The validated CYP induction HepaRG™ test method: Preparation for TG adoption

Miriam Jacobs on behalf of the GOLIATH team

UK Health Security Agency

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Status pre-validation of methods for metabolic disruption

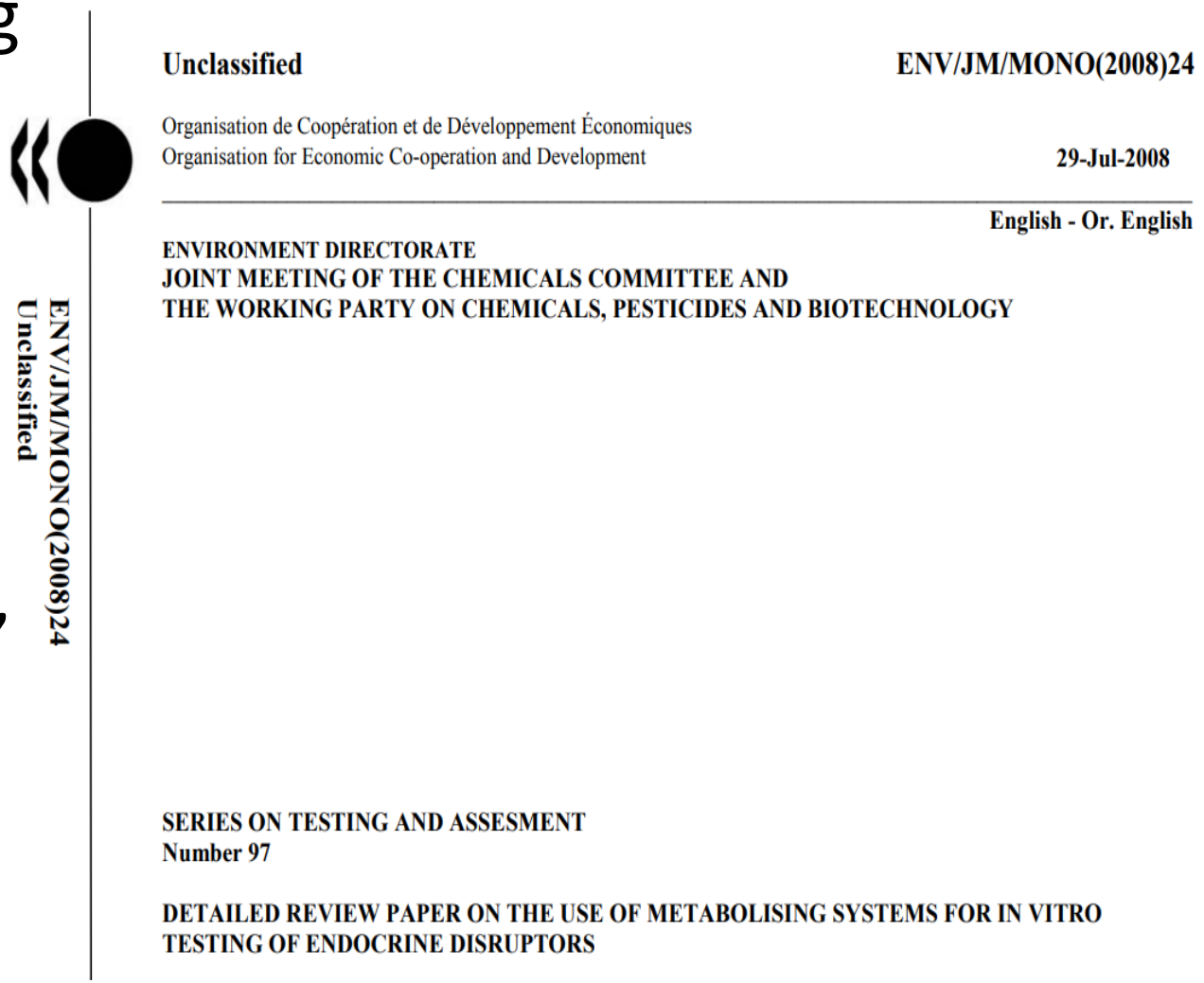
Steps in pre-validation process	CYP induction assay: chemical augment.	PPARα and PPARɣ reporter cells	hMSC differentiation to white adipocytes	HepaRG steatosis assay HPR116	Pancreatic beta cell function	Zebrafish model on metabolic disruption
Proficiency chemical selection	✓	✓	✓	✓	✓	
SOP and test definition available	✓	✓	✓	✓	✓	✓
Data evaluation	✓	✓	✓			
Labs recruited for transfer	✓	✓	✓			
Data from naive labs	✓	✓	✓			
Independent statistical analysis	✓	✓	✓			
Preliminary conclusion on test method predictivity	✓	✓	✓			
Prevalidation report	✓	✓	✓			

