EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

ESAC Request 2013-01

ECVAM Scientific Advisory Committee

(ESAC)

ECVAM REQUEST FOR ESAC ADVICE

on an ECVAM-led validation study on two in vitro hepatic human-derived test methods for assessing liver enzyme induction (CYP) as a readout for potential biotransformation following chemical exposure.

Title page information	
Abbreviated title of ESAC request	Validation project on CYP induction assay for the assessment of human metabolic competent hepatic test systems
ESAC REQUEST Nr.	2013-01
Template used for preparing request	EP 2.01
Date of finalising request	12.6.2013
Date of submitting request to ESAC	1) Preliminary request at ESAC 38 (18/19 June 2013) ~~2) Final request: 26/9 2013 (date of availability of the VSR clear)~~ 2) ESAC 39
Request discussed through	ESAC 38 (preliminary request) & 39 (final request)
Opinion expected at (date)	~~ESAC meeting 2Q 2014~~ EASC meeting 3Q 2014 (ESAC 40)
File name of this request	**ESAC REQUEST_2013-01-CYP-induction-final(2).doc**

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1. TYPE OF REQUEST

Request Type	Identify request ("YES")
R1 ESAC Peer Review **of a Prevalidation Study or Validation Study**	**YES**
If R1)applies please specify further:	
►Prevalidation Study	
►Prospective Validation Study	**YES, addressing the reliability and relevance of *human metabolic competent hepatic test systems using the CYP induction assay* following repeated exposure to chemicals over several days (2 &3 days)**
►Retrospective Validation Study	
►Validation Study based on Performance Standards	
R2 Scientific Advice on a test method submitted to ECVAM for validation **(e.g. the test method's biological relevance etc.)**	
R3 Other Scientific Advice **(e.g. on test methods, their use; on technical issues such as cell culturing, stem cells, definition of performance standards etc.)**	

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

Validation project on human-derived hepatic systems *(their reliability and relevance)* to investigate biotransformation and toxicity, by evaluation of cytochrome P450 induction competence

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Summary

Toxicokinetics: For chemical safety assessments relating to systemic toxicity, information on Absorption, Distribution, Metabolism, Excretion (ADME) is of key importance. Depending on the industrial sector, the terms "pharmacokinetics" or "toxicokinetics" have been used to describe the four ADME processes. This request addresses the metabolism ("biotransformation") aspect of ADME in the area of toxicity testing and hence related to toxicokinetics.

Hepatic metabolic competent assays as key complementary information: With toxicological testing increasingly relying on in vitro methods which, might have reduced metabolism capacity (i.e. the biotransformation of a chemical by liver enzymes), human derived metabolic competent hepatic test systems promise to deliver key information to complement, within integrated approaches, hazard and risk assessments of substances within integrated approaches based on non-animal methods.

EURL ECVAM Study on CYP induction in two hepatic test systems: To address the issue of hepatic metabolism, EURL ECVAM has conducted a validation project on human-derived hepatic systems (their reliability and relevance) to investigate biotransformation and toxicity, by evaluation of cytochrome P450 (CYP) induction competence. Two test systems have been used: primary cryopreserved human hepatocytes and the cryopreserved human HepaRG cell line. On these test systems biotransformation effects were assessed following repeated challenges with the test items (chemicals). The project, involving a laboratory ring trial, assessed the reliability and the relevance of these two test systems using test items with human clinical reference data regarding induction of the four chosen CYP isoforms. As human data of sufficient quality are only available for pharmaceuticals, all test items are pharmaceutical substances. Owing to the nature of the effect measured, relevance was not assessed through analysis of predictions of adverse effects but by evaluating to which extent in vitro CYP induction profiles reflected those derived from human reference data. This analysis was intended to provide information on the metabolic competence of the test system in view of the human situation.

The project is of high relevance for existing and expected regulatory needs and data requirements as it assesses to which extent human-derived hepatic test systems can be employed for investigating whether specific substances are likely to induce CYP enzymes. While subcellular metabolic fractions are often applied for short-term (acute) investigations (e.g. rat liver S9 fraction in the Ames test), the in vitro human CYP induction project provides information on longer-term repeated exposure.

Information on CYP induction can be used to address the following three aspects related to toxicity of substances:

(1) CYP induction as toxicity pathway in its own right: CYP induction in itself can lead to adverse effects by affecting biotransformation of endogenous (=non xenobiotic substances) and thus disturbing normal metabolism and physiological homoeostasis.

(2) CYP induction as an alert for potential biotransformation of xenobiotics: CYP induction may indicate that the inducing chemical itself may be subject to biotransformation, leading to toxic or non-toxic metabolites. It should be noted that the CYP induction assay does not provide information about the number or nature (toxic/non-toxic) of possible metabolites. To cover this aspect, a metabolite identification assay would be needed. However, CYP induction data may complement information from other in vitro methods that lack metabolic capacity. The CYP assay can be used to assess potential inducing capacity of chemicals irrespective of use class, i.e. including drugs, pesticides, cosmetic ingredients, household products etc. It provides information on the potential biotransformation to be expected. It can be used as an alert on potential biotransformation within in integrated approaches based on a suite of in vitro assays and other information sources providing predictions on absence or presence of a specific toxicity effect / adverse effect.

