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

Miriam Naomi Jacobs*, Barbara Kubickova† and Eugene Boshoff

Centre for Radiation, Chemical and Environmental Hazards (CRCE), Department of Toxicology, Public Health England (PHE), Harwell Science and Innovation Campus, Chilton, United Kingdom

Cytochrome P450 (CYP) enzymes play a key role in the metabolism of both xenobiotics and endogenous chemicals, and the activity of some CYP isoforms are susceptible to induction and/or inhibition by certain chemicals. As CYP induction/inhibition can bring about significant alterations in the level of in vivo exposure to CYP substrates and metabolites, CYP induction/inhibition data is needed for regulatory chemical toxicity hazard assessment. On the basis of available human in vivo pharmaceutical data, a draft Organisation for Economic Co-operation and Development Test Guideline (TG) for an in vitro CYP HepaRG test method that is capable of detecting the induction of four human CYPs (CYP1A1/1A2, 2B6, and 3A4), has been developed and validated for a set of pharmaceutical proficiency chemicals. However to support TG adoption, further validation data was requested to demonstrate the ability of the test method to also accurately detect CYP induction mediated by industrial and pesticidal chemicals, together with an indication on regulatory uses of the test method. As part of “GOLIATH”, a European Union Horizon-2020 funded research project on metabolic disrupting chemical testing approaches, work is underway to generate supplemental validated data for an additional set of chemicals with sufficient diversity to allow for the approval of the guideline. Here we report on the process of proficiency chemical selection based on a targeted literature review, the selection criteria and considerations required for acceptance of proficiency chemical selection for OECD TG development (i.e. structural diversity, range of activity, relevant chemical sectors, global

**Abbreviations:** AChE, Acetylcholinesterase; ADME, Absorption, Distribution, Metabolism, and Excretion; AhR, Aryl Hydrocarbon Receptor; B[a]P, Benzo-[a]-Pyrene; BPA, Bisphenol A; CAR, Constitutive Androstane Receptor; CYP, Cytochrome P450; DEET, N,N-Diethyl-m-Toluamide; DHMB, 2,3-Dihydroxy-4-Methoxybenzaldehyde; EDCs, Endocrine Disrupting Chemicals; EFSA, European Food Safety Authority; EROD, Ethoxyresorufin-O-Deethylase; HLM, Human Liver Microsomes; IATA, Integrated Approach to Testing and Assessment; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; LC-MS, Liquid Chromatography-Mass Spectrometry; LOAEL, Lowest Observed Adverse Effect Level; MIE, Molecular Initiation Event; NAM, New Approach Methodologies; OECD, Organisation for Economic Cooperation and Development; PBPK, Physiology-Based Pharmacokinetic; PFOA, Perfluorooctanoic Acid; PHH, Primary Human Hepatocytes; PK, Pharmacokinetics; POPs, Persistent Organic Pollutants; PPARs, Peroxisome Proliferator Activated Receptors; PXR, Pregnane X Receptor; QIVIVE, Quantitative In Vitro to In Vivo Extrapolation; REPS, Recombinant Enzyme Preparations; TBBPA, Tetra Brominated Bisphenol A; TG, OECD Test Guideline; WNT, OECD Working Group of National Coordinators of the Test Guideline Programme.
INTRODUCTION

The application of in vitro test method tools for chemical hazard assessment in relation to human health protection is often limited due to insufficient understanding of chemical metabolism, bioactivation or deactivation, and bioavailability.

The liver is a main site of Phase I and Phase II metabolism of endogenous and exogenous substances including nutrients, drugs and chemicals, but many other tissues (such as but not limited to the gut, kidney, placenta) in the body also have metabolism competency. Phase I metabolism encompasses the biochemical reactions that introduce reactive and polar groups into xenobiotic compounds by oxidation, reduction, or hydrolytic reactions. The major Phase I modifications are catalysed by a large family of Cytochrome P450 (CYP) enzymes (Lewis 2002), and in the first steps, CYPs may transform a xenobiotic into a harmless metabolite (detoxification) that can be easily eliminated via Phase II, or, vice versa, a non-toxic parent chemical may be transformed (metabolically bioactivated) into a toxic metabolite.

CYP induction, that is transcriptional activation/upregulation in CYP gene expression and protein levels, is first triggered by the binding of a chemical to specific nuclear receptors, these constitute the molecular initiation event (MIE). The Aryl hydrocarbon Receptor (AhR), is primarily responsible for the CYP1A and 1B family, the Pregnane X Receptor (PXR) for the CYP3A family and the Constitutive Androstane Receptor (CAR) for the CYP 2B family (Waxman 1999), and the Peroxisome Proliferator Activated receptors (PPARs) for the CYP4A family. These receptors also play major roles in regulating many physiological functions, including hormone and lipid regulation (Kliewer, Lehmann et al., 1999; Jacobs and Lewis 2002).

