28-32 summarize the results expressed as fold-inductions in the same cell samples originating from basal and positive control inducer wells. Table 28 provides a comprehensive picture of n-fold inductions in various experiments in three batches of cryoHepaRG cells in three laboratories. Tables 29 to 32 analyzed within batch and between batch variabilities of the experiments described in Table 27. Another set of tables on cryoheps 33-37.areshown in Tables

There is a considerable variability over time (i.e. within batch), in some cases 2- to 3-fold, but coefficients of variation were rarely over 50 %, usually between 20 and 40 % (Table 30a). It has to be stressed that the biological tool in these studies is a cell system, which has variable output dependent on time, place, operator, and numerous aspects inherent in the setup. In this respect the variabilities exemplified here seem acceptable and not conspicuously different from other cell systems and setups.

Table 28. Induction of enzyme activity in cryoHepaRG cells with positive control inducer (n-fold induction rate)

		Janssen			Pharmacelsus			EURL ECVAM		
	run	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036
CYP1A2 (BNF)	1	19.7	18.7	29.3	41.5	43.0	25.5	21.1	26.9	21.2
	2	41.8	25.4	51.4	58.0	61.7	21.9	5.8	71.8	37.3
	3	55.0	41.9	23.9	69.2	64.2	48.0	18.6	13.2	19.8
	4	56.1	50.2	14.1	94.1	46.5	62.9	12.3	13.9	8.4
	5	36.4	38.5	25.9	60.5	62.1	63.9	26.6	6.7	46.9
CYP2B6 (PB)	1	13.6	6.9	9.6	11.9	9.6	10.9	17.8	6.7	14.3
	2	12.1	9.8	12.3	17.0	14.1	8.6	17.6	13.0	14.1
	3	5.8	6.9	5.2	10.6	12.7	6.2	9.9	17.4	7.4
	4	9.4	5.8	3.7	17.6	9.8	5.9	14.7	12.1	9.6
	5	5.5	6.2	5.0	8.5	6.6	7.6	9.9	7.1	19.7
CYP3A4 (RIF)	1	5.9	10.0	5.9	6.0	7.1	6.1	12.0	6.4	9.3
	2	9.6	11.8	9.7	8.7	10.1	5.7	11.6	8.3	12.0
	3	4.7	12.3	4.8	15.7	18.8	10.5	7.3	7.6	7.7
	4	7.3	9.3	4.9	11.1	15.1	6.5	7.1	8.6	8.3
	5	4.6	10.6	7.3	12.3	27.5	9.0	9.0	6.8	8.8

Table 29. Within Batch Variability. Average +- STD of n-fold induction rates over runs taken from Table 28 (n-fold induction rate)

	Janssen			Pharmacelsus			EURL ECVAM		
	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036
CYP1A2 (BNF)	41.8 ± 15.0	35.0 ± 12.7	28.9 ± 13.8	64.7 ± 19.3	55.5 ± 9.9	44.4 ± 20.0	16.9 ± 8.1	26.5 ± 26.4	26.7 ± 15.3
CYP2B6 (PB)	9.3 ± 3.6	7.1 ± 1.6	7.1 ± 3.6	13.1 ± 4.0	10.6 ± 2.9	7.8 ± 2.0	14.0 ± 3.9	11.3 ± 4.5	13.0 ± 4.7
CYP3A4 (RIF)	6.5 ± 2.1	10.8 ± 1.2	6.5 ± 2.0	10.8 ± 3.7	15.7 ± 8.0	7.6 ± 2.1	9.4 ± 2.3	7.5 ± 1.0	9.2 ± 1.7

Table 30a. Within Batch Variability. Coefficient of Variation of n-fold induction rates over runs taken from Table 28 (n-fold induction rate)

	Janssen			Pharmacelsus			EURL ECVAM		
	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036	HPR116020	HPR116035	HPR116036
CYP1A2 (BNF)	36%	36%	48%	30%	18%	45%	48%	99%	57%
CYP2B6 (PB)	39%	22%	51%	31%	28%	26%	28%	40%	36%
CYP3A4 (RIF)	32%	11%	31%	34%	51%	28%	24%	13%	18%

Table 30b. Within Laboratory Variability for each batch. Coefficient of Variation of n-fold induction rates over runs taken from Table 28 (n-fold induction rate)

	HPR116020			HPR116035			HPR116036		
	Janssen	Pharmacelsus	EURL ECVAM	Janssen	Pharmacelsus	EURL ECVAM	Janssen	Pharmacelsus	EURL ECVAM
CYP1A2 (BNF)	36%	30%	48%	36%	18%	99%	48%	45%	57%
CYP2B6 (PB)	39%	31%	28%	22%	28%	40%	51%	26%	36%
CYP3A4 (RIF)	32%	34%	24%	11%	51%	13%	31%	28%	18%

Table 31. Between Batch Variability. Average +- STDEV of n-fold induction rates over runs, over batches taken from Table 28 (n-fold induction rate)

	Janssen	Pharmacelsus	EURL ECVAM
CYP1A2 (BNF)	35.2 ± 13.9	54.9 ± 17.9	23.4 ± 17.5
CYP2B6 (PB)	7.9 ± 3.1	10.5 ± 3.6	12.8 ± 4.2
CYP3A4 (RIF)	7.9 ± 2.7	11.3 ± 5.9	8.7 ± 1.8

Table 32. Between Batch Variability. Coefficient of Variation of n-fold induction rates over runs, over batches taken from Table 28 (n-fold induction rate)

	Janssen	Pharmacelsus	EURL ECVAM
CYP1A2 (BNF)	40%	33%	75%
CYP2B6 (PB)	39%	35%	33%
CYP3A4 (RIF)	34%	52%	21%

Table 33. Induction of enzyme activity in cryohep cells with positive control inducer (n-fold induction rate)

		AstraZeneca			KaLy-Cell			EURL ECVAM		
	run	B270808	S240408	S2406A	B270808	S240408	S2406A	B270808	S240408	S2406A
CYP1A2 (BNF)	1	9.9	3.5	9.9	21.8	22.6	27.8	6.9	9.0	33.3
	2	14.0	4.2	13.6	27.6	26.8	28.6	13.1	8.9	29.7
	3	20.5	22.8	18.4	44.3	8.6	27.6	17.4	9.1	34.3
	4	28.6	24.1	23.0	58.3	15.5	46.9	13.5	6.7	35.4
	5	22.8	10.3	21.6	7.3	8.9	24.2	14.3	10.5	22.9
	6	20.0	24.9	21.8	12.4		18.4	17.9	9.2	25.9
CYP2B6 (PB)	1	4.8	4.7	13.0	10.3	9.9	5.9	5.8	6.3	11.6
	2	6.0	5.7	12.5	10.3	9.2	8.7	5.0	4.6	12.3
	3	8.7	9.4	13.6	20.5	9.9	7.0	5.7	6.5	12.5
	4	9.2	9.6	14.8	25.1	10.8	13.2	5.9	0.0	12.7
	5	7.3	5.0	14.3	10.6	12.3	19.8	4.4	8.0	10.3
	6	8.9	10.0	21.3	13.6		18.2	5.1	7.4	10.3
CYP3A4 (RIF)	1	8.3	4.3	10.2	8.6	3.5	8.2	10.4	2.9	11.0
	2	7.2	3.8	8.7	9.2	2.7	5.9	10.9	3.0	10.1
	3	5.7	2.9	11.3	19.1	2.6	13.6	10.8	2.7	12.3
	4	6.4	4.0	15.7	13.5	2.9	23.7	11.1	2.9	10.8
	5	6.0	2.1	13.5	12.6	5.1	10.1	11.1	3.2	12.0
	6	7.1	2.8	15.6	16.7		8.0	11.1	2.8	15.5

Table 34. Within Batch Variability. Average +- STD of n-fold induction rates over runs taken from Table 33 (n-fold induction rate)

	AstraZeneca			KaLy-Cell			EURL ECVAM		
	B270808	S240408	S2406A	B270808	S240408	S2406A	B270808	S240408	S2406A
CYP1A2 (BNF)	19.3 ± 6.6	15.0 ± 10.1	18.0 ± 5.3	28.6 ± 19.5	16.5 ± 8.1	28.9 ± 9.6	13.9 ± 3.9	8.9 ± 1.2	30.3 ± 5.0
CYP2B6 (PB)	7.5 ± 1.8	7.4 ± 2.5	14.9 ± 3.2	15.1 ± 6.3	10.4 ± 1.2	12.1 ± 5.9	5.3 ± 0.6	5.5 ± 2.9	11.6 ± 1.1
CYP3A4 (RIF)	6.8 ± 0.9	3.3 ± 0.9	12.5 ± 2.9	13.3 ± 4.1	3.3 ± 1.0	11.6 ± 6.5	10.9 ± 0.3	2.9 ± 0.2	11.9 ± 1.9

Table 35a. Within Batch Variability. Coefficient of Variation of n-fold induction rates over runs taken from Table 33 (n-fold induction rate)

	AstraZeneca			KaLy-Cell			EURL ECVAM		
	B270808	S240408	S2406A	B270808	S240408	S2406A	B270808	S240408	S2406A
CYP1A2 (BNF)	34%	68%	29%	68%	49%	33%	28%	14%	17%
CYP2B6 (PB)	24%	34%	22%	42%	11%	49%	11%	53%	9%
CYP3A4 (RIF)	14%	26%	23%	31%	31%	56%	2%	6%	16%

Table 35b. Within Batch Variability. Coefficient of Variation of n-fold induction rates over runs taken from Table 33 (n-fold induction rate)

	B270808			S240408			S2406A		
	AstraZeneca	KaLy-Cell	EURL ECVAM	AstraZeneca	KaLy-Cell	EURL ECVAM	AstraZeneca	KaLy-Cell	EURL ECVAM
CYP1A2 (BNF)	34%	68%	28%	68%	49%	14%	29%	33%	17%
CYP2B6 (PB)	24%	42%	11%	34%	11%	53%	22%	49%	9%
CYP3A4 (RIF)	14%	31%	2%	26%	31%	6%	23%	56%	16%

Table 36. Between Batch Variability. Average +- STDEV of n-fold induction rates over runs, over batches taken from Table 33 (n-fold induction rate)

	AstraZeneca	KaLy-Cell	EURL ECVAM
CYP1A2 (BNF)	17.4 ± 7.4	25.2 ± 14.0	17.7 ± 10.0
CYP2B6 (PB)	9.9 ± 4.4	12.7 ± 5.2	7.5 ± 3.5
CYP3A4 (RIF)	7.5 ± 4.3	9.8 ± 6.1	8.6 ± 4.3

Table 37. Between Batch Variability. Coefficient of Variation of n-fold induction rates over runs, over batches taken from Table 33 (n-fold induction rate)

	AstraZeneca	KaLy-Cell	EURL ECVAM
CYP1A2 (BNF)	42%	56%	57%
CYP2B6 (PB)	44%	41%	46%
CYP3A4 (RIF)	57%	63%	50%

7.4 Reproducibility between batches and laboratories

This analysis is based on the experiments in five laboratories focusing on the concentration-dependent induction responses of four CYP-selective activities by test items in cryoHepaRG cells and human cryoheps in culture. Initial statistical analysis is presented in the Statistical Report, especially tables 15-58 and figures 13-56.

The basis for the subsequent assessment of the experiments referred to above is the assessment of the primary results according to the batches and laboratories. In an earlier phase of the study, the VMG tentatively decided on the following criteria for the significant induction:

*A test item is considered an inducer if a **≥2-fold** increase of enzymatic activity (of statistical significance) is measured. The criterion was a VMG decision, based on their experience with CYP activity. VMG pointed out that as a ≥2-fold increase is just a point information it is important to observe also a **dose response induction curve**. The VMG suggested that at least 2 out of the 6 concentrations should be above the background, to be sure data are relevant. The points should be clearly above the background signal.*

However, VMG did not consider the situation in which the increase is less than 2-fold, even if it is statistically significantly different from 1. It is obvious that statistically significant increases, even if <2-fold may suggest induction of lesser potency. Also, the significance of the form of concentration-response curve remained to be decided later when the results from decisive experiments have been collected. The application of the Hill equation to the test results was performed in an attempt to provide a formal background for the assessment of the concentration-response curve (see appendix 17). It has to be noted, however, that the fit to the Hill curve does not provide statistical significance when the actual curve is steeply increasing at the highest concentrations. In this case, if the curve is otherwise consistent, the induction status is reinforced.

It is clear that besides the statistical treatment of the induction results, there is also a relatively large component of judgemental assessment, especially in assessing the concentration-response curve and its consistency and irregularity. This assessment is based largely on the experiences of VMG members in their experimental work on induction of CYP enzymes in various circumstances.

The following criteria were used for making the decision about the classification of response.

Within batch

'Potent inducer' (marked ++ in table M4.1)
- a statistically significant ≥2-fold increase
- no major irregularities in concentration-response curve

'Weak inducer' (marked + in table M4.1)
- at least 2 statistically significant >1 and <2-fold increases
- one increases may be judged to be adequate, if such an increase occurs at the highest concentration in a consistent concentration-response curve
- no major irregularities in concentration-response curve

'Non-inducer' (marked – in table M4.1)
- no statistically significant increases in any activity point
- statistically significant increases in one or 2 points without apparent consistency
- major irregularities in concentration-response curve

Within laboratory

'Potent inducer'

- in at least two batches a statistically significant ≥2-fold increase
- a consistent concentration-response curve (visually and/or Hill analysis)

'Weak inducer'

- in one batch a statistically significant ≥2-fold increase and a consistent curve
- in at least 2 batches a statistically significant >1 and <2-fold increase
- occurrence of such increases at the highest concentrations in a reasonably consistent concentration-response curve
- no major irregularities in concentration-response curve

'Non-inducer'

- a statistically significant <2-fold increase in one batch, or several in batches displaying irregularities in concentration-response curves
- no statistically significant increases in any activity point
- major irregularities in concentration-response curve

The definion of 'weak inducer' is not very precise and only slight variations in this definition would cause differences in the final classification of a test item. There is a borderline between potent inducer and non-inducer, which is partially created by sporadic statistically significant points and irregularities in concentration-response curves. The interpretation of borderline cases is certainly vulnerable to various viewpoints and criticisms, but it is of importance to raise this problem and make it transparent in deciding whether a compound is an inducer or non-inducer.

The following tables present the batch and laboratory focused analysis of induction status of test items.

TABLE M4.1 Evaluation of concentration-dependent induction responses by test items according to batch and laboratory based on the application of the above mentioned more extensive criteria.

Omeprazole/HepaRG (based on table 15 and figure 13 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	++	Potent inducer	++H	Potent inducer	++H	Potent inducer
	HPR116035	++		++H		++	
	HPR116036	++H		++H		++H	
CYP2B6	HPR116020	+	Potent inducer	++H	Potent inducer	++	Potent inducer
	HPR116035	++H		+H		-	
	HPR116036	-		++		+	
CYP2C9	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	- (1 ss)*		- (1 ss)		-	
	HPR116036	-		-		-	
CYP3A4	HPR116020	-H	Weak inducer	+	Potent inducer	++H	Potent inducer
	HPR116035	+H		- (1 ss)H		++	
	HPR116036	- (1 ss, cc)		+		-	
Omeprazole/Cryoheps (based on table 16 and figure 14 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	++H	Potent inducer	++H	Potent inducer	++H	Potent inducer
	S240408	++		++		++H	
	S2406A	++H		++		++	
CYP2B6	B270808	+	Potent inducer	-	Potent inducer	++H	Potent inducer
	S240408	++H		-		++H	
	S2406A	++H		++		++	
CYP2C9	B270808	-	Non-inducer	- (1 ss)	Non-inducer	-	Non-inducer
	S240408	+		+		-	
	S2406A	-		-		-	
CYP3A4	B270808	++H	Potent inducer	+(1 ss, irr)	Potent inducer	++H	Potent inducer
	S240408	-		- (1 ss)		-H	
	S2406A	++H		++ (cc)H		++H	

Carbamazepine/HepaRG (based on table 19 and figure 17 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	++	Potent inducer	++H	Potent inducer	+ (cc)	Potent inducer
	HPR116035	++H		++H		+ (cc)	
	HPR116036	++H		++H		+ (cc)	
CYP2B6	HPR116020	++	Potent inducer	++H	Potent inducer	++	Potent inducer
	HPR116035	++H		++H		++	
	HPR116036	++H		++H		++H	
CYP2C9	HPR116020	++	Weak inducer	+H	Weak inducer	-	Weak inducer
	HPR116035	-		- (cc)		+ (1 ss, cc)	
	HPR116036	-H		-		+ (cc)	
CYP3A4	HPR116020	++	Potent inducer	++H	Potent inducer	++	Potent inducer
	HPR116035	++		++H		++H	
	HPR116036	++H		++H		++	
Carbamazepine/Cryoheps (based on table 20 and fig 18 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	-	Non-inducer	-	Weak inducer	+	Weak inducer
	S240408	+ (irr)		- (1ss, irr)		+	
	S2406A	-		++		+ (irr)	
CYP2B6	B270808	++	Potent inducer	-	Potent inducer	++	Potent inducer
	S240408	++		++		++	
	S2406A	++H		++		++	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-H	Non-inducer
	S240408	+		+ (irr)		-	
	S2406A	-(irr)		+ (irr)		-	
CYP3A4	B270808	++	Potent inducer	++	Potent inducer	++	Potent inducer
	S240408	++		++		++	
	S2406A	++H		++		++	

*qualifiers used: ss, (number of individual test values) statistically significant; cc, (concentration-response) curve consistent; irr, irregularities in concentration-response curves. Original values and curves can be found in appropriate tables and figures of the statistical report. H refers to a statistically significant fit of the experimental concentration-response curve to the Hill equation (see StatRep 2).