(3) CYP induction as an alert for potential biotransformation of other non-inducing xenobiotics contained in mixtures. CYP induction additionally may alert to the potential biotransformation of xenobiotics contained in mixtures, including xenobiotics that, by themselves, would not induce enzyme activity. This may complement testing information for an assessment of mixtures. By studying the effects of mixtures and their constituting components on CYP enzyme induction, the assay may help estimating and interpreting possible chemical-chemical/drug-drug interaction processes (e.g. inhibition, induction, etc.).

Notably, reliable test systems informing about CYP induction may also help refining cancer prevention and treatment strategies. Known carcinogens, pro-carcinogens, and chemotherapeutics have CYPs involved in their metabolic pathways.

In summary, the project may contribute to international harmonization of risk assessment in the field of repeated dose and also acute systemic toxicity, and may provide information on those specific metabolism pathways covered by the assay. The CYP induction assay moreover may deliver two standardized human plateable metabolic competent test systems that may be used for additional applications (e.g. metabolic clearance, metabolite identification of chemical compounds, metabolism-mediated systemic effects etc).

2) Background

Metabolism of xenobiotics might lead to biotransformation enzyme inhibition/induction, leading to a significant variation of the compound 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. CYP inhibition may thus 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 (induced de novo protein synthesis) plays a crucial role in accelerating the metabolism of chemical being exposed to, leading to inactivation or detoxification of these chemicals (e.g. clearance).

Due to the complex biological mechanism behind CYP induction (xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene), it has been used as a biological tool to assess the relevance of the two metabolic competent systems. Furthermore, CYP induction is a key building block in defining adverse outcome pathways based on nuclear receptor interactions as initial key event.

The CYP test method addresses CYP induction but does not provide information on CYP inhibition. In addition it provides information on cellular events (e.g. xenobiotic-nuclear receptor binding). It is conceivable that this assay is useful for a wide variety of chemical substances, independent of their use class (i.e. not only for new drugs but also for other compounds such as cosmetic ingredients, chemicals,

pesticides and mixtures).

At regulatory level, while EMA and FDA Guidelines require CYP induction assessment for new drugs, toxicokinetics in relation to safety assessment of a broad spectrum of chemicals (e.g. chemicals, cosmetics, pesticides) currently is not systematically addressed by standardized test methods: No OECD Test Guidelines exists for the use of sub-cellular fractions, primary cells and in silico methods to investigate biotransformation (including the induction of biotransformation enzymes). Although according to OECD TG 417, toxicokinetics should be evaluated in vivo using the rat as test system, the availability of human-based models would avoid uncertainties in inter-species variability. The importance of toxicokinetics and the need for reliable "longer-term" metabolic test systems in this field has been strongly supported by a panel of scientific experts invited by the European Commission (ECVAM/ DG SANCO) and by the OECD Draft Guidance Document 151.

Human hepatocyte-like cells (metabolically competent) may provide a test system resembling the human liver machinery. Primary human hepatocytes are recognised by FDA as an effective tool for assessing CYP induction potential and they reflect the donor to donor variability and polymorphisms. With a stable cell line, such as HepaRG® (which is considered to be derived from one single donor), expressing relevant enzymes and regulatory cellular mechanisms, a reproducible system is obtained generating comparable data at different laboratories and allowing building up a reliable data base. Compared to fresh hepatocytes, a stable cell line offers the following advantages: easy access and availability, more consistent response to inducers. HepaRG® cells could be used as a "longer term" cellular system for metabolism of xenobiotics with a low turnover. These substances are notoriously difficult to study in present systems using primary human hepatocytes, because of short viability and stability of the cells.

CYP enzymes have been shown as the primary biotransformation enzymes to be assessed since they are involved in the biotransformation of a large number of chemical compounds. However, the two metabolically competent test systems may be extended to further key enzymes involved in liver metabolism (e.g. other CYPs, phase II enzymes).

3) Study objectives and design

Study objective: The in vitro human CYP induction validation study assesses the reliability and the relevance of two human-derived test systems (I) the cryopreserved human hepatocytes and (II) the cryopreserved human HepaRG® cells, by measuring the cytochrome P450 (CYP) induction competence.

CYP enzyme activity: CYP induction is assessed at the level of protein activity (measuring the enzymatic transformation of substrate substances into known identifiable products) and not at messenger (mRNA) level. Indeed, it has been described that there is an apparent discrepancy between mRNA induction and protein activity and the lack of positive correlation between CYP activity and the specific CYP mRNA level. This phenomenon 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. In contrast to CYP mRNA levels, CYP enzyme activity is the functional endpoint of CYP induction and the clinically relevant basis of potential chemical-chemical interactions.

Test items selection: As the relevance of the two test systems is assessed by comparing the in vitro results of the study to the in vivo human data, the test items have been selected based on the availability of human in vivo data and at least one test item should trigger CYP induction following the binding to one of the main nuclear receptors (PXR, CAR or AhR).

The four CYPs (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) isoforms investigated are recommended by FDA and EMA Guidelines. Indeed, they are responsible of a high percentage of metabolism of clinically relevant chemicals and cover at least the main key event in CYP induction which is the chemical-receptor binding as they are induced via PXR (CYP2B6, CYP2C9 and CYP3A4), CAR (CYP2B6 and CYP3A4), AhR (CYP1A2). Two negative controls (no CYP inducer) and positive inducer controls have been included. Test items have been

coded and distributed by EURL ECVAM.