- OECD Projects: Detailed Review Paper on Metabolic Disruption (MD) and a conceptual IATA underway;
  - CYP induction draft TG 2025 ?

OECD Identified need to incl. metabolism for in vitro testing of EAS/EDs

Review background

- Early 2000s: initiation of DRP 97 at OECD by Belgium, with completion by EC JRC
- OECD adoption 2008



ENV/JM/MONO(2008)24
Unclassified

Unclassified **ENV/JM/MONO(2008)24**

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development **29-Jul-2008**

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

SERIES ON TESTING AND ASSESMENT
Number 97

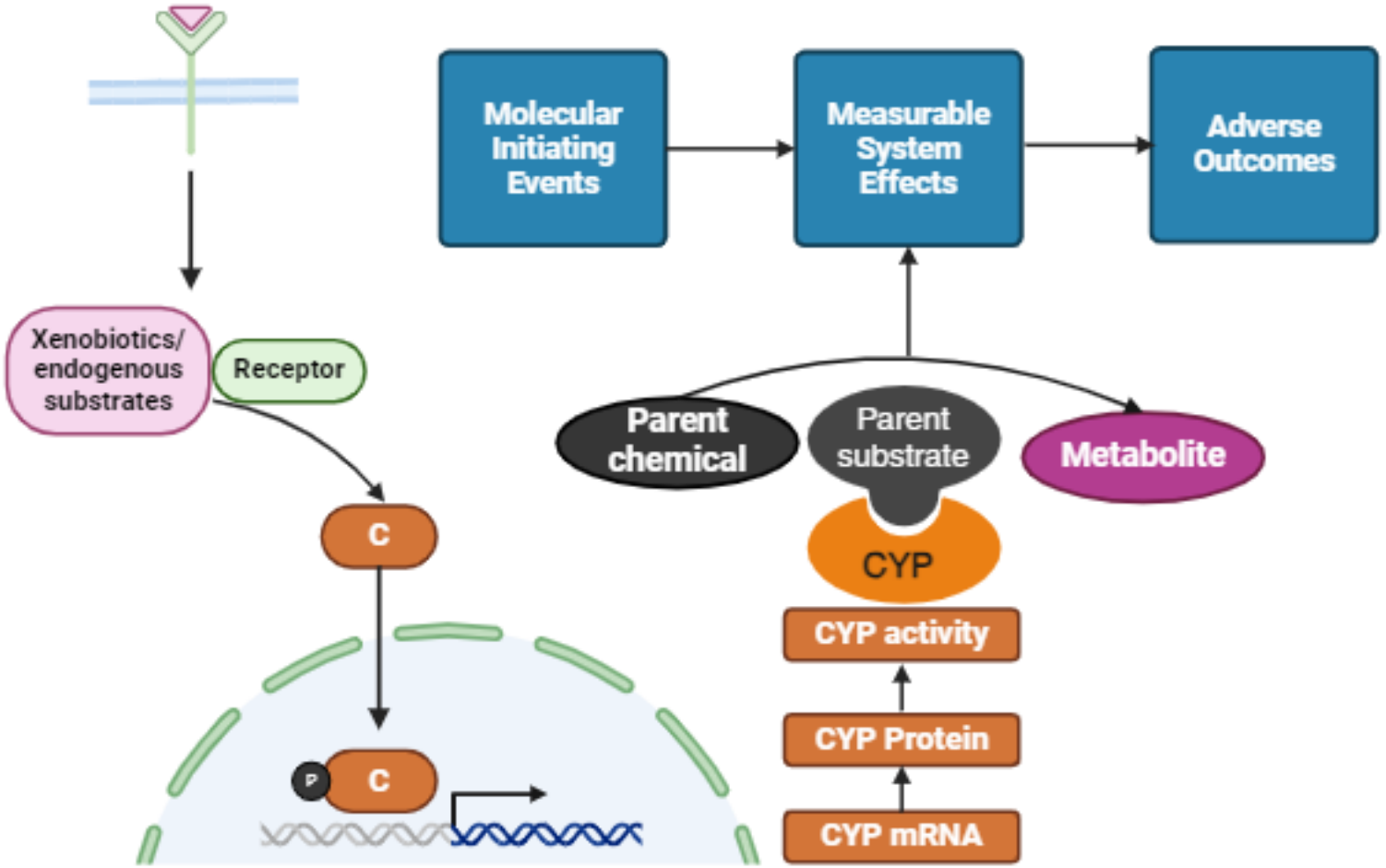
DETAILED REVIEW PAPER ON THE USE OF METABOLISING SYSTEMS FOR IN VITRO TESTING OF ENDOCRINE DISRUPTORS