Phenytoin/HepaRG (based on table 23 and figure 21 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	++	Potent inducer	++	Potent inducer	++H	Potent inducer
	HPR116035	++		++		+ (cc)	
	HPR116036	++		++		++	
CYP2B6	HPR116020	++	Potent inducer	++H	Potent inducer	++H	Potent inducer
	HPR116035	++		++		++	
	HPR116036	++H		++H		++H	
CYP2C9	HPR116020	++H	Potent inducer	+	Weak inducer	-	Non-inducer
	HPR116035	++		+		-	
	HPR116036	++H		+		+	
CYP3A4	HPR116020	++	Potent inducer	++	Potent inducer	++H	Potent inducer
	HPR116035	++		++		++	
	HPR116036	++		++		++	
Phenytoin/ Cryoheps (based on table 24 and figure 22 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	+ (1 ss, cc)	Potent inducer	-H	Potent inducer	++	Potent inducer
	S240408	+		+		++	
	S2406A	++		++		++	
CYP2B6	B270808	+	Potent inducer	-	Potent inducer	++	Potent inducer
	S240408	++		++		++	
	S2406A	++H		++		++H	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		- (1 ss)	
	S2406A	-		-		-	
CYP3A4	B270808	++H	Potent inducer	++H	Potent inducer	++	Potent inducer
	S240408	-H		++		++	
	S2406A	++H		++		++H	

Rifabutin/ Cryoheps (based on table 31 and fig 29 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	+	Weak inducer	+(irr)	Non-inducer	+	Weak inducer
	S240408	+ (ns, cc)		-		-	
	S2406A	+H		-		+	
CYP2B6	B270808	++	Potent inducer	+ (irr)	Non-inducer	+	Potent inducer
	S240408	-		-		++	
	S2406A	++		-		+	
CYP2C9*	B270808	+ (4 ss)	Weak inducer	+ (irr)	Weak inducer	+	Weak inducer
	S240408	+ (ns, cc)		+		+	
	S2406A	+ (6 ss)		+		+	
CYP3A4	B270808	++	Potent inducer	+ (irr)	Weak inducer	++	Potent inducer
	S240408	-		+		+	
	S2406A	++		+ (irr)		++	

*flat curves, but consistent

Sulfinpyrazone/HepaRG (based on table 33 and figure 31 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	++H	Potent inducer	++H	Potent inducer	+ (cc)	Potent inducer
	HPR116035	++		++H		+ (cc)H	
	HPR116036	++H		++H		++H	
CYP2B6	HPR116020	++	Potent inducer	++H	Potent inducer	-	Weak inducer
	HPR116035	++		+H		-	
	HPR116036	++H		+H		+ (cc)	
CYP2C9	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-(3 ss, irr)		-		-	
	HPR116036	-		-		-(3 ss, irr)	
CYP3A4	HPR116020	++H	Potent inducer	++H	Potent inducer	++H	Potent inducer
	HPR116035	++H		++H		++H	
	HPR116036	++H		++H		++H	
Sulfinpyrazone / Cryoheps (based on table 34 and fig 32 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	-	Weak inducer	+	Non-inducer	-	Weak inducer
	S240408	+		-		-	
	S2406A	+		-		++	
CYP2B6	B270808	++	Potent inducer	++	Potent inducer	++	Potent inducer
	S240408	++		+		++	
	S2406A	++		++		++	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	-		-		-	
CYP3A4	B270808	++H	potent inducer	++H	Potent inducer	++H	Potent inducer
	S240408	+		+		+H	
	S2406A	++H		++H		++H	

Bosentan/HepaRG (based on table 37 and figure 35 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	++	Potent inducer	++H	Potent inducer	++	Potent inducer
	HPR116035	++		++H		++	
	HPR116036	++		++H		+ (irr)	
CYP2B6	HPR116020	++	Potent inducer	++	Weak inducer	+	Potent inducer
	HPR116035	+		-		++H	
	HPR116036	-		-		-	
CYP2C9	HPR116020	+	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		+	
	HPR116036	-		-		-	
CYP3A4	HPR116020	++	Potent inducer	++H	Potent inducer	++	Potent inducer
	HPR116035	++		++H		++	
	HPR116036	++		++H		++	
Bosentan / Cryoheps (based on table 38 and fig 36 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	-	Non-inducer	++ (irr)	Weak inducer	-	Potent inducer
	S240408	-		-		-	
	S2406A	-		-		++	
CYP2B6	B270808	++	Potent inducer	+	Potent inducer	++H	Potent inducer
	S240408	++H		-		++H	
	S2406A	++H		++		++H	
CYP2C9	B270808	-	weak inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	++		-		-	
CYP3A4	B270808	++H	Potent inducer	+ (irr)H	Weak inducer	++H	Potent inducer
	S240408	++		+		++	
	S2406A	++		+ (irr)		++H	

Artemisinin/HepaRG (based on table 41 and figure 39 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		+	
	HPR116036	-		-		-	
CYP2B6	HPR116020	++	Potent inducer	++	Potent inducer	++	Potent inducer
	HPR116035	++		++		++	
	HPR116036	++		++		++	
CYP2C9	HPR116020	+ (2 ss)	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		+	
	HPR116036	-		-		-	
CYP3A4	HPR116020	-	Weak inducer	+	Non-inducer	-	Non-inducer
	HPR116035	-(4 ss)		-		-	
	HPR116036	-		-		-	
Artemisinin/ Cryoheps (based on table 42 and fig 40 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	+	Non-inducer	+	Non-inducer	++	Potent inducer
	S240408	-		-		++	
	S2406A	-		-		+	
CYP2B6	B270808	++	Potent inducer	+	Potent inducer	-	Potent inducer
	S240408	+		++		++	
	S2406A	++ (irr)		+		++	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		- (3 ss)		-	
	S2406A	-		-		-	
CYP3A4	B270808	+	Weak inducer	++	Potent inducer	++	Potent inducer
	S240408	-		++		+	
	S2406A	+		++		++	

Efavirenz / Cryoheps (based on table 20 and fig 43 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	-	Non-inducer	+	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	+ (1 ss)		-		- (3 ss)	
CYP2B6	B270808	++H	Potent inducer	+	Potent inducer	+H	Potent inducer
	S240408	++H		+H		+H	
	S2406A	++H		++ (irr)		++H	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	- (4 ss)		-(2 ss, irr)		-	
CYP3A4	B270808	++H	Potent inducer	++H	Potent inducer	++H	Potent inducer
	S240408	+H		+H		+H	
	S2406A	++		++H		++H	

Rifampicin/HepaRG (based on table 47 and figure 45 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	++	Potent inducer	++	Potent inducer	++	Potent inducer
	HPR116035	++		++		++	
	HPR116036	++		++		++	
CYP2B6	HPR116020	+	Weak inducer	++	Potent inducer	+	Potent inducer
	HPR116035	+		-		-	
	HPR116036	-		+		++	
CYP2C9	HPR116020	-	Non-inducer	-	Non-inducer	-	Weak inducer
	HPR116035	-		-		-	
	HPR116036	-		-		++	
CYP3A4	HPR116020	++	Potent inducer	++	Potent inducer	++	Potent inducer
	HPR116035	++		++		++	
	HPR116036	++		++		++	
Rifampicin/ Cryoheps (based on table 48 and fig 46 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	- (2 ss)	Non-inducer	++ (irr)	Non-inducer	-	Weak inducer
	S240408	-		-		-	
	S2406A	-		-		+	
CYP2B6	B270808	++	Potent inducer	++	Potent inducer	++	Potent inducer
	S240408	++		+		++	
	S2406A	++		+		++	
CYP2C9	B270808	+	Potent inducer	++	Potent inducer	++	Potent inducer
	S240408	++		+		++	
	S2406A	++		++		+	
CYP3A4	B270808	++	Potent inducer	++	Potent inducer	++	Potent inducer
	S240408	+		+		+	
	S2406A	++		++		++	

Metoprolol/HepaRG (based on table 51 and figure 49 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	-	Non-inducer	- (2 ss, irr)	Non-inducer	-	Non-inducer
	HPR116035	-		+ (irr)		-	
	HPR116036	-		-		-H	
CYP2B6	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-(6 ss, irr)		+ (5 ss, irr)		-	
	HPR116036	-		-		-H	
CYP2C9	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-(6 ss, irr)		+ (5 ss, irr)		-	
	HPR116036	-		-		-	
CYP3A4	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		+(5 ss, irr)		-	
	HPR116036	-		-		-	
Metoprolol/ Cryoheps (based on table 52 and fig 50 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	++H	Potent inducer	++	Potent inducer	-	Potent inducer
	S240408	++H		-		++	
	S2406A	++H		++H		++	
CYP2B6	B270808	-(2 ss, irr)	Non-inducer	-	Non-inducer	-H	Weak inducer
	S240408	-		-		-	
	S2406A	-		-		++	
CYP2C9	B270808	-	Non-inducer	-	Weak inducer	- (3 ss, cc)	Weak inducer
	S240408	-		-		+ (2 ss, cc)	
	S2406A	+ (3 ss, irr)		+ (2 ss, cc)H		+ (3 ss, cc)H	
CYP3A4	B270808	-	Non-inducer	+(2 ss, irr)	Weak inducer	-	Weak inducer
	S240408	-		-(2 ss, irr)		-	
	S2406A	-		++ (cc)		++ (cc)H	

Penicillin/HepaRG (based on table 27 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		-	
	HPR116036	-		-		+ (3 ss, irr)	
CYP2B6	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		-	
	HPR116036	-		-		-	
CYP2C9	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		-	
	HPR116036	-		-		- (3 ss, irr)	
CYP3A4	HPR116020	-	Non-inducer	-	Non-inducer	-	Non-inducer
	HPR116035	-		-		-	
	HPR116036	-		-		-	
Penicillin/ Cryoheps (based on table 28 and fig 18 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-(2 ss, irr)	
	S2406A	-		-		-	
CYP2B6	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-(4 ss, irr)	
	S2406A	-		-		-	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-(2 ss. irr)	Non-inducer
	S240408	-(2 ss, irr)		-		-	
	S2406A	-(2 ss, irr)		-		-(5 ss, irr)	
CYP3A4	B270808	-	Non-inducer	- (1ss, cc)	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	-		+ (2 ss, cc)		-	

Sotalol/HepaRG (based on table 55 and in figure 53 in StatRep)							
	Batch	Janssen		Pharmacelsus		EURL ECVAM	
CYP1A2	HPR116020	-	Non-inducer	-	Non-inducer	+(4 ss, irr)	Non-inducer
	HPR116035	-(2 ss, irr)		-		-(2 ss, irr)	
	HPR116036	-		-		-	
CYP2B6	HPR116020	-	Non-inducer	-	Non-inducer	+(4 ss, irr)	Non-inducer
	HPR116035	-(5 ss, irr)		-		-(2 ss, irr)	
	HPR116036	-		-		-	
CYP2C9	HPR116020	-	Non-inducer	-	Non-inducer	+4 ss, irr)	Non-inducer
	HPR116035	-		-		-	
	HPR116036	-(6 ss, irr)		-		-	
CYP3A4	HPR116020	-	Non-inducer	-	Non-inducer	+(5 ss, irr)	Non-inducer
	HPR116035	-		-		-(2 ss, irr)	
	HPR116036	-(5 ss, irr)		-		-	
Sotalol/ Cryoheps (based on table 56 and fig 54 in StatRep)							
	Batch	AstraZeneca		Kaly-Cell		EURL ECVAM	
CYP1A2	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	-		-		-	
CYP2B6	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	-		-		+(3 ss, irr)	
CYP2C9	B270808	-	Non-inducer	-	Non-inducer	-	Non-inducer
	S240408	-		-		-	
	S2406A	-		-		-	
CYP3A4	B270808	-	Non-inducer	+	Non-inducer	-	Non-inducer
	S240408	-		-(2 ss, irr)		-	
	S2406A	-		-		-(2 ss, irr)	

7.5 Between laboratory reproducibility

For the analysis presented below in Table M4.2, classification concordance among the laboratories of 3/3, 2/3, and 1/3 were extracted from Table M4.1, (which is based on the Statistical Report). The table M4.2 demonstrates that three (actually five altogether) different laboratories produce the same induction classification when performing the experiment with the same batch. In 66 % of the experiments with cryoHepaRG cells and 55% in cryoheps were judged to give the same induction class (potent, weak, non-inducer) in all laboratories and at least 2 out of three laboratories were concordant in >90% of the experiments. The concordance is marginally better with cryoHepaRG batches than with cryohep batches, which is quite expected.

Table M4.2. Reproducibility between laboratories of induction status assessments in 3 different cryoHepaRG and 3 different cryoheps batches per test item (10) and CYP activity (4). Inducer classifications are taken from the above table.

	Number of concordant classifications between laboratories (3) per test items (10) and CYP activity (4)			
cryoHepaRG batch	3/3 (all similar)	2/3	1/3 (all different)	
HPR116020	25 (62.5%)	12 (30%)	3 (7.5%)	40 (100%)
HPR116035	23 (57.5%)	12 (30%)	5 (12.5%)	40 (100%)
HPR116036	31 (77.5%)	7 (17.5%)	2 (5.0%)	40 (100%)
Combined	79 (66%)	27 (22.5%)	10 (8.3%)	120 (100%)
cryoheps batch				
B270808	25 (52%)	19 (40%)	4 (8%)	48 (100%)
S240408	27 (56%)	20 (42%)	1 (2%)	48 (100%)
S2406A	27 (56%)	17 (35%)	4 (8%)	48 (100%)
Combined	79 (55%)	56 (39%)	9 (6.3%)	144 (100%)

7.6 Summary and comments for each test items

Based on the statistical report and on the above within- and between-batch and within- and between-laboratory analyses (especially in Table M4.1), a number of basic characteristics of concentration-response curves for each test items are commented and tabulated in the following paragraphs.

Threshold refers to a concentration (µM) in which a statistically significant increase in enzyme activity over the control value (1) has been observed. In toxicology, certain benchmark limit

values, e.g. a concentration (dose) for 10 % response, are increasingly used, and such values could be useful also for the characterization of induction response.

2-fold (F2)/maximal induction refers to test item concentration(s) (µM) at which a statistically significant >2-fold or maximal (n-fold) induction response has been observed.
In the Statistical Report 2, experimental points have been fitted into the Hill equation, to provide EC50 values, a concentration producing a 50% response of the maximal response. Reliable EC50 values (for reliability, see the StatRep2) have been giving in the following tables and also in the summary table in section 5.

Maximal induction response refers to the highest n-fold increase of enzyme activity over the control value.

Response curve refers to a curve of responses (n-fold induction or enzyme activity) constructed over the whole range of concentrations of a test item. Consistency refers to a visually satisfactory concentration-response curve (sometimes with a downward tendency at the highest concentrations). Bell-shaped refers to a curve with a downward trend after a maximum. Irregularities are noted if they are conspicuous. It is clear that consistency of the concentration-response curve provides reliability to the evaluation and decision whether the test item is an inducer or not.

Induction status is a composite classification on the basis of batchwise assessment of induction potential shown in **TABLE M4.1**. It may be of importance to stress here that the classification of induction status into potent inducer, weak inducer, and non-inducer, based on results from the three laboratories, is even more judgemental than in the case of a single laboratory.