Test facilities: For each test system, three test facilities carried out the ring trial.

Study design: The CYP validation project followed the modular approach as defined by ECVAM and the OECD Guidance Document No. 34. The study was supervised by of the Validation Management Group (VMG), a group of internal and external experts in the field, who advised at each critical decision point (e.g. solubility, cytotoxicity, CYP induction). With respect to modular approach the CYP validation project provides information on module 1) test definition, module 2) within laboratory reproducibility, module 3) transferability and module 4) between laboratory reproducibility. In addition, the data generated provide preliminary information on module 5) predictive capacity for CYP induction against human CYP induction data; however, the number of chemicals tested is based on availability of such human in vivo data.

Module 4 - between laboratories reproducibility has been experimentally divided into three steps. In each steps the 3 test facilities involved per test system carried out different studies based on approved SOPs and study plans by VMG and reported each study by means of a study report.

1. Solubility: test items are investigated for their solubility in DMSO and for their solubility in the experimental conditions (cell culture medium, 37°C, 5%CO2) => the highest soluble concentrations have been used as starting concentrations in cytotoxicity experiments. This part has been run in parallel at EURL ECVAM by an independent team, as reference.
2. Cytotoxicity: the cytotoxic potential of test items is assessed => the highest non-cytotoxic concentration is used as starting concentrations in induction experiments.
3. Induction: the potential of test items to induce CYPs is assessed. After a recovery period, cells are exposed to the test items. Subsequently, the conversion of CYP1A2, CYP2B6, CYP2C9, CYP3A4 probe substrates is measured using a "cocktail approach", i.e. the functional CYP activities are all determined concurrently in one reaction ("n-in one"). Quantification of probe products is conducted by LC-MS analysis

Some parts of the CYP validation project have been carried out under GLP in the EURL ECVAM GLP Test Facility.

4) Results and conclusions of the validation project/study

This is the first project in its kind with the following objectives:

A. Assess the transferability, the reproducibility (within and between-lab) of two human CYP induction-in vitro methods, by evaluating the induction of four CYP enzyme activities (CYP1A2, CYP2B6, CYP2C9, CYP3A4)
   The two in vitro methods use the metabolically competent test systems
   - cryopreserved human HepaRG cells
   - cryopreserved human primary hepatocytes
B. Assess the predictive capacity using human CYP induction in vivo reference data.

The two human CYP induction in vitro test methods might be used within integrated approaches for information on Mode of Action and biotransformation for both substances and mixtures/products.

Since the human in vitro CYP validation study is the first project in its kind the VMG could not set specific targets a priori for each of the modules. The VMG evaluated the obtained information and as such draw ex-post conclusion based on the data generated.
In principle, it has to be recognised that induction is not an all-or-none response, but a quantitative concentration - and time-dependent complex process consisting of a number of steps to the ultimate response. Consequently the criteria for defining the 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).
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.

1 Test definition	The existing background documents and related scientific references for both the human CYP cryoHepaRG cell line induction in vitro method and the human cryopreserved primary hepatocytes induction in vitro method and the current validation trial findings adequately demonstrate the intended purpose, the need for, the status of development, and the mechanistic basis of the two human CYP induction in vitro methods. Improved, well-defined and described and robust standard operating procedures (SOPs) for both methods are available using a standard template. Both in vitro methods are defined and described in an orderly, logical, and systematic way and are the result from improvements of the initial SOP versions received by EURL ECVAM, from additional inquiries, from experimental and non-experimental investigations and studies. Acceptance criteria are included throughout both SOPs. Replicate experiments are appropriate defined for each experimental step in the in vitro method. Characterisation standards for the two human in vitro test systems based on scientific evidence and a description on how to evaluate these, in order to ensure that the test systems can be reliably used for testing, are included in the SOPs. A good in vitro method experimental design is used for the different steps used in the SOPs and adequate placing of test, reference and control items, allowed the generating of enough data to draw conclusions. Data recording, data analysis and all statistical methods and calculations used are adequately described and the SOPs include a clear description and definition of the statistical or non-statistical methods used to analyse the resulting data.
2 Within laboratory reproducibility	For the CYP1A2, CYP2B6, CYP3A4 and CYP2C9 enzymes investigated, a higher reproducibility was obtained for the human CYP cryoHepaRG cell line induction in vitro method than for the human cryopreserved primary hepatocytes induction in vitro method. This result can be expected because cryoHepaRG cell batches are 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. However, the variability could also originate from isolation, freezing and thawing procedures. In the present study, one of the cryohep batches, S240408, demonstrated borderline inducing 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. The analysis of basal activities and induction responses by positive control inducers indicated quite large variability of activities and responses within batches, between batches and within laboratories. 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.
3 Transferability	The human CYP induction in vitro methods were successfully transferred to naïve laboratories. The in vitro methods can be performed in any modern analytical laboratory and with minimum standards in cell culture (Good Cell Culture Practice). Experienced personnel can readily be trained in the test methods. The test definition/description aspects of the SOPs are clearly written, the execution of the experimental steps and the data analysis can be performed without difficulties. All apparatus and reagents needed for the execution of the two human CYP induction in vitro methods are readily available commercially. The cryoHepaRG® cells are nowadays available from different suppliers in Europe, USA, Japan and Brazil.
4 Between laboratory reproducibility	All the different laboratories produced the same induction classification (potent/weak inducer, non-inducer) of blind test items when performing the experiment with the same batch. 66 % of the experiments with the human CYP cryoHepaRG cell line induction in vitro method and 55% of the experiments with human CYP cryoheps induction in vitro method were judged to give the same induction class in all laboratories and at least 2 out of three laboratories for both human CYP induction in vitro methods were concordant in >90% of the experiments. Analysis of basal activity and induction results produced by different laboratories with the same batches indicated that concordance was dependent on test system used. The cryoHepaRG CYP induction in vitro method showed higher reproducibility for the 4 CYPS under investigation compared to cryoheps CYP induction in vitro method when using for the evaluation the same