HepaRG CYP induction test method

The assay evaluates CYP induction for 3 key human phase I metabolic enzymes: **CYP1A2**, **CYP2B6**, and **CYP3A4** (Bernasconi et al. 2019).

These enzymes cover activation of nuclear receptors responsible for majority of hepatic metabolism, **namely**

- **Aryl hydrocarbon Receptor (AhR; CYP1A2)**
- **Constitutive Androstane Receptor (CAR; CYP2B6)**
- **Pregnane X Receptor (PXR; CYP3A4)**
- Functional endpoint: induction of **catalytic enzyme activity** rather than gene expression



Bernasconi C, Pelkonen O, Andersson TB, et al. (2019) Validation of in vitro methods for human cytochrome P450 enzyme induction: Outcome of a multi-laboratory study. Toxicol In Vitro 60:212-228 doi:10.1016/j.tiv.2019.05.019

Test method validation timeline

Link to TSAR (Tracking System for Alternative methods towards Regulatory Acceptance)

- 2009: Test method submitted to TSAR; validation initiated via EURL ECVAM
- 2012: CYP induction validation study report published. OECD
- 2013-04: Project approved and included in the OECD TGP for the development of a Test Guideline on CYP enzyme induction in human-derived hepatic metabolic competent test systems.
- 2014-03: Final validation study report published. TSAR
- 2014-11: ESAC opinion and WG report: Endorsement at the 40th ESAC plenary meeting.
- 2020-06: Project suspended and moved to Annex 1 of the Work Plan of the OECD TGP.
- 2021-04: (on behalf of GOLIATH), UK take over lead: OECD Project 4.76: Test Guideline for the establishment on human-derived hepatic system to investigate biotransformation and toxicity of compounds by evaluation of CYP450 induction competence

H2020 Goliath deliverable: augmentation of chemical AD for HepaRG CYP induction test method

EURL ECVAM: validation using pharmaceuticals

Augmenting the chemical applicability domain beyond pharmaceuticals

Methodology:

1. Address chemical AD expansion by lit review support, e.g. in vitro human cell lines such as HepaRG, HuH7, HepG2, PHH, etc.