7.6.1 Omeprazole

CYP1A2 was induced with very high induction values by omeprazole in both cryoHepaRG and cryoheps cells. In cryoHepaRG cells, the concentration response was increasing with a similar pattern across batches whereas in cryoheps an increase is followed by a decrease at highest tested concentration 116 µM, the pattern is more batch dependent.

In cryoHepaRG cells, omeprazole started to induce activity (statistically different from solvent) at 1.43 µM and was a potent inducer (activity ≥2-fold, statistically significant) at 12.9 µM. The highest induction rate in three batches for cryoHepaRG was between 21 and 34.6 µM with similar levels across batches. All laboratories gave rather similar concentration-response curves.

In cryoheps, more batch-specific responses were observed, batch S2406A shows the highest induction rate (36.8), followed by batch B270808 (17.6) and the smallest response by S240408 (10.5). All laboratories classified omeprazole as a potent inducer, although the concentration for maximal induction varied between batches from 12.9 to 116 µM.

CYP2B6 was induced by omeprazole in both cryoHepaRG cells and cryoheps, but the induction rate was dependent on specific batches and was much lower that at CYP1A2.

In cryoHepaRG cells, the highest induction rate occurred at 38.6 µM with values 2.4 - 3.3 across batches. All laboratories produced a positive induction response (≥2-fold statistically significant) at least in one batch.

In cryoheps, the patterns and induction rates were batch-dependent. Higher induction values that in cryoHepaRG cells were observed. Batch B270808 had lowest induction (3.5), followed by batch S240408 (3.6) and batch S2406A with highest induction (12.7). A concentration dependent pattern was observed. All laboratories produced a positive induction response (≥2-fold statistically significant) at least in 2 batches.

CYP2C9 displayed rather flat concentration-response curves and no ≥2-fold induction was discernible in cryoHepaRG or primary hepatocyte cells. In cryoHepaRG cells, omeprazole was judged to be a non-inducer, Although in cryoheps all laboratories gave some hints of response, omeprazole was judged to be a non-inducer.

CYP3A4 was induced by omeprazole in cryoheps. The response in cryoHepaRG cells was judged to be potent, although some curves were not consistent.

In cryoHepaRG cells, the highest induction values were slightly above 2 in at least one or two batches in each laboratory. All laboratories produced a ≥2-fold induction response in at least one batch. Whether this is regarded as a positive or negative induction signal is somewhat problematic, but the concentration-dependency in many response curves would suggest a positive response.

In cryoheps the maximal induction was batch-dependent, batch S240408 has lowest induction (1.7), followed by batch B270808 (3.7) and batch S2406A with the highest induction (7.4). Concentration-dependent pattern is observed. All laboratories produced a roughly similar result.

Table M4.3. Omeprazole: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	1.43 – 4.29	12.9/38.6 – 116	21 – 34.6	consistent	Potent inducer
CYP2B6	12.9	38.6/38.6	2.4 – 3.3	consistent	Potent inducer
CYP2C9	no	no	no	flat	Non-inducer
CYP3A4	38.7	38.6	2	non-consistent	Potent inducer
Cryoheps					
CYP1A2	1.43	38.6 - 116	10.5 - 36.8	consistent	Potent inducer
CYP2B6	4.29 - 12.9	38.6 - 116	3.5 - 12.7	consistent (116 down)	Potent inducer
CYP2C9	no	no	no	flat	Non-inducer
CYP3A4	4.29 - 12.9	38.7 - 116	3.7 - 7.4	consistent (116 down)	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction by omeprazole in vivo using selective probe substrates (see also Table 2 of Background)

Although omeprazole is claimed to be one of a few clinically relevant CYP1A2 inducers (Hukkanen et al, 2012), it seems that it is rather weak at the best. In clinical drug-drug interactions, 120 mg omeprazole will produce a 30 % induction of caffeine clearance (Rost et al, 1994). However, at clinically relevant doses (20-60 mg) drug interaction as a result of an induction of CYP1A2 by omeprazole (or one of its enantiomers) is not confirmed (summarized in Andersson et al., 2001). Omeprazole has not been reported to induce CYP2B6, CYP2C9 or CYP3A *in vivo*.

It has been demonstrated that clinical outcomes of omeprazole depend on many factors such as the genotype status of CYP2C19 and CYP1A2. These factors may become important when attempting to perform pharmacokinetic *in vitro-in vivo* modelling and prediction.

Omeprazole at a dose of 40mg/mL once daily, gives rise to a $c_{max,unbound}$ value of 12 to 60 ng/mL (0.035 – 0.174 µM).

CYP2B6 and CYP3A4 induction detected here is in line with the finding that at high concentrations *in vitro* 100µM of omeprazole, a moderate increase of 400% of control in CYP3A4 mRNA in human hepatocytes was observed (Raucy et al, 2003).

7.6.2 Carbamazepine

CYP1A2 was induced by carbamazepine in both cryoHepaRG and cryoheps, but patterns and range of concentrations with significant induction were very different.

In cryoHepaRG cells, the concentration response was increasing with a similar pattern across batches. Carbamazepine started to induce activity in cryoHepaRG cells at 18.8 µM and being a potent inducer at and above 56.4 µM. The highest induction rate in three batches for cryoHepaRG was between 5.5 and 8.9 with similar levels across batches.

In cryoheps, a concentration pattern was flatter than in cryoHepaRG, the levels were batch dependent and highest induction rate was between 1.6 and 3.1.

CYP2B6 was induced by carbamazepine in both cryoHepaRG and cryoheps. The differences in patterns of concentration responses are similar to the situation of CYP1A2 with main difference in slightly higher induction rate.

Carbamazepine started to induce activities in cryoHepaRG cells at concentration 2.09 µM and was a potent inducer at and above 18.8 µM. The highest induction rate in three batches for cryoHepaRG was between 8.5 - 11.2.

In cryoheps, the concentration-response pattern is increasing, the levels are batch dependent, and the highest induction rate is between 3.3 and 8.9.

CYP2C9 displayed rather flat concentration-response curves. However, in certain batches and laboratories concentration-response curves were consistent and there was a hint of induction in the highest concentrations in both cryoHepaRG or cryohepscells.

CYP3A4 was also induced by carbamazepine in both cryoHepaRG and cryoheps cells. Patterns are similar to the situation described in CYP1A2 paragraph for cryoHepaRG, whereas for cryoheps concentration response is increasing with highest induction rate in batch S2406A (12.2), followed by batch B270808 (8.5) and lowest in batch S240408 (4.3).

Table M4.4. Carbamazepine: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
cryoHepaRG					
CYP1A2	18.8	56.4/169	5.5 – 8.9	consistent	Potent inducer
CYP2B6	2.09	18.8/56.4 - 169	8.5 – 11.2	consistent	Potent inducer
CYP2C9	56.4 - >40	169	1.5 – 2.5	consistent	weak inducer
CYP3A4	6.27 – 18.8	18.8/56.4 - 169	2.0 – 8.5	consistent	Potent inducer
Cryoheps					
CYP1A2	high (if present)	high (if present)	1.6 – 3.1	flat or irregular	Weak inducer
CYP2B6	2.09 – 6.27	2.09 – 6.27 (higher, batch-dependent)	3.3 – 8.9	mostly consistent	Potent inducer
CYP2C9	high (if present)	high (if present)	1.3 – 2.2	flat or irregular	Non-inducer
CYP3A4	2.09 – 6.27	6.27 – 18.8/56.4-169	4.3 – 12.2	consistent	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction in vivo by carbamazepin using selective probe substrates (see also Table 2 of Background)

In clinical studies, carbamazepine has been shown to reduce the levels of drugs that are substrates for CYP3A4, CYP2C9, CYP2B6, and CYP1A2 enzymes. Regarding CYP1A2, carbamazepine induced levels of CYP1A2 substrate olanzapine (Lucas et al, 1998). With respect to CYP3A4, coadministration of carbamazepine (600 mg/day; 14 days) decreases levels of simvastatin (Ucar et al, 2004), ethinyl estradiol (Doose et al, 2003), and cyclosporine (Cooney et al, 1995). Implicating CYP2C9, carbamazepine induced metabolism of phenytoin (Lai et al, 1992). Carbamazepine increased the clearance of efavirenz which is mostly metabolized by CYP2B6 (Ji et al, 2008).

Following a 18.4 mg/kg/day oral dose of carbamazepine a $c_{max,unbound}$ concentration of 2.4 µg/mL (10.2 µM) was determined (Ref: Goodman & Gilman 10th Edition 2001). This concentration of carbamazepine *in vivo* is in good agreement with the concentration *in vitro* that was able to induce CYP1A2 and 3A4 activity in HepaRG and cryopreserved human hepatocytes.

7.6.3 Phenytoin

CYP1A2 was induced by phenytoin in both cryoHepaRG and cryoheps with similar patterns and induction rates. In cryoHepaRG cells, phenytoin started to induce activity in cryoHepaRG cells at 12.2 µM and being a potent inducer at and above 36.5 µM. The highest induction rate in three batches for cryoHepaRG was between 5.4 and 8.3 with similar levels across batches. In cryoheps, the concentration-dependent response started at 36.5 µM with the highest induction rate between 3.7 and 7.9.

CYP2B6 was induced by phenytoin in both cryoHepaRG and cryoheps cells. The induction rate was higher and concentration range with significant induction was much wider than with CYP1A2. Phenytoin started to induce activity in cryoHepaRG cells at the lowest tested concentration 0.45 µM and became a potent inducer at and above 1.35 µM. The highest induction rate in three batches of cryoHepaRG was between 9.2 - 20.6. In cryoheps of two donors, phenytoin started to induce activity at the lowest tested concentration of 0.60 µM with highest induction rate in batch S2406A (15.9), followed by batch S240408 (9.6). Donor B270808 showed no response at two of the three laboratories (induction response at the highest tested concentration was 5.9-fold in one laboratory).

CYP2C9 displayed rather variable concentration-response curves in cryoHepaRG cells, in which the batch-wise classification ranged from non-inducer to potent inducer. Although classification in such a case remains rather uncertain, phenytoin was judged to be a weak inducer based on mostly consistent concentration-response curves. In cryoheps, phenytoin was classified as a non-inducer.

CYP3A4 was also induced by phenytoin in both cryoHepaRG and cryoheps. In cryoHepaRG cells, the concentration response was increasing with a similar pattern across batches. Phenytoin started to induce activity in cryoHepaRG cells at 4.05 µM and was a potent inducer at and above 12.2 µM. The highest induction rate in three batches for cryoHepaRG was between 6.2 - 11.4 with similar levels across batches. In cryoheps of two donors, phenytoin started to induce activity at concentrations of 1.80 and 5.40 µM with the highest induction rate in batch S2406A (10.5), followed by batch B270808 (8.6). Donor S240408 showed an induction response (6.6-fold) only at two highest concentrations..

Table M4.5. Phenytoin: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	>2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	12.2	36.5/30.0	5.4 – 8.3	consistent	Potent inducer
CYP2B6	0.45	1.35/4.05 - 36.5 (109)	9.2 – 20.6	consistent	Potent inducer
CYP2C9	4.05 – 12.2	12.2 – 36.5 (109)	1.3 – 3.0 (if present)	consistent	Weak inducer
CYP3A4	4.05	12.2	6.2 – 11.4	consistent	Potent inducer
Cryoheps					
CYP1A2	5.40 – 16.2	16.2 – 48.6/146	3.7 – 7.9	consistent	Potent inducer
CYP2B6	0.60 – 1.80	0.60 – 48.6/16.2 - 146	5.9 – 15.9	consistent	Potent inducer
CYP2C9	no	no	no	flat	Non-inducer
CYP3A4	0.60 – 16.2 (b)	1.80 – 16.2 (b)	6.6 – 10.5	consistent	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction in vivo by phenytoin using selective probe substrates (see also Table 2 of Background)

Clinically, phenytoin has been shown to reduce the levels of drugs that are substrates for CYP3A4, CYP2B6, CYP2C9, and CYP1A2 enzymes. For example, phenytoin reduced the levels of CYP1A2 substrate theophylline (Vestal et al, 1993). Regarding CYP3A4, phenytoin caused an increased elimination of carbamazepine (Lai et al, 1992), ethinylestradiol (Crawford et al, 1990), and benzodiazepines (Fridell et al, 2003). As an implication of CYP2C9 induction, phenytoin increased the metabolism of sirolimus, mainly eliminated by CYP2C9 (Fridell et al, 2003).

Following a 300 mg oral dose of phenytoin a therapeutic free concentration of $c_{max,unbound}$ value of 1.1 to 2.2 µg/mL (4.4 – 8.8 µM) was determined (c_{max},total 40-80 µM, Ref: Goodman & Gilman 10th Edition 2001). This concentration of phenytoin *in vivo* is in good agreement with the concentration *in vitro* that was able to induced CYP1A2, CYP2B6, and 3A4 activity in HepaRG cells and human hepatocytes.

7.6.4 Rifabutin

Rifabutin was tested only in cryoheps. Concentration-response curves were so variable, that the batch- and test facility-wise analysis was necessary to get any idea about the induction potential of rifabutin. The enzyme activity measurements for all CYPs in batch B270808 by one test facility (Kaly-Cell) showed systematically higher values across the whole concentration range tested. This would suggest a laboratory specific effect in that specific situation. For other batches, enzyme activities measured in this test facility did not deviate so conspicuously from others, but these curves provided a different view about induction potential as compared with other test facilities. Due to the above mentioned variabilities, threshold and maximal induction concentrations of rifabutin were highly dependent on batches and test facilities and basically the assessment had to rely upon results from 2 test facilities.

Rifabutin is an example of compound where the relative induction rate with respect to the (relevant) positive control provided a better assessment of its effect (see Statistical Report). However when responses are expressed in relative induction rates, the patters are very similar across all test facilities involved.

Table M4.6. Rifabutin: CYP induction in cryoheps.

Enzyme	threshold[1]	>2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
Cryoheps					
CYP1A2	?	2.2 – 6.7	2.2	flat or consistent	Weak inducer
CYP2B6	0.10 – 0.29 (b, ld)	0.29 - 0.87/ 2.62 – 7.87	3.0 – 3.3	flat or consistent (b,ld)	Potent inducer
CYP2C9	?	?	2.2	variable (b,ld)	Weak inducer
CYP3A4	0.10 (b/ld)	0.10/2.62 (b,ld)	2.2 – 10.0 (b,ld)	bell-shaped (b,ld)	Potent inducer

[1]threshold is a concentration (uM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) at which <2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.
b: batch-dependent
ld: laboratory-dependent

Reports on CYP induction *in vivo* by rifabutin using selective probe substrates (see also Table 2 of Background)

Clinically, rifabutin reduces the levels of drugs that are substrates for CYP3A4, like ethinylestradiol (LeBel et al, 1998) and CYP2B6 (Hsu et al, 2010).
Rifabutin is a rifamycin derivative like rifampicin. In vivo, the therapeutic blood concentration of the two compounds are quite different, with rifampicin much higher than rifabutin. Thus, inducing properties of rifabutin towards the drug metabolizing enzymes, in particular towards CYP3A, are less pronounced than rifampicin. Rifabutin and rifampicin are consistently able to induce the overall biotransformation of testosterone in a dose-dependent manner, with both fresh and cryopreserved human hepatocytes (2-fold by rifabutin, 4-fold by RIF) (Reinach et al, 1999).

Following a 600 mg oral daily dose of rifabutin a therapeutic free concentration of $c_{max,unbound}$ value of 109 ng/mL (0.13 µM) was determined (c_{max},total 6.5 µM, Ref: Goodman & Gilman 10th Edition 2001).

7.6.5 Sulfinpyrazone

CYP1A2 was strongly induced by sulfinpyrazone in cryoHepaRG cells and slightly in cryoheps.
In cryoHepaRG cells, sulfinpyrazone started to induce activity at 3.66 µM and became a potent inducer at and above 11.0 µM except in batch HPR116035 at EURL ECVAM. The highest induction rate in three batches of cryoHepaRG was between 2.8 and 3.5. Differences observed in induction rates across laboratories are not present when relative induction rates are calculated.
In cryoheps, induction was quite low and batch dependent. Highest induction rate values were in batch S2406A with values up to 3.5 (EURL ECVAM only). In any case, sulfinpyrazone was deemed to be a weak inducer.