	batches. The highest reproducibility value was observed for CYP3A4 (all batches ≥ 90%). 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 between laboratory reproducibility for the human CYP induction in vitro method is not satisfactory for one (Batch S240408) out of the three cryoheps' batches for the four CYPs. For this batch the lowest reproducibility value was observed for CYP2B6 (all 37%). The other two batches showed between laboratory reproducibility values between 61% and 80% (CYP2C9 excluded). At least qualitatively, CYP1A2-, 2B6 and 3A4-selective probe activities performed as expected in both in vitro methods both for the data generated with model inducers (rifampicin, phenobarbital and beta-napthoflavone at defined concentration) and with the test items evaluated at different concentrations. CYP2C9-selective probe activity was relative high in both cell systems and overall induction responses remained quite low. 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. Data generated give clear indications that the human CYP cryoHepaRG cell line induction in vitro method and the human cryopreserved primary hepatocytes induction in vitro method are relatively similar in their ability to detect and classify substances in terms of CYP1A2, CYP2B6, CYP3A4 and CYP2C9 induction.
5 Predictive capacity	The cryoheps CYP induction in vitro method has been regarded, as a gold standard for CYP induction studies for regulatory purposes. Therefore the comparison of the two CYP induction in vitro methods is of considerable interest. Classification of 10 test items into potent, weak and non-inducers was performed for both in vitro methods. In 28/40 (70%) the two in vitro methods were concordant in their classification. Interestingly, when the cryoheps CYP induction in vitro method classified a response as potent (18 cases), only 3 were discordant in cryoHepaRG cells CYP induction in vitro method (i.e. 16.7%). Out of 15 non-inducer classifications by the cryoheps CYP induction in vitro method, only 2 were discordant by cryoHepaRG cells CYP induction in vitro method (13.3%). Although this analysis took into consideration only the overall classification without regard of laboratories separately, it nevertheless gives some confidence that the human CYP cryoHepaRG cell line induction in vitro method and the human cryopreserved primary hepatocytes induction in vitro method are rather similar in their ability to detect and classify substances in terms of induction potentiality. The overall classifications of the blind test items on the basis of the two in vitro methods are in line with the in vivo knowledge on classification of these test items. 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 on the, applicability domain (pharmaceuticals). However, it is assumed that the human 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 the two human CYP induction in vitro methods. 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 capacity of the two human CYP induction in vitro methods to predict in vivo induction was based on the potency in vitro and the maximum in vivo plasma concentrations of each test item. The sensitivity (fraction of correctly predictive positive to all positive inducing compounds) and the specificity (fraction of correctly predicted negatives to all negatives (non-inducers)) in the clinic was calculated. There are two obvious conclusions to be drawn on the basis of these results: firstly, the number of test items is limited, impacting the statistical analysis, and secondly, knowledge about in vivo concentrations and in vivo induction properties of test items are needed for correct classification. The results indicated that human cryoHepaRG cells CYP induction in vitro method showed 100% sensitivity and specificity for the prediction of CYP1A2, CYP2B6 and CYP3A induction based on the results from the test itemd used in the present study. The human cryoheps CYP induction in vitro method also showed 100% sensitivity and specificity for CYP2B6 and CYP3A induction. However, the human cryoheps CYP induction in vitro method showed only 25% sensitivity for prediction of CYP1A2 induction since the method failed to predict induction of three test items known to induce CYP1A2 in vivo. The human cryoheps CYP induction in vitro method showed 100% specificity since this method predicted all CYP1A2 non-inducers to be negative.

References

References (Guidelines)

1. US FDA draft Guidance for Industry, Drug Interaction Studies —Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, February 2012.
2. European Medicine Agency (EMA), Guideline on the Investigation of Drug Interactions, CPMP/EWP/560/95/Rev. 1, 21 June 2012.
3. Hartung T., Bremer S., Casati S., Coecke S., Corvi R., et al. (2004) A modular approach to the ECVAM principles on test validity. Altern Lab Anim. 32: 467-472.
4. OECD (2005) OECD 34: 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.
5. OECD (2010) OECD Test No 417: Toxicokinetics. Guideline for the testing of chemicals. OECD, Paris, No. 417.
6. OECD (2011) Draft Guidance Document 151 in support of OECD Test Guideline 443 on an Extended One Generation Reproductive Toxicity Study. OECD, Paris, 151.