   (Approach presented to WNT in 2016, 2017 and supported by WNT)

2. Chemicals of specific interest: pesticides, biocides, contaminants, food additives

3. Chemicals of interest in Goliath: TBT, BPA (TBBPA, BPS/BPF), PFOA, TPP, phthalates, triclosan, OP's, benzophenone

- Proposed chemicals reviewed by 2 WNT NCs,
- and also peer reviewed in scientific literature


REVIEW article

Front. Toxicol., 20 June 2022

Sec. Regulatory Toxicology

https://doi.org/10.3389/ftox.2022.880818

Candidate Proficiency Test Chemicals to Address Industrial Chemical Applicability Domains for *in vitro* Human Cytochrome P450 Enzyme Induction

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Background and objectives

AIM: Augmentation of the CYP induction test method beyond that previously validated for pharmaceuticals (Bernasconi et al 2019) OECD Project 4.76

NIHR | Health Protection Research Unit in Environmental Exposures and Health at Imperial College London

Department for Environment Food & Rural Affairs

3 UK Health Security Agency

Third Naïve Laboratory Auxiliary CYP project

1 INRAE la science pour la vie, l'humain, la terre

2 Utrecht University

GOLIATH Testing metabolism disrupting chemicals

completed

1 Proficiency substances
Omeprazole
Carbamazepine
Phenytoin
Penicillin
Sulfinpyrazone
Bosentan
Artemisinin
Rifampicin
Metoprolol
Sotalol

2 10 Augmentation chemicals
Tebuconazole
Benfuracarb
Chlorpyrifos
N,N-diethyl-m-toluamide (DEET)
Permethrin
Fipronil
Triclosan
PFOA
Bisphenol A
Fludioxonil

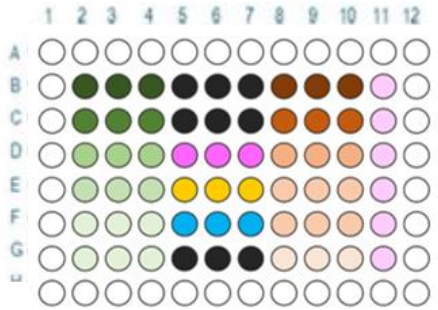
Jacobs MN, Kubickova B, Boshoff E. Candidate Proficiency Test Chemicals to Address Industrial Chemical Applicability Domains for *in vitro* Human Cytochrome P450 Enzyme Induction. Front Toxicol. 2022 Jun 20;4:880818.

CYP induction assay: SOP

Thawing and Seeding
Fri Day 1

HPR116 cells
7.2 x 10^4 cells/well
BIOPREDIC

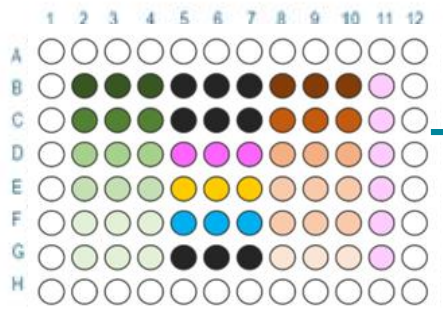
Treatments
Mon Day 4 t=0 h / Tue Day 5 t=24 h



CYP Induction

Test item exposure
48 h

Addition of probe substrate cocktail
Wed Day 6



Enzymatic probe substrate cocktail

1 h incubation

METABOLITE IDENTIFICATION
LC-MS/MS

INDUCER
2-fold incr.
2 consecutive conc's

Test items (6 serial dilutions)

Negative control (0.1% DMSO)

Medium solvent-free control

Positive controls (inducers)

Eg of analytical results: Proficiency Pharmaceuticals

Proficiency chemicals	Conclusion and comparison with expected result		
	CYP1A2	CYP2B6	CYP3A4
omeprazole	P	P	N
carbamazepine	P	P	P
phenytoin	P	P	P
penicillin G	N	N	N
sulfinpyrazone	P	P	P
bosentan	P	P	P
artemisinin	N	P	N
rifampicin	P	P	P
metoprolol	N	N	N
sotalol	N	N	N

Accepted proficiency ≥ 80% chemicals classified concordantly:

- Shown **successful proficiency (GREEN)** for **all** 3 CYP enzymes (100%)

Within-laboratory reproducibility (cell batches)

- 70% for CYP1A2 and CYP3A4
- 40% for CYP2B6 (more variability)