CYP2B6 was induced quite consistently by sulfinpyrazone in cryoHepaRG cells in two laboratories. Highest induction rate value was 3.3. The induction in cryoheps was much higher than in cryoHepaRG cells and patterns were similar across batches and laboraories. Sulfinpyrazone started to induce CYP activity in cryoheps at 3.66 µM and was classified as a potent inducer at and above 30.0 µM. The highest induction rate in three batches for cryoheps was between 6.4 - 12.9.

CYP2C9 was not inducible by sulfinpyrazone. Concentration-response curves were flat, negative, but did not show any major irregularities.

CYP3A4 was also induced by sulfinpyrazone in both cryoHepaRG and cryoheps.
In cryoHepaRG cells, the situation is similar to CYP1A2. Sulfinpyrazone started to induce activity in cryoHepaRG cells at 1.22 µM and was a potent inducer at and above 11.0 µM. The highest induction rate in three batches for cryoHepaRG was between 7.2 - 11.4. Differences observed at induction rates across test facilities were not present when relative induction rates were calculated.

In cryoheps concentration-dependent responses increased except in batch S240408 where almost a flat pattern was present. The highest induction rate was observed in batch S2406A (11.2), followed by batch B270808 (7.5) and the lowest rate was in batch S240408 (2.9).

Table M4.7. Sulfinpyrazone: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	>2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	3.66	11.0/33.0 – 98.9	2.8 – 3.5	consistent	Potent inducer
CYP2B6	3.66	11.0 - 33.0/98.9	no – 3.3	consistent	Potent inducer
CYP2C9	no	no		flat or irregular	Non-inducer
CYP3A4	1.22	11.0	7.2 – 11.4	consistent	Potent inducer
Cryoheps					
CYP1A2	variable (b, ld)	33.0 – 98.9 (b, ld)	2.7 - 3.5	variable	Weak inducer
CYP2B6	3.66	33.0	6.4 – 12.9	consistent	Potent inducer
CYP2C9	no	no		negative curve	Non-inducer
CYP3A4	1.22	3.66/11.0 - 33.0	2.9 – 11.2	consistent	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which <2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction *in vivo* by sulfinpyrazone using selective probe substrates (see also Table 2 of Background)

The ability *in vivo* of sulfinpyrazone to induce CYP activities is based on rather old studies. Sulfinpyrazone has been indicated as a CYP2C9 (Toon et a,l 1986) and CYP3A (Caforio et al, 2000) inducer. CYP1A2? However, the ability has been confirmed in a number of *in vitro* studies using both cryoheps and HepaRG cells (Kanebratt and Andersson 2008b). CYP3A4 in primary cultures of human hepatocytes was induced moderately about 2 to 3-fold by sulfinpyrazone (Luo et al, 2002)

Following a single oral dose of 100mg or 200mg sulfinpyrazone, peak plasma concentrations of 5-6µg/ml or 13-22µg/ml, corresponding to free concentration of $c_{max,unbound}$ value of 100 to 440ng/mL, were observed. Sulfinpyrazone has been shown to increase the rate of antipyrine oxidation like rifampicin (Barry and Feely 1990). In addition, activation of microsomal liver enzymes and resultant acceleration of metabolism lowers the plasma concentration of theophylline (Upton 1991).

7.6.6 Bosentan

CYP1A2 was induced by bosentan in cryoHepaRG cells with a hump shaped concentration dependent pattern. A potent induction started at thelowest tested concentration of 0.29 µM with levels between 1 and 3.75. Induction increases up to the peak at concentrations 2.60 – 7.80 µM followed by a decrease. The highest tested concentration 70 µM was not different from solvent control. The highest induction values at the peak were in the range of 5.1 - 7.6.
In cryoheps, CYP1A2 induction rate is quite low and flat except in the batch S2406A at EURL ECVAM laboratory, with a consistent concentration response curve with the highest value reaching 2.8. In any case, bosentan was deemed to be a non-inducer.

CYP2B6 was induced by bosentan in cryoHepaRG cells, a flatten hump shaped pattern was present. The highest induction rate values are 1.7 - 3.8 across batches tested.
In cryoheps cells, the induction values are higher than in cryoHepaRGs, highest values ranged between 4.8 and 7.0 across batches and mostly consistent curves.

CYP2C9 induction could not be very clearly evaluated, because of variable concentration-response curves and either flat or negative trajectories and a number of statistically significant increases. However, bosentan was deemed a non-inducer in both cell systems.

CYP3A4 induction in cryoHepaRG cells by bosentan had a similar pattern as in CYP1A2. Hump shaped concentration dependent induction started at the lowest tested concentration of 0.29 µM with most of the fold-induction values between 2.1 and 5.6. Peak values were between 7.6 - 12.3.

In cryoheps cells, the induction values were very much batch dependent. An increasing concentration-dependent pattern was present in all batches with following highest induction rate values: the highest induction rate in batch S2406A (15.5), followed by batch B270808 (12.9) and lowest in batch S240408 (3.1).

Table M4.8. Bosentan: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	>2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	0.29	2.60 – 7.80	5.1 – 7.6	consistent (bell-shaped)	Potent inducer
CYP2B6	0.29 - 0.87	2.60 - 7.80	1.7 – 3.8	flat-negative	Potent inducer
CYP2C9	0.29	no	2.2	flat-negative	Non-inducer
CYP3A4	0.29	0.29/2.60 – 23.4	7.6 – 12.3	consistent (bell-shaped)	Potent inducer
Cryoheps					
CYP1A2	?	?	2.8 (1)	flat, irregular	Weak inducer
CYP2B6	? (variable)	5.85	7.9 (variable)	irregularities, consistent	Potent inducer
CYP2C9	0.22 – 1.95	?	2.3 (1)	flat, irregularities	Non-inducer
CYP3A4	0.07 -	0.65 - 1.95/ 1.95 – 17.6	3.1 – 15.5	consistent (irregularities)	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction in vivo by bosentan using selective probe substrates (see also Table 2 of Background)

In vivo, during multiple-dosing regimens, decreases in plasma levels of bosentan (Cmax and AUC in the magnitude of 30% to 40%) and its metabolites have been observed in human subjects that can be explained by an approximately two-fold increase in systemic plasma clearance. At least part of this change in clearance is due to induction of CYP3A4 by bosentan treatment (Weber et al, 1999). Concomitant treatment with bosentan reduced the exposure of CYP3A4 substrates simvastatin (Dingemanse et al, 2003) and sildenafil (Paul et al, 2005) confirming that *in vivo* bosentan is also a mild inducer of CYP3A4.

Bosentan decreased AUC of R-warfarin by 38%, and AUC of S-warfarin by 29%. Because R-warfarin is mainly metabolized by CYP3A4, it is postulated that the effect of bosentan on R-warfarin plasma

levels is due to enzymatic induction of CYP3A4. In the same study, S-warfarin plasma levels were also decreased by bosentan. This could be due to induction of CYP2C9, the hepatic isozyme mainly responsible for the metabolism of S-warfarin (Weber et al, 1999)

Following a 500 mg oral dose of bosentan a therapeutic free concentration of $c_{max,unbound}$ value of 32 ng/mL (0.060 µM) was determined (c_{max},total 5.8 µM, Dingemanse et al, 2004). At least the lowest effective concentrations of bosentan *in vitro* experiments are in the same range.

In vitro, bosentan has been shown to be a mild inducer of cytochrome CYP2C9 and 3A4.

7.6.7 Artemisinin

Downward concentration-response patterns interfered the evaluation of artemisinin. The cause of these negative patterns is not know.

CYP1A2 was not induced by artemisinin in cryoHepaRG cells. A slightly decreasing pattern is observed. In cryoheps the induction values seems to be higher than in cryoHepaRG cells but levels are not consistent across labs nor across batches. Nevertheless, the highest values were reaching a level of 3-fold in one laboratory and overall artemisinin was deemed to be a weak inducer of CYP1A2 in cryoheps.

CYP2B6 was judged to be induced by artemisinin in cryoHepaRG cells, although a decreasing pattern was present. At the lowest concentration tested, 0.58 µM, the induction was about 3-fold except in batch HPR116035 in which it was about 5-6. Induction became negligible at concentration 15.7 µM and above.
In cryoheps,CYP2B6 was also induced by artemisinin. The concentration response had a similar decreasing pattern as in cryoHepaRG cells. The between laboratory variability of induction response was higher. Values seemed to be batch dependent too. The highest induction rates were between 2.2 and 6.7.

CYP2C9 activity was deemed not to be inducible by artemisinin, although a few statistically increased points were observed. Downward curves were prominent.

CYP3A4 induction cryoHepaRG by artemisinin was quite low and negligible except slightly higher values in batch HPR116035 in Janssen laboratory with values reaching 1.9-fold.
In cryoheps cells, a mild hump shaped concentration pattern for induction was present. Highest induction was in batch S2406A (3-4), followed by batch B270808 (2.6-3.5) and lowest induction response in batch S240408 (1.5-2.5).

Table M4.9. Artemisinin: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	no	no		negative	Non-inducer
CYP2B6	0.58	0.58	2.4 – 6.4	negative, high values in smaller concentrations	Potent inducer
CYP2C9	no	no		negative	non-inducer
CYP3A4	no	no		negative	non-inducer
Cryoheps					
CYP1A2	0.58 - 1.75 (b,ld)	0.49 – 15.7 (b,ld)	3.1 (b,ld)	negative, bell-shaped (2)	Weak inducer
CYP2B6	0.58 - 1.75	0.58 – 1.75	2.4 – 6.8	negative, bell-shaped, high values in smaller concentrations	Potent inducer
CYP2C9	no	no		negative	Non-inducer
CYP3A4	0.58 – 1.75	5.25 – 47.2	5.5	bell-shaped, negative	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve. Consistent refers to a visually satisfactory concentration-response curve (sometimes with a downward tendency at the highest concentrations. Bell-shaped refers to a curve with a downward trend after a maximum.

Reports on CYP induction *in vivo* by artemesinin using selective probe substrate (see also Table 2 of Background)

In vitro, artemisinin is a potential inducer of P450 enzymes: the most inducible are CYP2B6 and CYP3A4, which are believed to be the main enzymes involved in the autoinduction of artemisinin metabolism (Xing et al, 2012). Artemisinin is a potent inhibitor of CYP2B6 with Ki of 5.7 µM which may explain the lack of induction effect in HepaRG cells (Susan et al, 2011). HepaRG cells are also more metabolically competent than cryoheps, HepaRG cells may thus produce a larger amount of aretemsinin metabolites which may be CYP inhibitors and affect the CYP specific measurement in the the present study.

Artemisinin induces the N-demethylation of S-mephenytoin probably by an increased capacity of CYP2B6 but not CYP2C9 (Simonsson et al, 2003). Artemisinin did not change the CYP3A dependent formation omeprazole sulfone formation or the cortisol metabolic ratio indicating no effect on CYP3A activity (Svensson et al, 1998). However other studies indicate that artemisinin may induce CYP3A4 based on the midazolam etabolite/parent ratio (Asimus et al, 2007).

Following a single 500mg oral dose of antimalarial artemisinin, plasma concentrations most often exceed 200 µg/L.corresponding to a therapeutic free concentration of $c_{max,unbound}$ value of 40 ng/mL (0.14 µM) was determined (Balint 2001, de Vries and Dien 1996). Effective concentrations in HepaRG cell and cryoheps are not too different as compared to the above concentration.

7.6.8 Efavirenz

Efavirenz was tested for induction in cryoheps only.

CYP1A2 was not induced by efavirenz. The only exception were 3 single concentrations at which induction measured went above 2-fold but these higher values were not confirmed/present in other test facilities.

CYP2B6 was induced by efavirenz in cryoheps. The highest values are observed in batch S2406A (4.5-12.1), followed by batch B270808 (1.6-5) and batch S240408 (2-4.3). A relative response rates do not show differences observed in induction rate values. See graph/table below.

CYP2C9 was deemed not to be an inducer based on flat or irregular concentration response curves.

CYP3A4 induction by efavirenz was the highest among 4 CYPs. Highly variable, but mostly quite consistent concentration induction patterns were observed with different batches. Differences between batches seemed not substantial when relative induction rates were considered. Highest induction of 8- to 15-fold was in batch S2406A and batch B270808, whereas a high response in one laboratory (Kaly-Cell) in batch S2406A reached even a level of 40. Lowest induction response in batch S240408 (2-3.4).

Table M4.10. Efavirenz: CYP induction in cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
Cryoheps					
CYP1A2	no	no		flat, irregularities	non-inducer
CYP2B6	1.56 – 4.69	4.69/14.1 (but variable)	1.6 – 12.1 (b, ld)	bell-shaped, irregularities	Potent inducer
CYP2C9	no	no		flat	non-inducer
CYP3A4	0.52 – 1.56	variable	2.0 – 40 (b, ld)	consistent	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction *in vivo* by efivarenz using selective probe substrate (see also Table 2 of Background)

Efavirenz has been shown *in vivo* to induce CYP3A4, CYP2B6 (Robertson et al, 2008), and CYP2C19 activities (Michaud et al, 2012, Koo et al, 2007; Mouly et al, 2002)

Following a single 600 mg oral dose of efavirenz, plasma concentrations of 4µg/mL.(12.7 µM) corresponding to a therapeutic free concentration of $c_{max,unbound}$ value of 20 ng/mL (0.063 µM) was determined (Goodman & Gilman 10th Edition 2001).

7.6.9 Rifampicin

Evaluation of induction potential of rifampicin was somewhat problematic due to rather high, but flat (concentration-dependently) concentration response relationship. However, highly significant values were taken indicative of an induction response, although clear concentration-response curves were not displayed.

Rifampicin shows quite flat curves across the concentration range, which were however judged to indicate potent induction response in both cell lines tested. In cryoHepaRG, the fold-induction values for CYP1A2 are 2-10, CYP2B6 1.5-3.8 (except EURL ECVAM reported values for batch HPR116036 reaching levels of 7), CYP3A4 3-10.5.

In cryoheps, a batch dependent pattern is present. CYP1A2 induction was low except batch B270808 in Kaly-Cell test facility (2.5-5.3) and batch S2406A in EURL ECVAM (1.6 -2.3). CYP2B6 was

induced in slightly increasing pattern with most of values in range 3-19. CYP3A4 induction by rifampicin is very batch dependent, similar values at same concentration range for batches B270808 and S2406A, values between 6-16, whereas batch S240408 is lower (but significant) with values in range 2.3-3.4.

Table M4.11. Rifampicin: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	0.20	0.20 – 0.60 / 1.80 – 16.2	10.4	Slightly bell-shaped	Potent inducer
CYP2B6	0.20 – 0.60	0.60 – 1.80 / 1.80 – 5.40	3.8 – 7.1	Slightly bell-shaped	Potent inducer
CYP2C9	No	No	1.3 – 3.3	Flat, irregular	Non-inducer
CYP3A4	0.20	0.20 – 0.60 / 1.80 – 16.2	10.9	Consistent (irregularities)	Potent inducer
Cryoheps					
CYP1A2	?	?	2.4 – 5.3	Flat, irregular	Non-inducer
CYP2B6	0.20	0.20 / 16.2 – 48.6	2.7 – 50.7	Consistent (irregularities)	Potent inducer
CYP2C9	0.20 – 0.60	1.8 – 16.2	2.7 – 5.1	Consistent (irregularities)	Potent inducer
CYP3A4	0.20	0.20 / 0.60 – 48.6	2.5 – 18.4	Flat (high level)	Potent inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

Reports on CYP induction *in vivo* by rifampicin using selective probe substrate (see also Table 2 of Background)

Rifampicin is a classical PXR-ligand and inducer, which was used also as a reference positive inducer in this validation process. There exists a number of clinical studies demonstrating the induction of CYP1A2 (Kwara et al, 2011), CYP2B6 (Kwara et al, 2011, Bardictch-Crovo et al, 1999), CYP3A4 (Barditch-Crovo et al, 1999), and CYP2C9 (Heimark et al, 1987, Kirby et al, 2011).

Following a single 600mg oral daily dose of rifampicin, plasma concentrations of 6.5µg/mL (7.93 µM) corresponding to a therapeutic free concentration of $c_{max,unbound}$ value of 0.65 to 2µg/mL (0.79 to 2.44 µM) was determined (Goodman & Gilman 10th Edition 2001)

Some clinical studies have also reported that rifampicin treatment enhances the clearance of drugs eliminated by CYP2C9, such as phenytoin (Kay et al, 1985), suggesting that rifampicin induces CYP2C9 expression in vivo. However, induction of CYP2C9 with this prototypic inducer was not observed in HepaRG or cryopreserved hepatocytes in the present study. In a recent report large variability in CYP 2C reponse to rifampicin treatment between batches of cryoheps was reported by Yajima et al. 2014 (Yajima et al, 2014). CYP2C9 mRNA was not induced by rifampicin in 2 out of 8 hepatocyte lots.