References on the test systems and CYP induction / inhibition

7. Abadie-Viollon C., Martin H., Blanchard N., Pekthong D., Bachellier P., Mantion G., Heyd B., Schuler F., Coassolo P., Alexandre E. and 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, 346-356.
8. Abass K., Turpeinen M. and Pelkonen O. (2009) An evaluation of the cytochrome P450 inhibition potential of selected pesticides in human hepatic microsomes. J Environ Sci Health B. 44, 553-563.
9. Andersson T.B., Kanebratt K.P. and Kenna J.G. (2012) The HepaRG cell line: a unique in vitro tool for understanding drug metabolism and toxicology in human. Expert Opin Drug Metab Toxicol. 8, 909-920.
10. 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. ATLA 27, 579-638.
11. Gripon P., Rumin S., Urban S., Le Seyec J., Glaise D., Cannie I., Guyomard C., Lucas J., Trepo C., and Guguen-Guillouzo C.(2002) Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 26, 15655-15660.
12. Guillouzo A., Corlu A., Aninat C., Glaise D., Morel F. and 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.
13. Kanebratt K.P. and Andersson T.B. (2008) HepaRG Cells as an in Vitro Model for Evaluation of Cytochrome P450 Induction in Humans. Drug Metab Dispos. 36, 137-45.
14. McGinnity D.F., Zhang G., Kenny J.R., Hamilton G.A., Otmani S., Stams K.R., Haney S., Brassil P., Stresser D.M. and Riley R.J. (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, 1259-1268.

15. Richert L., Abadie C., Bonet A., Heyd B., Mantion G., Alexandre E., Bachellier P., Kingston S., Pattenden C., Illouz S., Dennison A., Hoffmann S. and 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. Toxicology in Vitro 24, 335–345.

16. Silva J.M., Day S.H. and Nicoll-Griffith D.A. (1999) Induction of cytochrome-P450 in cryopreserved rat and human hepatocyte. Chemico-Biological Interactions 121, 49–63.

References as supporting documents for current practice and Mode Of Action of CYP Induction

17. 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.

18. 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.

19. Fuhr U. (2000) Induction of drug metabolising enzymes: pharmacokinetic and toxicological consequences in humans. Clin Pharmacokinet. 38, 493-504.

20. Kozawa M., Honma M. and Suzuki H. (2009) Quantitative prediction of in vivo profiles of CYP3A4 induction in humans from in vitro results with a reporter gene assay. Drug Metab Dispos. 37, 1234-1241

21. Jiunn Lin H. (2006) CYP Induction-Mediated Drug Interactions: in Vitro Assessment and Clinical Implications. Pharmaceutical Research 23, 1089-1116.

22. Jossé R., Aninat C., Glaise D., Dumont J., Fessard V., Morel F., Poul J.M., Guguen-Guillouzo C. and Guillouzo A. (2008) Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metab Dispos. 36, 1111-1118.

23. Sinz M., Wallace G. and Sahi J. (2008) Current industrial practices in assessing CYP450 enzyme induction: preclinical and clinical. AAPS J. 10, 391-400.

24. Tompkins L.M. and Wallace A.D. (2007) Mechanisms of cytochrome P450 induction. J Biochem Mol Toxicol.21, 176-181.

25. Waxman D.J. (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. Arch Biochem Biophys. 369, 11-23.

References on toxicokinetics and on reliable and relevant metabolically competent test system

26. Adler S., Basketter D., Creton S., Pelkonen O., van Benthem J. et al. (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects-. Arch Toxicol. 85, 367-485.

27. Blaauboer B.J., Boobis A.R., Castell J.V., Coecke S., Groothuis G.M.M., Guillouzo A., Hall T.J, Hawksworth G.M., Lorenzon G., Miltenburger H.G., Rogiers V., Skett P., Villa P. and Friedrich J. (1194) The Practical Applicability of Hepatocyte Cultures in Routine Testing. The Report and Recommendations of ECVAM Workshop 1 ATLA 22, 231-241.

28. Coecke S., Ahr H., Blaauboer B.J., Bremer S., Casati S., Castell J., Combes R., Corvi R., Crespi C.L., Cunningham M.L., Elaut G., Eletti B., Freidig A., Gennari A., Ghersi-Egea J.F., 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 J.A. 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.

29. Coecke S., Blaauboer B.J., Elaut G., Freeman S., Freidig A., Gensmantel N., Hoet P., Kapoulas V.M., Ladstetter B., Langley G., Leahy D., Mannens G., Meneguz A., Monshouwer M., Nemery B., Pelkonen O., Pfaller W., Prieto P., Proctor N., Rogiers V., Rostami-Hodjegan A., Sabbioni E., Steiling W., van de Sandt .JJ. (2005) Toxicokinetics and metabolism. Altern Lab Anim. 33, 147-175.

30. Coecke S., Pelkonen O., Leite S.B., Bernauer U., Bessems J.G., Bois F.Y., Gundert-Remy U., Loizou G., Testai E. and Zaldívar J.M. (2012) Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. Toxicol In Vitro 4 (in press).