The CYP1A, 1B, 3A and 2E subfamilies are responsible for the bioactivation of the majority of xenobiotics. For example, with repeated exposure, many chemical carcinogens are bioactivated by CYP1A, indeed such chemicals selectively induce this family, thus exacerbating their carcinogenicity (Ioannides and Lewis 2004; Rendic and Guengerich 2021). Pharmaceuticals, nutrients and some industrial chemicals are mostly activated by CYP3A4, and dietary nutrients/contaminants by CYP1A2, 2E1, and 3A4 (Jacobs and Lewis 2002; Jacobs, Nolan et al., 2005; Hakkola, Hukkanen et al., 2020; Rendic and Guengerich 2021 and references therein). Endogenous substances that are usually components of physiological processes, are mainly activated by CYP1A, 1B1, and 3A enzymes (Rendic and Guengerich 2021). The latter recent review analysing the catalytic activity of CYP families in relation to catalytic bioactivation showed predominant participation of CYP3A4, 1A2, and 1A1, followed by CYP2E1 and 1B1. CYPs 2C9, 2D6, 2A6, 2C19, and 2B6 also having significant participation.

Phase II metabolism often involves the further conjugation of the metabolite with polar molecules, such as sulphate, amino acids, glutathione or glucuronic acid, facilitated by various transferases, generating metabolites that are more soluble to facilitate elimination.

Phase I CYP enzyme induction therefore plays a pivotal initial role in the metabolism of both xenobiotics and endogenous chemicals and constitutes a sensitive biomarker for metabolic competence of in vitro test systems. Chemically mediated induction and/or inhibition of CYPs can lead to marked changes in CYP substrate and metabolite concentrations, and in vitro CYP induction and inhibition data are currently commonly used to predict potential CYP mediated clinical drug interactions for pharmaceuticals (EMA 2012; US FDA, 2020) and are compiled in pharmaceutical CYP induction/inhibition databases (e.g. SIMCYP (Marsousi, Desmeules et al., 2018)).

Thus CYP induction and inhibition data are also needed for a wide number of human health endpoints both for pharmaceutical therapeutic and chemical hazard assessment purposes, for instance ranging from hormone and fatty acid metabolism (Kliewer, Lehmann et al., 1999), hepatotoxicity, and steatosis (Massart, Begriche et al., 2022) to, in inflammatory responses (Rubin, Janefeldt et al., 2013), and non-genotoxic carcinogenicity (Ioannides and Lewis 2004; Jacobs, Colacci et al., 2016).

Furthermore, the greater inclusion of CYP induction/inhibition data into chemical hazard assessment will facilitate the shift from regulatory reliance on animal in vivo testing to New Approach Methodologies (NAMs) that refer to and include a battery of relevant in vitro and in silico tools. While the incorporation of metabolic capacity into in vitro genotoxicity testing has been routinely conducted for several decades, in contrast, although discussed a lot, it has not progressed very quickly for other human health endpoints. Illustrations of how the tests could be combined within the OECD conceptual frameworks and Integrated Approaches to Testing and Assessment (IATA) have been developed (Jacobs, Laws et al., 2013; Jacobs, Colacci et al., 2020).

Whilst many chemicals are metabolized by CYPs, enzyme induction data are the focus of the HepaRG CYP enzyme induction test method (Bernasconi, Pelkonen et al., 2019). The need for human CYP induction/inhibition test data to improve

Keywords: CYP, P450, validation, test guideline, HepaRG, metabolism
the predictive accuracy of in vitro test methods and in silico tools for chemical toxicological hazard assessment, was established by the OECD Test Guideline Programme over 14 years ago, particularly in relation to endocrine disruption (Jacobs, Janssens et al., 2008; OECD 2008). OECD member countries recommended that the human CYP enzyme induction test method was the optimum metabolism test method to take forward for OECD Test Guideline (TG) purposes, and on this basis, validation activities were later initiated and completed for a test method for an in vitro human hepatocyte cell line CYP enzyme induction assay. The assay is capable of detecting an induction of the enzymatic activity of four CYP isoforms (CYP1A1/1A2, 2B6, and 3A4) (Bernasconi, Pelkonen et al., 2019), and was developed and validated using pharmaceutical chemicals. These CYP enzymes are commonly involved in metabolizing drugs and environmental toxicants (Esteves, Rueff et al., 2021), and in producing pharmacokinetic (PK) interactions of medicines (EMA 2012; Ooka, Lynch et al., 2020; US FDA, 2020).