7.6.10 Metoprolol

In cryoHepaRG cells, metoprolol did not show significant induction across CYP and batches tested. The only isolated exception was batch HPR116035 at Pharmacelsus laboratory where the induction reaches 2.5 values for all CYP tested.

In cryoheps, metoprolol showed significant induction responses especially in CYP1A2, where the fold induction for the highest tested concentration of 150 µM increases up to 3.6-fold, and in CYP3A4 induction in batch S2406A, where an increasing concentration dependent induction pattern was present. The highest values were very variable across laboratories, between 1.6 and 10.6. There was also positive responses in CYP2B6 and CYP2C9.

Table M4.12. Metoprolol: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	no	no	no	flat	Non-inducer
CYP2B6	no	no	no	flat	Non-inducer
CYP2C9	no	no	no	flat	Non-inducer
CYP3A4	no	no	no	flat	Non-inducer
Cryoheps					
CYP1A2	49.9 - 150	49.9 - 150	3.6	consistent	Potent inducer
CYP2B6	no	no	5.0 (1)	flat, except 1	Weak inducer
CYP2C9	no	no		flat, except 1	Weak inducer
CYP3A4	no	no	1.6 – 10.6	flat, except 1	Weak inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

7.6.11 Penicillin

Penicillin doesn't show any significant induction of 4 enzymes confirmed across batches and laboratories, both for cryoHepaRG and cryoheps.

Table M4.13. Penicillin: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	no	no	no	flat	Non-inducer
CYP2B6	no	no	no	flat	Non-inducer
CYP2C9	no	no	no	flat	Non-inducer
CYP3A4	no	no	no	flat	Non-inducer
Cryoheps					
CYP1A2	no	no	no	flat	Non-inducer
CYP2B6	no	no	no	Flat	Non-inducer
CYP2C9	no	no	no	Flat	non-inducer
CYP3A4	no	112 (1 ld, 2 b)	1.5 – 2.1	Flat, consistent	Non-inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

7.6.12 Sotalol HCl

Sotalol HCL did not induce any significant activity in any of 4 CYPs tested. Some isolated statistically significant induction values showed up but in most cases do not exceed value 1.5.

Table M4.14. Sotalol: CYP induction in cryoHepaRG and cryohep cells.

Enzyme	threshold[1]	≥2 – fold/max[2]	maximal n-fold[3]	response curve[4]	inducer status
CryoHepaRG					
CYP1A2	no	no	no	Flat, irregular	non-inducer
CYP2B6	no	no	no	Flat, irregular	non-inducer
CYP2C9	no	no	no	Flat, irregular	non-inducer
CYP3A4	no	no	no	Flat, irregular	non-inducer
Cryoheps					
CYP1A2	no	no	no	Flat, irregular	non-inducer
CYP2B6	no	no	no	Flat, irregular	non-inducer
CYP2C9	no	no	no	Flat, irregular	non-inducer
CYP3A4	no	no	no	Flat, irregular	non-inducer

[1]threshold is a concentration (µM) in which a statistically significant increase in enzyme activity has been observed.
[2]concentration(s) (µM) at which ≥2-fold/maximal induction response has been observed.
[3]maximal induction response expressed as an n-fold increase of activity over control value.
[4]consistency of the concentration-response curve.

7.7 VMG conclusion on module 4

Since the human in vitro CYP validation study is the first project in its kind the VMG could not set specific targets apriori for each of the modules. The VMG evaluated the obtained information and as such draw ex-post conclusion based on the data generated.

Criteria for the classification of induction response

Induction response is a complicated time- and inducer-dependent process; consequently the criteria for defining inducer status consist of both objective statistically definable measures (extent of induction, statistically significant increase) and subjectively (at least for now) definable measures (form of the concentration – response curve, any irregularities, distribution of statistically significant increases along concentration points, fit to Hill curve). At the current stage the classification includes a certain measure of subjective decision, unless it is possible to repeat experiments or to modify the experimental setup (e.g. range of concentrations).

Variability

Especially the analysis of basal activities and induction responses by positive control inducers indicated quite large variability of activities and responses within batches, between batches, within laboratories and between laboratories, over the duration of the validation study. However, among separate experiments, standard deviations of single experimental concentrations were quite reasonable and allowed for the statistical treatment of a majority of concentration – response relationships resulting in significant induction responses.

Reproducibility between batches

It is convincingly demonstrated that human cryoheps display large variabilities in their CYP-associated activities. In the present study, one of the batches, S240408, demonstrated only borderline effects with the prototypic CYP3A4 inducers rifampicin and phenobarbital, and this low inducibility was repeated with test items, especially with strong CYP3A4 inducers such as carbamazepine and phenytoin.

Although the overall between-batch variability in cryoHepaRG cells seemed to be a bit smaller than in cryoheps, even with cryoHepaRG it is advisable to use several batches, because induction response seems to vary between batches (passages), especially with respect to CYP3A4.

Consideration on 'sporadic' findings

Because between batch and between laboratory variability is inevitable, assessment of 'sporadic' cases should be considered. With artemisinin (see fig 40, CYP3A4, Stat Report), the VMG had to consider what is the practical relevance of a consistent positive finding in one batch (or perhaps two) in one test facility among otherwise negative or irregular concentration-response relationships? The recommendations in the FDA guidance is that if the compound results in an induction according to preset criteria in one out of three batches of hepatocytes the compound is regarded as an inducer and a clinical DDI study is needed.

Another 'sporadic' finding was with an assumed negative control metoprolol: what is the significance of one positive consistent curve (one batch, one facility) for one activity (CYP2B6)?
Also with CYP3A4: what is the significance of two positive consistent curves (one batch, two facilities)? If the compound were a new test item under pharmaceutical development, the FDA recommendation is pretty obvious: an in vivo investigation is required.

Reproducibility between laboratories

Analysis of activity and induction results produced by different laboratories with the same batches indicated that concordance was dependent on test system used used.

CryoHepaRG showed higher reproducibility for the 4 CYPS under investigation compared to cryoheps. The VMG concluded that the BLR is satisfactory for all CYPs. The highest reproducibility value was observed for CYP3A4 (all ≥ 90%).

CryoHeps showed lower reproducibility for the 4 CYPS under investigation compared to cryoHepaRGs. Based on the information generated, and not having the availability of such historical data for other similar ring trials (since this validation project was the first in its kind) , the VMG concluded that the BLR is not satisfactory for one (Batch S240408) out of the three batches for the four CYPs. For this batch the lowest reproducibility value was observed for CYP2B6 (all 37%). The other two batches showed BLR values between 61% and 80% (CYP2C9 excluded).

Considerations of different CYP-selective activities

At least qualitatively, CYP1A2-, 2B6 and 3A4-selective probe activities performed as expected in both cell systems and with model inducers and test items. CYP2C9-selective probe activity was relative high in both cell systems and overall induction responses remained quite low. However, the reason for this problem may be that the CYP2C9 induction is less well defined and its molecular basis is not elucidated to the extent than those of other CYP-selective probe activities employed here.

Comparison between cryoheps and cryoHepaRG test systems

Cryoheps has been regarded, despite their obvious limitations, as a gold standard for *in vitro* cellular studies of metabolism-related processes. Therefor the comparative performance of the cell systems is of considerable interest. Classification of 10 test items into potent, weak and non-inducers was performed for both cell systems. In 28/40 (70%) the two cell systems were concordant in their classification. Interestingly, when cryoheps classified a response as potent (18 cases), only 3 were discordant in cryoHepaRG cells (i.e. 16.7%). Out of 15 non-inducer classifications by cryoheps, only 2 was discordant by cryoHepaRG cells (13.3%). Although this analysis took into consideration only the overall classification without regard of laboratories separately, it nevertheless gives some confidence that cryoHepaRG and cryoheps are rather similar in their ability to detect and classify substances in terms of induction potentiality.

2-fold induction: should this threshold be changed in the future or is it the right one?

In principle, it has to be recognized that induction is not an all-or-none response, but a quantitative concentration- and time-dependent process consisting of a number of steps to the ultimate response. Finally the significance of the response is determined by the consequences, i.e. clinically significant interaction, manifest adverse reaction etc. Theoretically, induction response should preferably be characterized by quantitative measures such as EC50, F2, Emax, Tmax, perhaps a threshold concentration such as BMDL10 or corresponding. These quantitative coefficients could be determined to any response, receptor binding, mRNA, enzyme protein, activity, more distal biomarker. A 2-fold induction response is a useful point for calculations of statistical significance and it helps in assessing, interpreting and extrapolating the response, but it is possible to select other thresholds

Inhibition and induction

Enzyme activity measurements are vulnerable to inhibition with artemisinin as a possible example in these studies. mRNA measurements may be used and an additional source of information to complement activity measurements and in cases such as artemisinin have the necessary information to take solid decisions. Also *in vitro* tests to assess the inhibitory potency of test items and their metabolites should be easily performed.

8 PREDICTIVE CAPACITY (MODULE 5)

Background

Because test items and reference inducers (except for the positive CYP1A2 control beta naphthoflavone) and non-inducers were pharmaceuticals, the described study design provides direct evidence about applicability domain to only pharmaceuticals. However, it is assumed that the CYP induction method is generic, i.e. any substance which has a capacity to activate a nuclear receptor directly by binding or indirectly by other routes, could be detected as an inducer by this CYP induction method. In this context the two human *in vitro* CYP induction methods can be used to assess compounds belonging to different chemical domains and use classes.

The CYP induction in vitro method has been proposed and was accepted as a candidate for regulatory use and as such is currently listed on the OECD work programme to develop a performance-based OECD test guideline for the human in vitro CYP induction methods.

The validation project clearly provides the necessary information on the essential test method components of the two in vitro methods to allow to start drafting the first version of a performance based test guideline for CYP induction in vitro methods.

The project clearly gives inside on potential **performance standards** that can be used:

- ***In vitro method definition/description related standards*** are the elements that are part of the SOPs of the 2 in vitro methods that are essential to understand and to carry out hu,man CYP induction methods using a SOP that enables to give information on solubility, cytotoxicity and CYP induction itself. The *in vitro* methods clearly describe the essential requirements related to the test systems and the equipment necessary to be used. .
- ***Physical standards*** that can be proposed are those compounds that have been tested in both in vitro methods that have known physicochemical and mechanistic characteristics (PXR, CAR, AhR nuclear receptor-xenobiotic interaction) and for which are solid, high quality human *in vivo* CYP induction data avaible and have a good predictive capacity. Such compounds can be as such representative physical standards for in vivo human CYP induction, covering the four CYPs investigated in this validation project.
- ***Methodological standards*** refer to the standard methodology used to provide specific essential information and provide evidendence of good characterisation of the test systems for the basal and induced enzymatic activities for the four CYPS.

Such standards will guide the end-users of the OECD performance-based test guideline ultimately to obtain the information necessary that can be introduced in harmonised reporting formats for in vitro methods.

Details on predictive capacity

An overview of the predicted classification and the reference classification for CYP3A4, CYP2B6 and CYP1A2 in both cryoHepaRG cells and in cryoheps is presented in Tables M5.1-6. CYP2C9 was

not included in the analysis because the *in vivo* (and also *in vitro*) literature was fragmentary and inadequate for definite analysis. Sensitivity and specificity analysis is presented under each table. The predicted classification is the overall inducer classifications for each test substance and CYP provided in Tables M4.3 to M4.14.

There are two obvious conclusions to be drawn on the basis of these results: first of all, the numbers of reference inducers and non-inducers are small affecting naturally the statistical analysis, and secondly, knowledge about *in vivo* concentrations of test items is needed for correct classification in many cases.

Although the numbers are small and consequently sensitivity analysis remains less than satisfactory, it is fair to say that overall classifications on the basis of *in vitro* studies are in line with *in vivo* knowledge of classification of test items. It is also fair to say that *in vivo* classifications themselves remain less than perfect, which is natural in the light of limitations of human clinical studies.
To predict induction from the *in vitro* results a simple corellatation analysis was applied using Cmax *in vivo* values and the concentration in the cell system resulting in 2-fold induction of the CYP enzyme activity. A ratio above 0.5 was judged to predict an *in vivo* induction response of the CYP enzyme. This is a rather conservative approach indicating that a concentration *in vitro* resulting in 2-fold induction of the CYP enzyme activity will be relevant if this concentration is half the Cmax value. Usually Cmax/EC50 values are used but for many substances a full dose response curve is not obtained why such an approach is difficult to apply. A similar approach using F2 values was applied by Kanebratt and Andersson (2008) using data from HepaRG cells. Recently several approaches to predict CYP3A induction was described by Einholf (Einholf et al, 2014). Prediction of *in vivo* induction of other CYP enzymes than CYP3A from *in vitro* data has been less reported mainly because of fewer compounds are reported to induce these enzymes and lack of relevant clinical information.

Towards a step-wise approach
It is envisaged that a step wise approach might be a pragmatic way to evaluate/use the in vitro methods:
1: Decide whether the compound is an inducer in one of the two human *in vitro* CYP induction methods subject of this validation project for one or more of the four CYP isoforms according to the criteria discussed in this document (two fold induction and a statistically significant increase)
2: Calculate a Cmax or F2 in hepaRG. For primary human hepatocytes this aspect needs further elaboration.
3: Relate the potency value to the Emax or AUC of the test item
4: tabulate the data together with the in vivo induction information for the test item
5: decide what ratio (e.g. Emax/F2) is a positive signal for the specific test system. For the primary human hepatocytes; this needs further elaboration.
6: calculate the sensitivity and specificity of the two cell systems. Sensitivity is defined as the fraction of correctly predictive positive to all positive inducing compounds in clinic and specificity is defined as the fraction of correctly predicted negatives to all negatives (non-inducers) in the clinic.

Table M5.1 Prediction of CYP3A4 in vivo induction category (inducer/non-inducer) on the basis of the cryoHepaRG experiment and concentrations of test items in vitro (F2 = 2-fold induction) and in vivo (C_{max})

	Induction in vitro	F2 (µM)	In vivo C_{max} (µM)	C_{max}/F2	Prediction In vivo inducer	Induction shown in vivo	True Positive predictivity	True Negative predictivity	
Omeprazole	++	38,6	0.68-3.5	0.09-0.017	No*	No		y	
Carbamazepine	++	18,8	39	2	Yes	yes	Y		
Phenytoin	++	12,2	40-80	3.2-6.5	Yes	Yes?	Y		
Sulfinpyrazone	++	11	45	4,1	Yes	Yes	Y		
Bosentan	++	0,29	5,8	20	yes	Yes	Y		
Artemisinin	-	Nv***	1.0-2.0	nv	No	Yes and No**		Y	
Rifampicin	++	0.2-0.6	8.0-12.0	40-13	Yes	Yes	Y		
metoprolol	-	nv	0.14-0.38	nv	No	No		y	
Penicillin G	-	nv	36	nv	No	No		y	
Sotalol	-	nv	2	nv	No	No		y	

*it has to be stressed that for this prediction it is necessary to know (or estimate in a reliable way) the C_{max} concentration of the test item. Without this knowledge the prediction would be 'yes'.

** Artemesinin has been shown to be both an inducer and non-inducer of CYP3A in clinical studies using different probe substrates

***no value

HepaRG

Sensitivity= TP/(TP+FN) 5/5+0=1

Specificity= TN/TN+FP) 5/5+0=1.0

Table 5M.2 Prediction of CYP3A4 induction category (inducer/non-inducer) on the basis of the cryoheps experiment and concentrations of test items in vitro (F2 = 2-fold induction) and in vivo (C_{max})

	Induction in vitro	F2 (µM)	In vivo C_{max} (µM)	C_{max}/F2	Prediction In vivo inducer	Induction shown in vivo	True Positive predictivity	True Negative predictivity
Omeprazole	++	38.7-117	0.68-3.5	0.017-0.09	No*	No		Y
Carbamazepine	++	6.27 – 18.	39	6.2-2.2	Yes	yes	Y	
Phenytoin	++	1.80 – 16.2	40-80	2.4-44	Yes	yes?	Y	
Rifabutin	++	0.1	0.44	4.4	Yes	Yes	Y	
Sulfinpyrazone	++	3,66	45	12,2	Yes	Yes	Y	
Bosentan	++	0.65 - 1.95	5,8	2.9-8.9	yes	Yes	Y	
Efavirenz	++	14.1	9.1-12.6	0.65-0.89	Yes?	Yes	Y?	
Artemisinin	++	5.25 – 47.2	1.0-2.0	0.02-0.38	No*	Yes and No***		Y
Rifampicin	++	0,2	8.0-12.0	40-60	Yes	Yes	Y	
metoprolol	+	nv	0.14-0.38	nv	No	No		Y
penicillin	-	112	36	0,32	No	No		Y
Sotalol	-	nv	2	nv	No	No		Y

*it has to be stressed that for this prediction it is necessary to know (or estimate in a reliable way) the C_{max} concentration of the test item. Without this knowledge the prediction would be 'yes'.