31. OECD (2008) Detailed Review Paper on the Use of Metabolising Systems for In Vitro Testing of Endocrine Disruptors. OECD Series on Testing and Assessment: Testing for Endocrine Disrupters, OECD, Paris, No. 97

References on cocktail approach and substrate specificity

32. Gerin B., Dell'aiera S., Richert L., Smith S. and Chanteux H. (2012) Assessment of cytochrome P450 (1A2, 2B6, 2C9 and 3A4) induction in cryopreserved human hepatocytes cultured in 48-well plates using the cocktail strategy. Xenobiotica. 15: 1-6.

33. Kanebratt K.P., Diczfalusy U., Bäckström T., Sparve E., Bredberg E., Böttiger Y., Andersson T.B. and Bertilsson L. (2008) Cytochrome P450 induction by rifampicin in healthy subjects: determination using the Karolinska cocktail and the endogenous CYP3A4 marker 4beta-hydroxycholesterol. Clin Pharmacol Ther. 84: 589-594.

34. Turpeinen M., Uusitalo J., Jalonen J. and Pelkonen O. (2005) Multiple P450 substrates in a single run: rapid and comprehensive in vitro interaction assay. Eur J Pharm Sci. 24, 123-132.

References on in vitro-in vivo extrapolation

35. Darnell M., Ulvestad M., Ellis E., Weidolf L. and Andersson T.B. (2012) In vitro evaluation of major in vivo drug metabolic pathways using primary human hepatocytes and HepaRG cells in suspension and a dynamic three-dimensional bioreactor system. J Pharmacol Exp Ther. 343, 134-44.

36. Donato M.T., Hallifax D., Picazo L., Castell J.V., Houston J.B., Gomez-Lechón M.J. and Lahoz A. (2010) Metabolite formation kinetics and intrinsic clearance of phenacetin, tolbutamide, alprazolam, and midazolam in adenoviral cytochrome P450-transfected HepG2 cells and comparison with hepatocytes and in vivo. Drug Metab Dispos. 38, 1449-1455.

37. Ilic K., Hawke R.L., Thirumaran R.K., Schuetz E.G., Hull J.H., Kashuba A.D., Stewart P.W., Lindley C.M. and Chen M.L. (2012) The Influence of Sex, Ethnicity, and CYP2B6 Genotype on Bupropion Metabolism as an Index of Hepatic CYP2B6 Activity in Humans. Drug Metab Dispos. 13.

38. Wattanachai N., Tassaneeyakul W., Rowland A., Elliot D.J., Bowalgaha K., Knights K.M. and Miners J.O. (2012) Effect of albumin on human liver microsomal and recombinant CYP1A2 activities: impact on in vitro-in vivo extrapolation of drug clearance. Drug Metab Dispos. 40, 982-989.

References on CYP and receptors

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

40. 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

41. 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.

42. Surapureddi S., Rana R., Goldstein J.A. (2011). NCOA6 differentially regulates the expression of the CYP2C9 and CYP3A4 genes. Pharmacol Res. 63:405-413.

References on inter-individual variability

43. Nakajima M., Yokoi T. (2011). microRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. Pharmacol Ther. 131:330-7.
44. Richert L., Abadie C., Bonet A., Heyd B., Mantion G., Alexandre E., Bachellier P., Kingston S., Pattenden C., Illouz S., Dennison A., Hoffmann S. and 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. Toxicology in Vitro 24, 335–345.

References on CYP m-RNA and CYP activity

45. 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.
46. Nakajima M. and Yokoi T. (2011). microRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. Pharmacol Ther. 131:330-7.
47. 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.
48. Aguiar M., Masse R., and Gibbs B.F. (2005). Regulation of cytochrome P450 by posttranslational modification. Drug Metabolism reviews 37:379-404.
49. Fahmi O.A. and Ripp S.L. (2010) Evaluation of models for predicting drug-drug interactions due to induction. Expert Opin Drug Metab Toxicol. 6, 1399-1416.
50. Aguiar M., Masse R. and Gibbs B.F. (2005) Regulation of cytochrome P450 by posttranslational modification. Drug Metab Rev. 37, 379-404.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does ECVAM require advice on the current issue?*	The opinion of ESAC on the CYP induction validation study should support EURL ECVAM with respect to the **development of an EURL ECVAM Recommendation** on the two test systems. The EURL ECVAM Recommendation will address (1) the **scientific basis** of the test systems/test methods [a) cryopreserved human hepatocytes and b) cryopreserved human HepaRG® cells] and the CYP induction readout performed in these test methods. Test definition will be described with a view to the **relevance of the test systems** for contributing to assessment of biotransformation and toxicity; (2) the **reproducibility and transferability** of the assays as assessed during the study; (3) the **relevance/predictive capacity** for human CYP induction **as assessed during the study** (appropriateness of reference data); (4) the assays' applicability and possible limitations. ESAC's advice should enable EURL ECVAM to conclude, within its EURL ECVAM Recommendation, on the **potential adequacy of the two test methods for routine testing** to predict human CYP induction in support of regulatory applications (e.g. future EU test method, OECD (Performance-Based) Test Guideline), outlining the possible **complementary information** that will be required, in addition to data from the test methods assessed, to gain insight into biotransformation and toxicological Mode of Action. The advice of ESAC should support ECVAM with respect to an analysis of possible remaining **data gaps** that need to be addressed in view of determining the test methods' potential use and usefulness within integrated approaches for biotransformation for both substances and mixtures/products.