Threshold cut-off is set at 2-fold

Cell-batch discordant results

Snapshot of analytical results: Augmentation chemicals

Augmentation chemicals	Conclusion and comparison with expected result		
	CYP1A2	CYP2B6	CYP3A4
Tebuconazole	P	P	P
Benfuracarb	P	P	P
Chlorpyrifos	P	N	P
DEET	P	P	P

Cell-batch discordant results

Accepted proficiency ≥ 80% chemicals classified concordantly:

- Shown **successful proficiency (GREEN)** for **all** 3 CYP enzymes (100%)

Within-laboratory reproducibility (cell batches)

- 100% for CYP1A2 and CYP2B6
- 75% for CYP3A4 (more variability) – ***ONLY Chlorpyrifos***

Threshold cut-off is set at 2-fold

OECD DRP 97 (2008): Long term goals: Regulatory safety applications of the test method

- **Generation of genetically-engineered mammalian cell lines containing steroid hormone NRs, their response elements and reporter genes, plus genes expressing specific enzymes (e.g. P450,** SULTs etc) need assessment for feasibility for HTS and subsequent submission for (pre) validation.

  Such multiple expressing cell lines could then be utilised to investigate the metabolic activation and detoxification of potential EAS *in vitro*.

CYP induction assays can be combined with EAT in vitro TGs

Whether a substance is an ED or not can currently only be definitely established by careful *in vivo* experiments, however *in silico* and *in vitro* screening, with the incorporation of metabolism would result in a more adequate prioritisation for further testing.

- If a substance is suspected of being a potential ED on the basis of *in vitro* tests using enzymes and molecular targets of human origin, and the substance is going to be finally tested *in vivo*, it is advised to first confirm activity *in vitro,* with materials obtained from the animal species and strain that will be used for the *in vivo* test.

  - Finally: longer term goals will need to be revisited on a regular basis, as the information base develops…

Incorporation of metabolism *in vitro* testing at Level 2 of the OECD CF for EDCs

Improve performance of QIVIVE/PBPK models, metab. & PK databases with expansion of CYP induct. data

Species-specific metabolism information

CYP Induction/Inhibition Assays Species Relevant **Metabolite Profiling Assays**

CYP induction data to support *in vivo* dose range finding experiments studies for the parent chemical and metabolites.

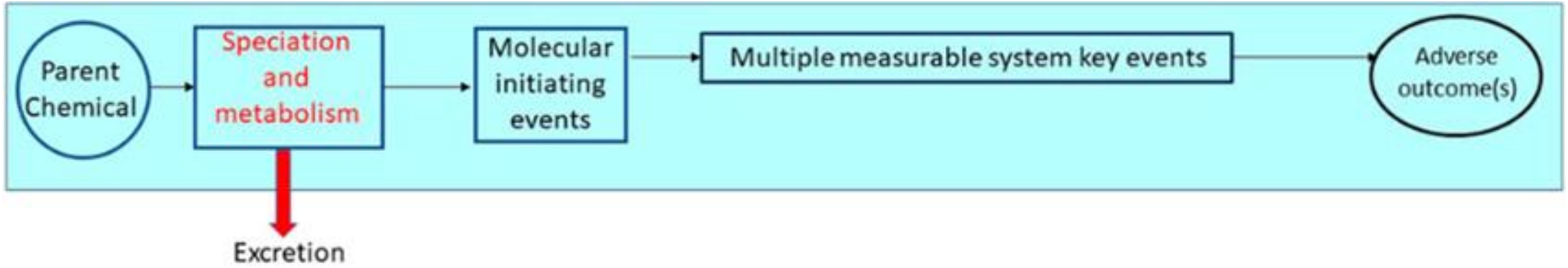
LEVEL 1 – Existing Data (incl. physiochemical and toxicity data) and Existing or New Non-Test Information (incl. read-across and **PK ADME** and other types of *in silico* data)