** Artemesinin has been shown to be both an inducer and non-inducer of CYP3A in clinical studies using different probe substrates

Human Hepatocytes:

Sensitivity= TP/(TP+FN) 7/7+0=1.0

Specificity= TN/TN+FP) 5/5+0=1.0

Table 5M.3 Prediction of CYP2B6 in vivo induction category (inducer/non-inducer) on the basis of the cryoHepaRG experiment and concentrations of test items in vitro (F2 = 2-fold induction) and in vivo (C_{max})

	Induction in vitro	F2 (µM)	In vivo C_{max} (µM)	C_{max}/F2	Prediction In vivo inducer	Induction shown in vivo	True Positive predictivity	True Negative predictivity
Omeprazole	++	38,6	0.68-3.5	0.017-0.09	No*	No		Y
Carbamazepine	++	18,8	39	2,1	Yes	Yes	Y	
Phenytoin	++	1.35	40-80	29.6-59	Yes	Yes	Y	
Sulfinpyrazone	+	11.0-33.0	45	1.3-4.1	Yes	?**		
Bosentan	++	2.60-7.80	5,8	0.7-2.2	Yes	?**		
Artemisinin	++	0.58	1.0-2.0	1.7-3.4	Yes	Yes	Y	
Rifampicin	++	0.6-1,80	8.0-12.0	4.4-20	Yes	Yes	Y	
metoprolol	-	nv	0.14-0.38	nv	No	No		Y
Penicillin G	-	nv	36	nv	No	No		Y
Sotalol	-	nv	2	nv	No	No		Y

*it has to be stressed that for this prediction it is necessary to know (or estimate in a reliable way) the C_{max} concentration of the test item. Without this knowledge the prediction would be 'yes'.
** No information on in vivo effects

HepaRG

Sensitivity= TP/(TP+FN) 4/4+0=1

Specificity= TN/TN+FP) 4/4+0=1

Table 5M.4 Prediction of CYP2B6 induction category (inducer/non-inducer) on the basis of the cryoheps experiment and concentrations of test items in vitro (F2 = 2-fold induction) and in vivo (C_{max})

	Induction in vitro	F2 (µM)	In vivo C_{max} (µM)	C_{max}/F2	Prediction In vivo inducer	Induction shown in vivo	True Positive predictivity	True Negative predictivity
Omeprazole	++	38.6 - 116	0.68-3.5	0.0006-0.09	No*	No		Y
Carbamazepine	++	2.09 – 6.27	39	66.2-18.7	Yes	Yes	Y	
Phenytoin	++	0.60 – 48.6	40-80	0.8-133	Yes	Yes	Y	
Rifabutin		0.29-0.87	0.44	1.5-2.0	Yes	Yes	Y	
Sulfinpyrazone	++	33	45	1,3	Yes	?**		
Bosentan	++	5,85	5,8	1	yes	?**		
Efavirenz		4.69	9.1-12.6	1.9-2.7	yes	Yes	Y	
Artemisinin	++	0.58 – 1.75	1.0-2.0	0.4-3.4	Yes	Yes	Y	
Rifampicin	++	0,2	8.0-12.0	40-60	Yes	Yes	Y	
metoprolol	-	nv	0.14-0.38	nv	No	No		Y
penicillin	-	nv	36	nv	No	No		Y
Sotalol	-	nv	2	nv	No	No		Y

*it has to be stressed that for this prediction it is necessary to know (or estimate in a reliable way) the C_{max} concentration of the test item. Without this knowledge the prediction would be 'yes'.
** No information on in vivo effects

Human Hepatocytes:

Sensitivity= TP/(TP+FN) 6/6+0=1

Specificty= TN/TN+FP) 4/4+0=1

Table 5M.5 Prediction of CYP1A2 in vivo induction category (inducer/non-inducer) on the basis of the cryoHepaRG experiment and concentrations of test items in vitro (F2 = 2-fold induction) and in vivo (C_{max})

	Induction in vitro	F2 (µM)	In vivo C_{max} (µM)	C_{max}/F2	Prediction In vivo inducer	Induction shown in vivo	True Positive predictivity	True Negative predictivity
Omeprazole	++	12,9	0.68-3.5	0.05-0.3	No*	No		y
Carbamazepine	++	54,6	39	0,7	Yes	yes	Y	
Phenytoin	++	36,5	40-80	1.1-2.2	Yes	Yes	Y	
Sulfinpyrazone	++	11	45	4	Yes	Yes?	Y?	
Bosentan	++	2.60 – 7.80	5,8	0.45-0.74	No*	?		
Artemisinin	-	nv	1.0-2.0		No	No		Y
Rifampicin	++	0.20 – 0.60	8.0-12.0	13-60	Yes	Yes	Y	
metoprolol	-	nv	0.14-0.38	nv	No	No		y
Penicillin G	-	nv	36	nv	No	No		y
Sotalol	-	nv	2	nv	No	No		y

*it has to be stressed that for this prediction it is necessary to know (or estimate in a reliable way) the C_{max} concentration of the test item. Without this knowledge the prediction would be 'yes'.

HepaRG

Sensitivity= TP/(TP+FN) 4/4+0=1

Specificity= TN/TN+FP) 5/5+0=1

Table 5M.6 Prediction of CYP1A2 induction category (inducer/non-inducer) on the basis of the cryoheps experiment and concentrations of test items in vitro (F2 = 2-fold induction) and in vivo (C_{max})

	Induction in vitro	F2 (µM)	In vivo C_{max} (µM)	C_{max}/F2	Prediction In vivo inducer	Induction shown in vivo	True Positive predictivity	True Negative predictivity	False Negative
Omeprazole	++	38.6-118	0.68-3.5	0.005-0.09	No*	No		Y	
Carbamazepine	+	No value	39	Novalue		yes			Y
Phenytoin	++	16.2 – 48.6	40-80	0.8-4.9	Yes	yes?	Y		
Rifabutin		2.2-6,7	0.44	0.06-0.2	No	No		Y	
Sulfinpyrazone	+	33.0 – 98.9	45	1,3	Yes	?			
Bosentan	+	?	5,8	No value	No	No*		Y	
Efavirenz	-	?	9.1-12.6	?	no	no		Y	
Artemisinin	+	0.49 – 15.7	1.0-2.0	4-0,06	Y	no			Y
Rifampicin	+	No value	8.0-12.0	No value	No	Yes			Y
metoprolol	++	nv	0.14-0.38	nv	No	No		Y	
penicillin	-	nv	36	nv	No	No		Y	
Sotalol	-	nv	2	nv	No	No		Y	

*it has to be stressed that for this prediction it is necessary to know (or estimate in a reliable way) the C_{max} concentration of the test item. Without this knowledge the prediction would be 'yes'.

Human Hepatocytes:

Sensitivity= TP/(TP+FN) 1/1+3=0.25

Specificty= TN/TN+FP) 7/7+0=1

9 SUMMARY OF THE SENSITIVITY AND SPECIFICITY ANALYSIS

The present study shows that cryoHepaRG cells and cryoheps are equally good to indicate whether a compound will be an inducer *in vivo* based on a qualitative correlation analysis using F2 value (the concentration resulting in 2-fold induction of the enzyme activity) from the *in vitro* cell system and Cmax concetrations *in vivo*. The sensitivity and specificity was 100% for both cell system by the drug substances used in the present evaluation.

Methods to predict induction of CYP1A2 and CYP2B6 is less practiced since fewer compounds have been documented *in vivo* to induce these enzymes. However if we apply the same model as for CYP3A the prediction of CYP2B6 by cryoHepaRG cells and cryoheps also reached 100% sensitivity and 100% specificity.

The cryoHepaRG cells showed a similar high sensitivity and specificity (100%) for CYP1A2 induction. However cryoheps only showed a 25% sensitivity for CYP1A2 induction based on the results that 3 of the 4 compounds known to induce CYP1A2 *in vivo* did not give a clear CYP1A2 induction result in cryoheps. The specificity was however 100%, since all the compounds not showing induction *in vivo* did not indicate a significant induction of CYP1A in cryoheps.

The weak induction of CYP2C9 in all conducted experiments, reflects the clinical situation. In clinical studies CYP2C9 induction by rifampicin is much lower than CYP3A4 induction. For this reason FDA, EMA and the pharmaceutical industry excluded CYP2C9 induction assessment from the induction battery. Furthermore, it is considered to be a minor problem and always secondary to induction of CYP3A4.

In conclusion: cryoHepaRG cells showed 100% sensitivity and specificity for the prediction of CYP1A2, CYP2B6 and CYP3A induction based on the results from the compounds used in the present study. Cryoheps also showed 100% sensitivity and specificity for CYP2B6 and CYP3A induction. However primary human hepatocytes showed only 25% sensitivity for prediction of CYP1A2 induction since the cells failed to predict induction by three compounds known to induce CYP1A2 *in vivo*. Cryoheps showed 100% specificity since the cells predicted all CYP1A2 non-inducers to be negative.

CYP3A4 activity				
		HepaRG cells		
		Inducers	Non-inducers	Undetermined/not tested
In vivo	Inducers 7 or 8*	5	1	2
	Non-inducers 3 or 4*	0	4	
	Undetermined 1	1	0	

*Artemesinin has been shown to be both an inducer and non-inducer of CYP3A in clinical studies using different probe substrates

CYP3A4 activity				
		Cryoheps		
		Inducers	Non-inducers	Undetermined
In vivo	Inducers 7 or 8	8	0	
	Non-inducers 3 or 4*	0	2	
	Undetermined 1	1	0	

*Artemesinin has been shown to be both an inducer and non-inducer of CYP3A in clinical studies using different probe substrates

10 REFERENCES

Abadie-Viollon C, Martin H, Blanchard N, Pekthong D, Bachellier P, Mantion G, Heyd B, Schuler F, Coassolo P, Alexandre E, Richert L. (2010) Follow-up to the pre-validation of a harmonised protocol for assessment of CYP induction responses in freshly isolated and cryopreserved human hepatocytes with respect to culture format, treatment, positive reference inducers and incubation conditions. Toxicol In Vitro. 24(1):346-56.

Abass K, Lämsä V, Reponen P, Küblbeck J, Honkakoski P, Mattila S, Pelkonen O, Hakkola J. (2012) Characterization of human cytochrome P450 induction by pesticides. Toxicology 294(1):17-26.

Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM. (2011) Alternative (non - animal) methods for cosmetics testing: current status and future prospects-2010. Arch Toxicol. 85(5):367-485.

Aguiar M, Masse R, and Gibbs BF. (2005) Regulation of cytochrome P450 by posttranslational modification. Drug Metabolism reviews 37:379-404.

Alexandre E, Baze A, Parmentier C, Desbans C, Pekthong D, Gerin B, Wack C, Bachellier P, Heyd B, Weber JC, Richert L. (2012) Plateable cryopreserved human hepatocytes for the assessment of cytochrome P450 inducibility: experimental condition-related variables affecting their response to inducers. Xenobiotica. 42(10):968-79

Andersson T. (1996) Pharmacokinetics, metabolism and interactions of acid pump inhibitors. Focus on omeprazole, lansoprazole and pantoprazole. Clin Pharmacokinet. 31(1):9-28.

Andersson T, Hassan-Alin M, Hasselgren G, Röhss K. (2001) Drug interaction studies with esomperazole, the (s)-isomer of omeprazole Clin Pharmacokinet. 40(7):523-37.

Andersson TB, Kanebratt KP, Kenna JG. (2012) The HepaRG cell line: a unique in vitro tool for understanding drug metabolism and toxicology in human. Expert Opin Drug Metab Toxicol. 8(7):909-20.

Andreasen AH, Brøsen K, Damkier P. (2007) A comparative pharmacokinetic study in healthy volunteers of the effect of carbamazepine and oxcarbazepine on cyp3a4. Epilepsia. 48(3):490-6.

Aninat C, Piton A, Glaise D, Le Charpentier T, Langouët S, Morel F, Guguen-Guillouzo C, Guillouzo A. (2006) Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab Dispos. 34(1):75-83.

Asimus S, Elsherbiny D, Hai TN, Jansson B, Huong NV, Petzold MG, Simonsson US, Ashton M. (2007) Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects. Fundam Clin Pharmacol. 21(3):307-16.

Bao Ting Zhu (2010) On the General Mechanism of Selective Induction of Cytochrome P450 Enzymes by Chemicals: Some Theoretical Considerations. Expert Opin Drug Metab Toxicol. 6, 483–494.

Backman JT, Granfors MT, Neuvonen PJ. (2006) Rifampicin is only a weak inducer of CYP1A2-mediated presystemic and systemic metabolism: studies with tizanidine and caffeine. Eur J Clin Pharmacol. 62(6):451-61.

Baird WM, Hooven LA, Mahadevan B. (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ Mol Mutagen. 45(2-3):106-14.

Balint GA. (2001) Artemisinin and its derivatives: an important new class of antimalarial agents. Pharmacol Ther. 90(2-3):261-265.

Barditch-Crovo P, Trapnell CB, Ette E, Zacur HA, Coresh J, Rocco LE, Hendrix CW, Flexner C. (1999) The effects of rifampin and rifabutin on the pharmacokinetics and pharmacodynamics of a combination oral contraceptive. Clin Pharmacol Ther. 65(4):428-38.

Barry M, Feely J. (1990) Enzyme induction and inhibition. Pharmacol Ther.48(1):71-94.

Bernus I, Dickinson RG, Hooper WD, Eadie MJ. (1994) Early stage autoinduction of carbamazepine metabolism in humans. Eur J Clin Pharmacol. 47(4):355-60.

Birkett DJ, Miners JO, Attwood J. (1983) Evidence for a dual action of sulphinpyrazone on drug metabolism in man: theophylline-sulphinpyrazone interaction. Br J Clin Pharmacol. 15(5):567-9.

Blake CM, Kharasch ED, Schwab M, Nagele P. (2013) A meta-analysis of CYP2D6 metabolizer phenotype and metoprolol pharmacokinetics. Clin Pharmacol Ther. 94(3): 394-399

Blaschke TF, Skinner MH. (1996) The clinical pharmacokinetics of rifabutin. Clin Infect Dis. 22: S15-21.

Caforio AL, Gambino A, Tona F, Feltrin G, Marchini F, Pompei E, Testolin L, Angelini A, Dalla Volta S, Casarotto D. (2000) Sulfinpyrazone reduces cyclosporine levels: a new drug interaction in heart transplant recipients. Heart Lung Transplant. 19(12):1205-8

Cerec V, Glaise D, Garnier D, Morosan S, Turlin B, Drenou B, Gripon P, Kremsdorf D, Guguen-Guillouzo C, and Corlu A (2007) Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. Hepatology 45:957–967.

Chetty M, Miller R, Seymour MA. (1998) Phenytoin auto-induction. Ther Drug Monit. 20(1):60-2.

Chu V, Einolf HJ, Evers R, Kumar G, Moore D, Ripp S, Silva J, Sinha V, Sinz M and Skerjanec A. (2009) In vitro and in vivo induction of cytochrome p450: a survey of the current practices and recommendations: a pharmaceutical research and manufacturers of America perspective. Drug Metab Dispos. 37:1339-1354.

Chen Y, Ferguson SS, Negishi M, Goldstein JA. (2004) Induction of human CYP2C9 by rifampicin, hyperforin, and phenolbarbital is mediated by the pregnane X receptor. J Pharmacol Exp Ther. 308:495–501.

Choi S.Y., Koh K.H. and Jeong H. (2013) Isoform-specific regulation of cytochromes P450 expression by estradiol and progesterone. Drug Metab Dispos 41:263-269.