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed in view of achieving the objective of the advice?*	1) **DESIGN & CONDUCT OF STUDY:** The ESAC is requested to review whether the study was conducted appropriately in view of the objective of the study. The study objective was to assess: (1) **reproducibility** of the test method within laboratories (WLR) (2) **transferability** of the test methods to other laboratories (3) **reproducibility** of the test methods between laboratories (BLR) (4) **relevance/predictive capacity** of the *in vitro* test methods for biotransformation of substances as compared to human clinical data from pharmaceuticals. (5) **the applicability domain and possible limitations** of the test method. The selection of the test substances and analyses of possible reasons in case results *in vitro* are not matching the human reference data should be carefully reviewed. When reviewing the design and conduct of the study, the following issues should

	be addressed in particular: (a) Clarity of the test definition (module 1) (b) Clarity of the definition of the study objective and study management (c) Appropriateness of the study design & execution in view of the study objectives, *inter alia:* ○ Is the **number of tested chemicals** sufficient for the purposes of the study? ○ Are the **reference data** (which are associated with the test chemicals) appropriate and of good quality in view of assessing in particular the predictive capacity? Should additional reference data (potentially also for the same substances) but from *other reference sources* been included? Where there selection criteria? Was the selection scientifically justified? ○ Was the identification of **validation chemicals** conducted in an appropriate manner (i.e. presence or absence of selection criteria, justification etc.)? ○ Is the **effect range (e.g. range of CYPs) of the selected chemicals/reference data** appropriate for the purpose of the study? ○ In case of **gaps** (chemical class etc.) – are these justified? ○ Is the **number of laboratories** sufficient? (d) Appropriateness of the **study execution** (e.g. were there pre-defined test acceptance criteria, were these respected? How were exceptions / deviations handled? Were provisions specified for retesting? Was the number of repetitions sufficient? etc.) (e) Appropriateness of the **statistical analysis** used for analysing WLR, transferability, BLR and relevance / predictive capacity. 2) **CONCLUSIONS OF STUDY:** The ESAC is requested to assess whether the conclusions, as presented in the material made available to ESAC are **substantiated by the information generated in the study** and are **plausible with respect to existing information and current views** (e.g. literature). In particular: (a) Are the conclusions on **reproducibility** (WLR and BLR) as well as transferability justified and plausible? (b) Are the conclusions on **relevance / predictive capacity** justified and plausible with respect to the reference data, other existing information and with respect to the intended use of the test methods. (c) Are there **possible gaps between study design and study conclusions** which remain to be addressed in view of the suggested conclusions / use (see also point 3)? (d) Do the data generated with the **validation set of chemicals together with possible available existing data** provide sufficient information on the **applicability and possible limitations** of the test method, in particular in view of its potential use within integrated approaches to support biotransformation and toxicological Mode of Action.

	3) **SUGGESTED USE OF THE TEST METHOD:** The ESAC is requested (a) to evaluate, on the basis of the data summarised in the validation study report, the intended use of the test method and its readiness to **serve as a reference point for defining performance criteria** for routine assessment, i.e. for developing a Performance-Based Test Guideline (PBTG). (b) to make additional recommendations (as required) on the proper scientific use of the test method, possibly within integrated approaches taking specific aspects of this method into account (e.g. applicability, technical limitations etc.), (c) to identify possible further information required (i.e. are there data gaps or gaps with regard to mechanistic understanding ?) to be able to determine the potential use and usefulness of the test method within integrated approaches, duly considering the need to text chemicals and mixtures.

4.3 TIMELINES

Timelines concerning this request *When does ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	Q3 2014 (i.e. ESAC40 meeting, autumn 2014)
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	**NO**
	Request to be presented to ESAC at ESAC plenary meeting	ESAC 39

5. ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request *Does the advice require an ESAC working group, an ESAC rapporteur etc.?*	**Structure(s) required**	**Required according to ECVAM? (YES/NO)**
	S1 ESAC Rapporteur	
	S2 ESAC Working Group	**YES, already set-up**
	S3 Invited Experts	
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above (S1-S3):	

5.2 DELIVERABLES AS PROPOSED BY ECVAM

Deliverables *What deliverables (other than the ESAC opinion) are required for addressing the request?*	**Title of deliverable other than ESAC opinion**	**Required? (YES/NO)**
	D1 ESAC Rapporteur Report and draft opinion	
	D2 ESAC Peer Review Report and draft opinion	**YES**
	If other than above (D1-D2):	

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

To be completed once the final validation study documentation has been adopted by the Validation Management Team.