LEVEL 2 – In Vitro Assays Providing Data About Selected Endocrine Mechanism(s) / Pathway(s). Estrogen (OECD TG 493) and androgen (US EPA TG OPPTS 890.1150) receptor binding; estrogen (OECD TG 455), androgen (OECD TG 458), and retinoid receptor transactivation; steroidogenesis (OECD TG 456); aromatase activity (US EPA TG OPPTS 890.1200); and thyroid disruption (e.g. thyroperoxidase inhibition, transthyretin binding, sodium iodide symporter etc). Also includes other receptor assays as appropriate, such as hPlacentox test method, measuring the P2X7 receptor activation, and robust HTP screens

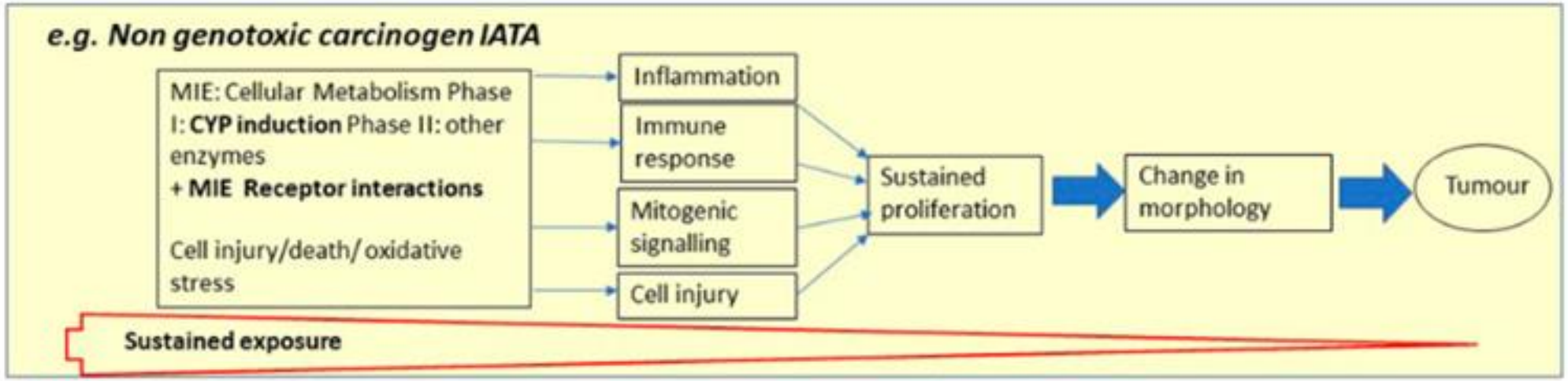
LEVEL 3 – *In Vivo* Assays Providing Data About Selected Endocrine Mechanism(s) /Pathways

LEVEL 4 – *In Vivo* Assays Providing Data on Adverse Effects on Endocrine Relevant Endpoints

LEVEL 5 – *In Vivo* Assays Providing More Comprehensive Data on Adverse Effects on Endocrine Relevant Endpoints Over More Extensive Parts of the Life Cycle of the Organism. Includes 2 mammalian and 6 non-mammalian OECD or US EPA TG assays



Application to multiple complex Integrated Approaches to Testing and Assessment



CYP Assay types
Bioassay/enzymology
S9 addition
HepaRG™ Phase I CYP induction: 1A1, 1A2, 3A4, 2B6, Phase II metabolism
CYP 2E1, CYP4A

Bioassay/enzymology
Inflammation related polymorphisms: Receptor-CYP's and CYPs 4A, 4F

Conclusions

1. Successful establishment of the CYP induction assay in 3 labs

2. Chemical Augmentation work shows **100% concordance** with literature

3. WLR is **more uncertain** for **CYP2B6**

Future directions

- **Preparation of manuscripts and combined reports**
- **2025-2026 Submission to the OECD Test Guideline Programme for review and potential TG adoption**

- Testing of additional augment. chemicals to further expand the chemical AD
- Optimise HepaRG cell line for Phase II metabolism capabilities
- Potential for inclusion in metabolism databases for hazard assessment eg Metapath
- Inclusion in PARC

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Chemical selection

- UKHSA: Eugene Boshoff, Barbara Kubickova

WNT chemical selection review:

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- Knud Laadegard Pedersen (DK)

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