Coecke S, Rogiers V, Bayliss M, Castell J, Doehmer J, Fabre G, Fry J, Kern A and Westmoreland C. (1999) The Use of Long-term Hepatocyte Cultures for Detecting Induction of Drug Metabolising Enzymes: The Current Status ECVAM Hepatocytes and Metabolically Competent Systems Task Force Report 1. ATLA 27, 579-638.

Coecke S, Ahr H, Blaauboer BJ, Bremer S, Casati S, Castell J, Combes R, Corvi R, Crespi CL, Cunningham ML, Elaut G, Eletti B, Freidig A, Gennari A, Ghersi-Egea JF, Guillouzo A, Hartung T, Hoet P, Ingelman-Sundberg M, Munn S, Janssens W, Ladstetter B, Leahy D, Long A, Meneguz A, Monshouwer M, Morath S, Nagelkerke F, Pelkonen O, Ponti J, Prieto P, Richert L, Sabbioni E, Schaack B, Steiling W, Testai E, Vericat JA and Worth A. (2006)

Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. Altern Lab Anim. 34: 49-84.

Coecke S, Pelkonen O, Leite SB, Bernauer U, Bessems JG, Bois FY, Gundert-Remy U, Loizou G, Testai E and Zaldívar JM. (2013) Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. Toxicol In Vitro 27(5):1570-7.

Cooney GF, Mochon M, Kaiser B, Dunn SP, Goldsmith B. (1995) Effects of carbamazepine on cyclosporine metabolism in pediatric renal transplant recipients. Pharmacotherapy 15(3):353-356.

Crawford P, Chadwick DJ, Martin C, Tjia J, Back DJ, Orme M. The interaction of phenytoin and carbamazepine with combined oral contraceptive steroids. (1990) Br J Clin Pharmacol.30(6):892-6.

Cui X, Palamanda J, Norton L, Thomas A, Lau YY, White RE, Cheng KC. (2002) A high-throughput cell-based reporter gene system for measurement of CYP1A1 induction. J Pharmacol Toxicol Methods. 47(3):143-51.

Denison MS, Nagy SR. (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol. 43:309-34.

Desbans C, Hilgendorf C, Lutz M, Bachellier P, Zacharias T, Weber JC, Dolgos H, Richert L, Ungell AL. (2014) Prediction of fraction metabolized via CYP3A in humans utilizing cryopreserved human hepatocytes from a set of 12 single donors. Xenobiotica. 44(1):17-27.

de Vries PJ, Dien TK. (1996) Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. Drugs52(6):818-836.

Diaz D, Fabre I, Daujat M, Saint Aubert B, Bories P, Michel H, and Maurel P. (1990) Omeprazole is an aryl hydrocarbon-like inducer of human hepatic cytochrome P450. Gastroenterology 99:737–747.

Dickinson RG, Hooper WD, Patterson M, Eadie MJ, Maguire B. (1985) Extent of urinary excretion of p-hydroxyphenytoin in healthy subjects given phenytoin. Ther Drug Monit. 7(3):283-9.

Dingemanse J, Schaarschmidt D, van Giersbergen PL. (2003) Investigation of the mutual pharmacokinetic interactions between bosentan, a dual endothelin receptor antagonist, and simvastatin. Clin Pharmacokinet. 42(3):293-301.

Dingemanse J, van Giersbergen PL. (2004) Clinical pharmacology of bosentan, a dual endothelin receptor antagonist. Clin Pharmacokinet. 43(15):1089-115.

Doose DR, Wang SS, Padmanabhan M, Schwabe S, Jacobs D, Bialer M. (2003) Effect of topiramate or carbamazepine on the pharmacokinetics of an oral contraceptive containing norethindrone and ethinyl estradiol in healthy obese and nonobese female subjects. Epilepsia44(4):540-549.

EC (2010). EC Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

EC (2009). EC Regulation 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products.

EC (2006). EC Regulation 1907/2006 of the European Parliament and of the Council of 18 december 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

EFSA European Food Safety Authority (2011) Draft Guidance for submission for food additive evaluations.

Einolf HJ, Chen L, Fahmi OA, Gibson CR, Obach RS, Shebley M, Silva J, Sinz MW, Unadkat JD, Zhang L, Zhao P. (2014) Evaluation of Various Static and Dynamic Modeling Methods to Predict Clinical CYP3A Induction Using In Vitro CYP3A4 mRNA Induction Data. Clin Pharmacol Ther. 95(2):179-88.

Elsherbiny DA, Asimus SA, Karlsson MO, Ashton M, Simonsson US. (2008) A model based assessment of the CYP2B6 and CYP2C19 inductive properties by artemisinin antimalarials: implications for combination regimens. J Pharmacokinet Pharmacodyn.35(2):203-17.

EMA European Medicines Agency (2012). Guideline on the Investigation of Drug Interactions.

Ernest CS 2nd, Hall SD, Jones DR. (2005) Mechanism-based inactivation of CYP3A by HIV protease inhibitors. J Pharmacol Exp Ther. 312(2):583-91.

FDA Food and Drug Administration (2012). Guidance for Industry Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations.

Fellay J, Marzolini C, Decosterd L, Golay KP, Baumann P, Buclin T, Telenti A, Eap CB. (2005) Variations of CYP3A activity induced by antiretroviral treatment in HIV-1 infected patients. Eur J Clin Pharmacol. 60(12):865-73.

Fahmi OA, Kish M, Boldt S, Obach RS. (2010) Cytochrome P450 3A4 mRNA is a more reliable marker than CYP3A4 activity for detecting pregnane X receptor-activated induction of drug-metabolizing enzymes. *Drug Metab Dispos*. 38(9):1605-11.

Fridell JA, Jain AK, Patel K, Virji M, Rao KN, Fung J. (2003). Phenytoin decreases the blood concentrations of sirolimus in a liver transplant recipient: a case report. Ther Drug Monit. 25(1):117-119

Gerets HH, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, et al. (2012) Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol Toxicol. 28(2):69-87.

Giao PT, de Vries PJ. (2001) Pharmacokinetic interactions of antimalarial agents. Clin Pharmacokinet. 40(5):343-73.

Gibson GG, Plant NJ, Swales KE, Ayrton A, El-Sankary W. (2002) Receptor-dependent transcriptional activation of cytochrome P4503A genes: induction mechanisms,species differences and interindividual variation in man. Xenobiotica. 32(3):165-206.

Goodwin B, Hodgson E, D'Costa DJ, Robertson GR, Liddle C. (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. Mol Pharmacol. 62(2):359-65.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C. (2002) Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 26;99(24):15655-60.

Guengerich FP. (2011) Mechanisms of drug toxicity and relevance to pharmaceutical development. Drug Metab Pharmacokinet. 26(1):3-14.

Guillouzo A, Corlu A, Aninat C,Glaise D, Morel F, Guguen-Guillouzo C. (2007) The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-Biological Interactions* 168:66–73.

Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, Gribaldo L, Halder M, Hoffmann S, Roi AJ, Prieto P, Sabbioni E, Scott L, Worth A, Zuang V. (2004) A modular approach to the ECVAM principles on test validity. Altern Lab Anim. 32(5):467-72.

Hauder J, Winkler S, Bub A, Rüfer CE, Pignitter M, and Somoza V. (2011) LC-MS/MS Quantification of Sulforaphane and Indole-3-carbinol Metabolites in Human Plasma and Urine after Dietary Intake of Selenium-Fortified Broccoli. J Agric Food Chem. 59(15):8047-57.

He M, Rettie AE, Neal J, Trager WF. (2001) Metabolism of sulfinpyrazone sulfide and sulfinpyrazone by human liver microsomes and cDNA-expressed cytochrome P450s. Drug Metab Dispos. 29(5):701-11.

Heimark LD, Gibaldi M, Trager WF, O'Reilly RA, Goulart DA. (1987) The mechanism of the warfarin-rifampin drug interaction in humans. Clin Pharmacol Ther. 42(4):388-94.

Herman D, Locatelli I, Grabnar I, Peternel P, Stegnar M, Lainscak M, Mrhar A, Breskvar K, Dolzan V. (2006) The influence of co-treatment with carbamazepine, amiodarone and statins on warfarin metabolism and maintenance dose. Eur J Clin Pharmacol. 62(4):291-6.

Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG. (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev. 1:159-234.

Hodgson E, Rose RL. (2007) Human metabolic interactions of environmental chemicals. J Biochem Mol Toxicol. 21(4):182-6.

Honkakoski P, Negishi M. (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. Biochem J. 347:321-37.

Houston JB, Rowland-Yeo K, Zanelli U. (2012) Evaluation of the novel in vitro systems for hepatic drug clearance and assessment of their predictive utility. Toxicol In Vitro. 26(8):1265-71.

Hsu O, Hill CJ, Kim M, Tan B, O'Brien JG. (2010) Decreased plasma efavirenz concentrations in a patient receiving rifabutin. Am J Health Syst Pharm.67(19):1611-4.

Hukkanen J. (2012) Induction of cytochrome P450 enzymes: a view on human in vivo findings. Expert Rev Clin Pharmacol. 5(5):569-85.

Ji P, Damle B, Xie J, Unger SE, Grasela DM, Kaul S. (2008) Pharmacokinetic interaction between efavirenz and carbamazepine after multiple-doseadministration in healthy subjects. J Clin Pharmacol. 48(8):948-56.

Jossé R, Aninat C, Glaise D, Dumont J, Fessard V, Morel F, Poul JM, Guguen-Guillouzo C, Guillouzo A. (2008) Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metab Dispos. 36(6):1111-8.

Kanebratt KP, Andersson TB. (2008a) Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. Drug Metab Dispos. 36(7):1444-52.

Kanebratt KP, Andersson TB. (2008b) HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans. Drug Metab Dispos. 36(1):137-45.

Kanebratt KP, Diczfalusy U, Bäckström T, Sparve E, Bredberg E, Böttiger Y, Andersson TB, Bertilsson L. (2008c) Cytochrome P450 induction by rifampicin in healthy subjects: determination using the Karolinska cocktail and the endogenous CYP3A4 marker 4beta-hydroxycholesterol. Clin Pharmacol Ther. 84(5):589-94.

Kay L, Kampmann JP, Svendsen TL, Vergman B, Hansen JE, Skovsted L, Kristensen M. (1985) Influence of rifampicin and isoniazid on the kinetics of phenytoin. Br J Clin Pharmacol. 20(4):323-6.

Ketter TA, Jenkins JB, Schroeder DH, Pazzaglia PJ, Marangell LB, George MS, Callahan AM, Hinton ML, Chao J, Post RM. (1995) Carbamazepine but not valproate induces bupropion metabolism. J Clin Psychopharmacol. 15(5):327-33.

Kirby BJ, Collier AC, Kharasch ED, Dixit V, Desai P, Whittington D. (2011) Complex drug interactions of HIV protease inhibitors 2: in vivo induction and in vitro to in vivo correlation of induction of cytochrome P450 1A2, 2B6, and 2C9 by ritonavir or nelfinavir. Drug Metab Dispos.;39(12):2329-37.

Kirchheiner J, Meisel C, Heesch C, Bauer S, Keulen T, Henckel H, Roots I, Brockmuller J. (2004) Metoprolol pharmacokinetics and pharmacodynamics in carriers of CYP2D6 genotypes predicting ultra-rapid metabolism. Clinical Pharmacol. and Ther. 75: 19.

Knockaert L1, Fromenty B, Robin MA. (2011) Mechanisms of mitochondrial targeting of cytochrome P450 2E1: physiopathological role in liver injury and obesity. FEBS J. 278(22):4252-60.

Koo HL, Hamill RJ, Andrade RA. (2007) Drug-drug interaction between itraconazole and efavirenz in a patient with AIDS and disseminated histoplasmosis. Clin Infect Dis.;45(6):77-79.

Kwara A, Tashima KT, Dumond JB, Poethke P, Kurpewski J, Kashuba AD. (2011) Modest but variable effect of rifampin on steady-state plasma pharmacokinetics of efavirenz in healthy African-American and Caucasian volunteers. Antimicrob Agents Chemother. 55(7):3527-33.

Lai ML, Lin TS, Huang JD. (1992) Effect of single- and multiple-dose carbamazepine on the pharmacokinetics of diphenylhydantoin. Eur J Clin Pharmacol. 43(2):201-3

Lin JH. (2006) CYP induction-mediated drug interactions: in vitro assessment and clinical implications. Pharm Res. 23(6):1089-116.

LeBel M, Masson E, Guilbert E, Colborn D, Paquet F, Allard S. (1998) Effects of rifabutin and rifampicin on the pharmacokinetics of ethinylestradiol and norethindrone. J Clin Pharmacol. 38(11):1042-1050.

LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, Richert L. (2005) Isolation and culture of primary human hepatocytes. Methods Mol Biol.290:207-29.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. J Clin Invest. 102(5):1016-23.

Le Vee M, Jigorel E, Glaise D, Gripon P, Guguen-Guillouzo C, Fardel O. (2006) Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. Eur J Pharm Sci. May;28(1-2):109-17.

Loboz KK, Gross AS, Williams KM, Liauw WS, Day RO, Blievernicht JK, Zanger UM, McLachlan AJ. (2006) Cytochrome P450 2B6 activity as measured by bupropion hydroxylation: effect of induction by rifampin and ethnicity. Clin Pharmacol Ther. 80(1):75-84.

López-Cortés LF, Ruiz-Valderas R, Viciana P, Alarcón-González A, Gómez-Mateos J, León-Jimenez E, Sarasanacenta M, López-Pua Y, Pachón J. (2002) Pharmacokinetic interactions between efavirenz and rifampicin in HIV-infected patients with tuberculosis. Clin Pharmacokinet41(9):681-90.

Lucas RA, Gilfillan DJ, Bergstrom RF. (1998) A pharmacokinetic interaction between carbamazepine and olanzapine: observations on possible mechanism. Eur J Clin Pharmacol. 54(8):639-43

Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E, Gan LS. (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. Drug Metab Dispos. 30(7):795-804.

Ma Q, Lu AY. (2007) CYP1A induction and human risk assessment: an evolving tale of in vitro and in vivo studies. Drug Metab Dispos. 35(7):1009-16.

Magnusson MO, Dahl ML, Cederberg J, Karlsson MO, Sandström R. (2008) Pharmacodynamics of carbamazepine-mediated induction of CYP3A4, CYP1A2, and Pgp as assessed by midazolam, caffeine, and digoxin. Clin Pharmacol Ther. 84(1):52-62.

Maronpot RR, Yoshizawa K, Nyska A, Harada T, Flake G, Mueller G, Singh B, Ward JM. (2010) Hepatic enzyme induction: histopathology. Toxicol Pathol. 38(5):776-95.

Martignoni M, Groothuis GM, de Kanter R. (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol. 2(6):875-94.

Masahiko N, Honkakoski P. (2000) Induction of drug metabolism by nuclear receptor CAR: molecular mechanisms and implications for drug research. Eur J Pharm Sci. 11(4):259-64.

McDonnell WM, Scheiman JM, Traber PG. (1992) Induction of cytochrome P450IA genes (CYP1A) by omeprazole in the human alimentary tract. Gastroenterology. 103(5):1509-16.

McGinnity DF, Zhang G, Kenny JR, Hamilton GA, Otmani S, Stams KR, Haney S, Brassil P, Stresser DM, Riley RJ. (2009) Evaluation of multiple in vitro systems for assessment of CYP3A4 induction in drug discovery: human hepatocytes, pregnane X receptor reporter gene, and Fa2N-4 and HepaRG cells. Drug Metab Dispos. 37(6):1259-68.

Medhi B, Patyar S, Rao RS, Byrav D S P, Prakash A. (2009) Pharmacokinetic and toxicological profile of artemisinin compounds: an update. Pharmacology. 84(6):323-32.

Michaud V, Ogburn E, Thong N, Aregbe AO, Quigg TC, Flockhart DA. (2012) Induction of CYP2C19 and CYP3A activity following repeated administration of efavirenz in healthy volunteers. Clin Pharmacol Ther. 91(3):475-482.

Miller M, Cosgriff J, Kwong T, Morken DA. (1990) Influence of phenytoin on theophylline clearance. Clin Pharmacol Ther. 35(5):666-9.

Miners JO, Birkett DJ. (1998) Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. Br J Clin Pharmacol. 45(6):525-38.

Moreland TA, Park BK, Rylance GW. (1982) Microsomal enzyme induction in children: the influence of carbamazepine treatment on antipyrine kinetics, 6 beta-hydroxycortisol excretion and plasma gamma-glutamyltranspeptidase activity. Br J Clin Pharmacol. 14(6):861-5.