Count	Description of document	Already available? (YES/NO)	File name
1	List of documents that can be made available to ESAC on request	Yes	
2	Project Plan Describes the CYP Induction validation project, the involved laboratories and the study design.	Yes	Appendix 02_Project Plan.pdf
3	Minutes of the meeting with VMG and test facilities involved in the Validation project	Yes	Face to Face meeting 2010: minutes.pdf
4	Explanatory document on the chemicals (test items) coding and distribution	Yes	Appendix 04_test items coding and distribution.pdf
5	Explanatory document on solubility testing performed by nephelometer at EURL ECVAM	Yes	Appendix 05_solubilitybynephelometry.pdf
6	Standard Operating Procedure for Cytochrom P450 Induction in CryoHepaRG_n-in-one_96well; version 02 of 01 June 2012	Yes	Appendix 06_SOP_Cytochrom P450 Induction in CryoHepaRG_n-in-one_96well).pdf
7	Standard Operating Procedure for Cytochrom P450 Induction in human cryopreserved hepatocytes_n-in-one_48well) ; version 08 of 05 November 2012	Yes	Appendix 07_SOP_Cytochrom P450 Induction in human cryopreserved hepatocytes_n-in-one_48well).pdf
8	Summary of revisions of the cryoHepaRG SOP	Yes	Appendix 08_Revisions of the SOP cryoHepaRG.pdf
9	Summary of revisions of the cryoheps SOP	Yes	Appendix 09_Revisions of the SOP cryoheps.pdf
10	Report from the statistician on the cytotoxicity positive control (chlorpromazine) results obtained at the lead laboratory (27 September 2011)	Yes	Appendix 10_chlorpromazine additional results to module 1 27sept2011.pdf
11	Report from the statistician on the cytotoxicity positive control (chlorpromazine) results performed on six cryoheps batches by the lead	Yes	Appendix 11_chlorpromazine concentration finding 27feb2012.pdf

	laboratory (27 February 2012)		
12	Amendment to the report issued on February 27th 2012 (Appendix 11). Dealing with two issues: (I) outlier identification, (II) acceptance criterion on the positive control for cytotoxicity in cryoheps	Yes	Appendix 12_chlorpromazine concentration finding 2april2012.pdf
13	Statistical analyses on data generated during Module 4 –CYP induction: within-laboratory and between-laboratory analysis	Yes	Appendix 13_CYP Statistical report.pdf
14	Core document regarding the objectives, the scientific background, the outcome and the VMG conclusions of the CYP Induction validation project	Yes	CYP_validation project report_FINAL 20140314.pdf
15	Study plan on Module 3-transferability (13.07.2010) test method: CYP Induction cryoHepaRG	Yes	Appendix 15_HepaRG_Trial Study plan Module 3 13.07.2010.pdf
16	Report from the lead laboratory on the Transferability Module for CYP induction on cryoHepaRG cells (07 November 2011)	Yes	Appendix 16_HepaRG_Report Transferability 07 November 2011.pdf
17	Agenda of the Transfer workshop organized by the lead laboratory on CYP induction in CryoHepaRG cells	Yes	Appendix 17_Agenda CryoHepaRG Transfer workshop.pdf
18	Agenda of the Transfer workshop organized by the lead laboratory on CYP induction in Cryoheps	Yes	Appendix 18_Agenda Cryoheps Transfer workshop.pdf
19	Report containing Hill curve fits to cryoHepaRG and croheps data	Yes	Appendix 19_CYP statistical report_Hill.pdf

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 38th meeting on 18 & 19 June 2013, the ESAC plenary unanimously decided to establish an ESAC Working Group "CYP induction" charged with the detailed scientific review of a study on two in vitro hepatic human-derived test methods for assessing liver enzyme induction as a readout for potential biotransformation following chemical exposure.

7.2 TITLE OF THE ESAC WORKING GROUP

Full title:
ESAC WG on CYP induction assay to evaluate metabolic competence of two human-derived hepatic test systems.

Abbreviated title:
ESAC WG CYP induction

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific review of the validation study concerning the reliability and relevance of the in vitro CYP induction test methods. The review needs to address the questions put forward to ESAC by ECVAM.

The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions of the Validation Management Team (VMT) are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLE OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Coordinator a detailed **ESAC Working Group Report** outlining its analyses and conclusions**.** A reporting template has been appended (Appendix 1) intended to facilitate the drafting of the report.

The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The Coordinator has proposed timelines which should be agreed upon during the first Teleconference (Item 1 in the table):

Item	Date/time	Description	Action / Deliverable
1	Early April	Meeting of WG in Ispra	• Explanation of review process • Work distribution • Further timelines • First rough draft of WG report (observations) and ESAC opinion
2	April, May, June, July	Teleconferences as needed	• Consensus finding on potential contentious issues • Clarifications
4	Early July 2014	Final ESAC WG report and ESAC opinion available	WG report and ESAC opinion

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

When preparing the final ESAC WG report to address these questions, the ESAC WG is requested to use a pre-defined reporting template. This template (see appendix 1) follows ECVAM's modular approach and addresses to which extent the standard information requirements have been addressed by the study. The template allows moreover for addressing the issues specific studies outlined in section 4.2. The Coordinator will provide guidance if necessary.

APPENDIX 1 REPORTING STRUCTURE FOR THE *ESAC WG REPORT*

The following suggested structure follows the ECVAM information requirements ("modules") for scientific review following validation and allows at the same time for the description of the analysis and conclusions concerning more specific questions. A template (ESAC WG consensus report template) has been created and will be made available to the ESAC.

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable. However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

The current template is

TEMPLATE_ESAC-WG_REPORT-v6.doc