Mouly S, Lown KS, Kornhauser D, Joseph JL, Fiske WD, Benedek IH, Watkins PB. (2002) Hepatic but not intestinal CYP3A4 displays dose-dependent induction by efavirenz in humans. Clin Pharmacol Ther. 72(1):1-9.

Mwinyi J., Cavaco I., Yurdakok B., Mkrtchian S. and Ingelman-Sundberg M. (2011) The ligands of estrogen receptor α regulate cytochrome P4502C9 (CYP2C9) expression. J Pharmacol Exp Ther. 338:302-309

Nakajima M. and Yokoi T. (2011) microRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. Pharmacol Ther. 131:330-7.

Nelson E, Powell JR, Conrad K, Likes K, Byers J, Baker S, Perrier D. (1982) Phenobarbital pharmacokinetics and bioavailability in adults. J Clin Pharmacol. 22(2-3):141-148.

Ngaimisi E, Mugusi S, Minzi OM, Sasi P, Riedel KD, Suda A, Ueda N, Janabi M,Mugusi F, Haefeli WE, Burhenne J, Aklillu E. (2010) Long-term efavirenz autoinduction and its effect on plasma exposure in HIV patients. Clin Pharmacol Ther. 88(5):676-84.

Obach RS, Walsky RL, Venkatakrishnan K. Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug-drug interactions. (2007) Drug Metab Dispos. 35(2):246-55.

OECD (2005). Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD Guidance on testing and assessment. OECD, Paris, No. 34.

OECD (2010). 417: Guidelines for the testing of chemicals: toxicokinetics

OECD (2012). Draft Guidance Document 151 in support of OECD Test Guideline 443 on an Extended One Generation Reproductive Toxicity Study.

Ohnhaus EE, Park BK. (1979) Measurement of urinary 6-beta-hydroxycortisol excretion as an in vivo parameter in the clinical assessment of the microsomal enzyme-inducing capacity of antipyrine, phenobarbitone and rifampicin. Eur J Clin Pharmacol. 26;15(2):139-45.

O'Reilly RA. (1974) Interaction of sodium warfarin and rifampin. Studies in man. Ann Intern Med. 81(3):337-40.

Oscarson M, Zanger UM, Rifki OF, Klein K, Eichelbaum M, Meyer UA. (2006) Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. Clin Pharmacol Ther. 80(5):440-456.

Pantuck EJ, Pantuck CB, Garland WA, Min BH, Wattenberg LW, Anderson KE, Kappas A, Conney AH. (1979) Stimulatory effect of brussels sprouts and cabbage on human drug metabolism. Clin Pharmacol Ther. 25(1):88-95.

Parker AC, Pritchard P, Preston T, Choonara I. (1998) Induction of CYP1A2 activity by carbamazepine in children using the caffeine breath test. Br J Clin Pharmacol. 45(2):176-8.

Parkinson A, Ogilvie BW (2010) Biotransformation of xenobiotics. In: Casarett and Doull's Toxicology: The Basic Science of Poisons, 7th edition, Ed by C.D.Klaassen. McGraw-Hill, New York and London, pp. 161-304.

Paul GA, Gibbs JS, Boobis AR, Abbas A, Wilkins MR. (2005) Bosentan decreases the plasma concentration of sildenafil when coprescribed in pulmonary hypertension. Br J Clin Pharmacol. 60(1):107-112.

Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J, Raunio H. (2008) Inhibition and induction of human cytochrome P450 enzymes: current status. Arch Toxicol. 82(10):667-715.

Perucca E, Grimaldi R, Frigo GM, Sardi A, Mönig H, Ohnhaus EE. (1988) Comparative effects of rifabutin and rifampicin on hepatic microsomal enzyme activity in normal subjects. Eur J Clin Pharmacol. 34(6):595-9.

Preissner S, Kroll K, Dunkel M, Senger C, Goldsobel G, Kuzman D, Guenther S, Winnenburg R, Schroeder M, Preissner R. (2010) SuperCYP: a comprehensive database

on Cytochrome P450 enzymes including a tool for analysis of CYP-drug interactions. Nucleic Acids Res. 38: 237-43.

Raucy JL. (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. Drug Metab Dispos. 31(5):533-539

Reed GA, Peterson KS, Smith HJ, Gray JC, Sullivan DK, Mayo MS, Crowell JA, Hurwitz A. (2005) A phase I study of indole-3-carbinol in women: tolerability and effects. Cancer Epidemiol Biomarkers Prev. 14(8):1953-60.

Reinach B, de Sousa G, Dostert P, Ings R, Gugenheim J, Rahmani R. (1999) Comparative effects of rifabutin and rifampicin on cytochromes P450 and UDP-glucuronosyl-transferases expression in fresh and cryopreserved human hepatocytes. Chem Biol Interact. 121(1):37-48.

Rhodes SP., Otten JN, Hingorani GP, Hartley DP. (2011) Simultaneous assessment of cytochrome P450 activity in cultured human hepatocytes for compound-mediated induction of CYP3A4, CYP2B6, and CYP1A2 Journal of Pharmacological and Toxicological Methods 63:223–226 .

Richert L, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P, LeCluyse E. (2002) Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. Toxicol In Vitro. 16(1):89-99.

Richert L, Abadie C, Bonet A, Heyd B, Mantion G, Alexandre E, Bachellier P, Kingston S, Pattenden C, Illouz S, Dennison A, Hoffmann S, Coecke S. (2010) Inter-laboratory evaluation of the response of primary human hepatocyte cultures to model CYP inducers - a European Centre for Validation of Alternative Methods (ECVAM) - funded pre-validation study. Toxicol In Vitro. 24(1):335-45.

Robertson SM, Maldarelli F, Natarajan V, Formentini E, Alfaro RM, Penzak SR. (2008) Efavirenz induces CYP2B6-mediated hydroxylation of bupropion in healthy subjects. J Acquir Immune Defic Syndr. 49(5):513-9.

Robson RA, Miners JO, Wing LM, Birkett DJ. (1984) Theophylline-rifampicin interaction: non-selective induction of theophylline metabolic pathways. Br J Clin Pharmacol.18(3):445-8.

Rodriguez-Antona C, Ingelman-Sundberg M. (2006) Cytochrome P450 pharmacogenetics and cancer. Oncogene. 13;25(11):1679-91.

Roymans D, Annaert P, Van Houdt J, Weygers A, Noukens J, Sensenhauser C, Silva J, Van Looveren C, Hendrickx J, Mannens G, Meuldermans W. (2005) Expression and induction potential of cytochromes P450 in human cryopreserved hepatocytes. Drug Metab Dispos.;33(7):1004-16.

Rost KL, Brösicke H, Heinemeyer G, Roots I. (1994) Specific and dose-dependent enzyme induction by omeprazole in human beings. Hepatology. 20(5):1204-12.

Sevior DK, Pelkonen O, Ahokas JT. (2012) Hepatocytes: the powerhouse of biotransformation. Int J Biochem Cell Biol. 44(2):257-61.

Shimada T. (2006) Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. Drug Metab Pharmacokinet. 21(4):257-76.

Silva JM, Day SH, Nicoll-Griffith DA. (1999) Induction of cytochrome-P450 in cryopreserved rat and human hepatocytes. Chemico-Biological Interactions 121:49–63.

Simonsson US, Jansson B, Hai TN, Huong DX, Tybring G, Ashton M. (2003) Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9. Clin Pharmacol Ther. 74(1):32-43.

Slattery JT, Kalhorn TF, McDonald GB, Lambert K, Buckner CD, Bensinger WI, Anasetti C, Appelbaum FR. (1996) Conditioning regimen-dependent disposition of cyclophosphamide and hydroxycyclophosphamide in human marrow transplantation patients. J Clin Oncol. 14(5):1484-94.

Staiger C, Schlicht F, Walter E, Gundert-Remy U, Hildebrandt R, de Vries J, Wang NS, Harenberg J, Weber E. (1983) Effect of single and multiple doses of sulphinpyrazone on antipyrine metabolism and urinary excretion of 6-beta-hydroxycortisol. Eur J Clin Pharmacol. 25(6):797-801.

Strolin Benedetti M, Pianezzola E, Brianceschi G, Jabes D, Della Bruna C, Rocchetti M, Poggesi I. (1995) An investigation of the pharmacokinetics and autoinduction of rifabutin metabolism in mice treated with 10 mg/kg/day six times a week for 8 weeks. J Antimicrob Chemother. 36(1):247-51.

Sueyoshi T, Kawamoto T, Zelko I, et al. (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. J Biol Chem. 274:6043–6.

Surapureddi S., Rana R., Goldstein J.A. (2011) NCOA6 differentially regulates the expression of the CYP2C9 and CYP3A4 genes. Pharmacol Res. 63:405-413.

Svensson US, Ashton M, Trinh NH, Bertilsson L, Dinh XH, Nguyen VH, Nguyen TN, Nguyen DS, Lykkesfeldt J, Le DC. (1998) Artemisinin induces omeprazole metabolism in human beings. Clin Pharmacol Ther. 64(2):160-7.

Takagi S., Nakajima M., Mohri T., Yokoi T. (2008) Post transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. J Biol Chem. 283:9674-9680.

Tompkins LM and Wallace AD. (2007) Mechanisms of cytochrome P450 induction. J Biochem Mol Toxicol. 21:176-181.

Toon S, Low LK, Gibaldi M, Trager WF, O'Reilly RA, Motley CH, Goulart DA.(1986) The warfarin-sulfinpyrazone interaction: stereochemical considerations. J. Clin Pharmacol Ther. 1986 Jan;39(1):15-24.

Turpeinen M, Tolonen A, Chesne C, Guillouzo A, Uusitalo J, Pelkonen O. (2009) Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. Toxicol In Vitro. 23(4):748-53.

Turpeinen M, Raunio H, Pelkonen O. (2006) The functional role of CYP2B6 in human drug metabolism: substrates and inhibitors in vitro, in vivo and in silico. Curr Drug Metab. 7:705-714.

Ucar M, Neuvonen M, Luurila H, Dahlqvist R, Neuvonen PJ, Mjörndal T (2004) Carbamazepine markedly reduces serum concentrations of simvastatin and simvastatin acid. Eur J Clin Pharmacol.59(12):879-82.

Upton RA. (1991) Pharmacokinetic interactions between theophylline and other medication (Part I). Clin Pharmacokinet. 20(1):66-80.

US Environmental Protection Agency (2011) Integrated approaches to testing and assessment strategy: use of new computational and molecular tools.

van Giersbergen PL, Halabi A, Dingemanse J. (2002a) Single- and multiple-dose pharmacokinetics of bosentan and its interaction with ketoconazole. Br J Clin Pharmacol. 53(6):589-95.

van Giersbergen PL, Treiber A, Clozel M, Bodin F, Dingemanse J. (2002b) In vivo and in vitro studies exploring the pharmacokinetic interaction between bosentan, a dual endothelin receptor antagonist, and glyburide. Clin Pharmacol Ther. 71(4):253-62.

Vestal RE, Cusack BJ, Crowley JJ, Loi CM. (1993) Aging and the response to inhibition and induction of theophylline metabolism. Exp Gerontol. 28(4-5):421-33.

Vinken M, Landesmann B, Goumenou M, Vinken S, Shah I, Jaeschke H, Willett C, Whelan M, Rogiers V (2013) Development of an Adverse Outcome Pathway from drug-mediated bile salt export pump inhibition to cholestatic liver injury. Toxicological Sci. 136(1):97-106.

Xing J, Kirby BJ, Whittington D, Wan Y, Goodlett DR. (2012) Evaluation of P450 inhibition and induction by artemisinin antimalarials in human liver microsomes and primary human hepatocytes. Drug Metab Dispos. 40(9):1757-1764.

Wadelius M, Darj E, Frenne G, Rane A. (1997) Induction of CYP2D6 in pregnancy. Clin Pharmacol Ther. 62(4): 400-407.

Walter E, Staiger C, de Vries J, Weber E, Bitzer W, Degott M, Jüngling K. (1982) Enhanced drug metabolism after sulfinpyrazone treatment in patients aged 50 to 60 years. Klin Wochenschr. 60(22):1409-13.

Wang H, Faucette S, Moore R, Sueyoshi T, Negishi M, LeCluyse E. (2004) Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. J Biol Chem. 279(28):29295-301.

Wang Y., Liao M., Hoe N., Acharya P., Deng C., Krutchinsky A.N. and Correia M.A.(2009) A role for protein phosphorylation in cytochrome P450 3A4 ubiquitin-dependent proteasomal degradation. J Biol Chem. 284:5671-5684.

Wang YM, Ong SS, Chai SC, Chen T. (2012) Role of CAR and PXR in xenobiotic sensing and metabolism. Expert Opin Drug Metab Toxicol. 8(7):803-17.Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z. (2003) The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. J Pharmacol Exp Ther. 306(1):287-300.

Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z. (2003) The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. J Pharmacol Exp Ther. 13:287–300.

Weber C, Gasser R, Hopfgartner G. (1999a) Absorption, excretion, and metabolism of the endothelin receptor antagonist bosentan in healthy male subjects. Drug Metab Disp. 27:810–815.

Weber C, Banken L, Birnboeck H, Schulz R. (1999b) Effect of the endothelin-receptor antagonist bosentan on the pharmacokinetics and pharmacodynamics of warfarin. J Clin Pharmacol. 39(8):847-54.

Weber C, Schmitt R, Birnboeck H, Hopfgartner G, Eggers H, Meyer J, van Marle S, Viischer HW, Jonkman JH. (1999c) Multiple-dose pharmacokinetics, safety, and tolerability of bosentan, an endothelin receptor antagonist, in healthy male volunteers. J Clin Pharmacol. 39(7):703-14.

Werk EE Jr, Macgee J, Sholiton LJ. (1964) Effect of diphenylhydantoin on cortisol metabolism in man. J Clin Invest. 43:1824-35.

Wietholtz H, Zysset T, Kreiten K, Kohl D, Büchsel R, Matern S. (1989) Effect of phenytoin, carbamazepine, and valproic acid on caffeine metabolism. Eur J Clin Pharmacol. 36(4):401-6.

Wietholtz H, Zysset T, Marschall HU, Generet K, Matern S. (1995) The influence of rifampin treatment on caffeine clearance in healthy man. J Hepatol. 22(1):78-81.

Williamson KM, Patterson JH, McQueen RH, Adams KF Jr, Pieper JA. (1998) Effects of erythromycin or rifampin on losartan pharmacokinetics in healthy volunteers. Clin Pharmacol Ther. 63(3):316-23.

Williams ML, Wainer IW, Embree L, Barnett M, Granvil CL, Ducharme MP. (1999) Enantioselective induction of cyclophosphamide metabolism by phenytoin. Chirality. 11(7):569-74.

Wing LM, Miners JO, Lillywhite KJ. (1985) Verapamil disposition-effects of sulphinpyrazone and cimetidine. Br J Clin Pharmacol. 19(3):385-91.

Yajima K, Uno Y, Murayama N, Uehara S, Shimizu M, Nakamura C, Masahiro KI, Yamazaki H. (2014) Evaluation of 23 Lots of Commercially Available Cryopreserved Hepatocytes for Induction Assays of Human Cytochromes P450 Drug Metab Dispos dmd.113.056804; published ahead of print February 19, 2014, doi:10.1124/dmd.113.056804

Zahno A, Brecht K, Morand R, Maseneni S, Török M, Lindinger PW, Krähenbühl S. (2011) The role of CYP3A4 in amiodarone-associated toxicity on HepG2 cells. BiochemPharmacol 81(3):432-41

Zanelli U, Caradonna NP, Hallifax D, Turlizzi E, Houston JB. (2012) Comparison of cryopreserved HepaRG cells with cryopreserved human hepatocytes for prediction of clearance for 26 drugs. Drug Metab Dispos. 40(1):104-10.

Zanger UM, Turpeinen M, Klein K, Schwab M. (2008) Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. Anal Bioanal Chem. 392(6):1093-1108.

Zhou SF, Zhou ZW, Yang LP, Cai JP. (2009) Substrates, inducers, inhibitors and structure-activity relationships of human Cytochrome P450 2C9 and implications in drug development. Curr Med Chem. 16(27):3480-675.

Zilly W, Breimer DD, Richter E. (1975) Induction of drug metabolism in man after rifampicin treatment measured by increased hexobarbital and tolbutamide clearance. Eur J Clin Pharmacol. 9(2-3):219-27.