The test method utilizes the metabolic capacity of differentiated cryopreserved immortalized human HepaRG cells coupled with analytical liquid chromatography and mass spectrometry (LC-MS) to quantify the induction of the CYP enzymes based on model substrate conversion (Bernasconi, Pelkonen et al., 2019). To improve human relevance, the study was designed on the basis of human CYP induction evidence, rather than the more plentiful in vivo rodent data. There was sufficient relevant human CYP induction data to allow for an assessment of the human translation potential of the test method, available only for pharmaceuticals (JRC TSAR, 2009). At that time there were no human in vivo data available for other regulatory sector classes (not least because there are ethical issues with respect to pesticide and contaminant testing in controlled human studies). In 2019, a draft TG was submitted for review to the OECD that was based upon the successfully performed and peer reviewed validation data generated using pharmaceutical chemicals. Review feedback was received from the OECD Working Group of National Coordinators of the TG Programme (WNT) that an essential requirement for the approval of the draft TG would be the provision of supporting validation data generated with additional proficiency chemicals representative of chemicals used in other relevant sectors, including industrial chemicals and pesticides. This was because some members of the WNT did not consider that the chemical applicability domain of pharmaceuticals tested in the original validation, gave sufficient coverage of the industrial chemical applicability domain that this test method is intended to be applied to. Due to the lack of primary human data for non-pharmaceuticals, the WNT accepted a compromise proposal on how to utilise the wider chemical metabolism data in the scientific literature, using metabolism data generated from relevant human cell lines, for example. This data was utilised to generate a chemical selection list, to supplement the reference/proficiency chemical list in the original HepaRG CYP enzyme induction test method validation, and the review and selection process is described in this paper. This list of suitable proficiency chemicals is the basis for augmenting the chemical applicability domain of the test method. This additional validation data is being generated on the HepaRG CYP enzyme induction test method, within “GOLIATH”, a European Union Horizon-2020 funded research project on metabolic disrupting chemical testing approaches.

Here we provide details on the selected chemicals, the method and supporting data used for selecting the chemicals, and an overview of intended regulatory applications of the test method.

METHODOLOGY

Criteria Used for the Identification of an Initial Selection Pool of Candidate Validation Chemicals

The chemical and structural diversity of the proficiency chemicals used for (pre)validation needs to address both the chemical applicability domain of the chemical Universe for which the test method is intended to predict endpoint-specific toxicity, but also be structurally relevant for the biological role of the endpoint. In addition, in many cases, where known, natural and endogenous ligands should be included in the chemical selection (Waxman 1999; Jacobs and Lewis 2002) as these are the ligands that the anthropogenic chemicals of concern are mimicking.

A targeted reiterative but not systematic literature search was carried out to identify an initial selection pool of candidate proficiency chemicals for which human CYP modulation data was available for CYP1A1/1A2, CYP2B6, and CYP3A4, and that belonged to OECD TG Programme relevant chemical classes (including industrial chemicals, pesticides, and food additives). Data sources from both human cell line in vitro and human in vivo studies relating to specified CYP induction and directly related receptors were sourced and critically evaluated, but due to the general scarcity of in vivo human studies carried out with non-pharmaceutical chemicals, only human in vitro data was available for most chemicals. Support that this would be an acceptable approach to take was first established within the OECD WNT, given the scarcity of human in vivo data. In some cases, it was appropriate to use rodent data for weight of evidence support.

On this regulatory acceptable basis, between 2017 and 2021, Scopus and pubmed search engines were queried with respect to human relevant data, cytochrome P450, and chemicals, including pesticides/bacteriocides and excluding pharmaceuticals as these are already addressed in the draft TG. The results were filtered and prioritised for human relevant data including human relevant cell lines, e.g. HepaRG, HepG2, and the relevant CYPs in the draft validated TG, together with relevant references within the papers. These were critically reviewed and double checked by the authors and then external regulatory experts for the OECD TG Programme (WNT).

In the validated test method, overall CYP enzymatic activity is quantified in an in vitro human hepatocyte cell line (cryopreserved HPR116 differentiated HepaRG cells) before and after pretreatment with test chemicals, by measuring the
rate of metabolic conversion of substrates that are selective for CYP1A1/1A2 (phenacetin to acetaminophen), CYP2B6 (bupropion to hydroxybupropion), and CYP3A4 (midazolam to 1'-hydroxymidazolam) using a LC-MS analytical technique (Bernasconi, Pelkonen et al., 2019). In such cell systems, the overall effect on enzyme activity is dependent on the extent to which a chemical up or downregulates not only CYP mRNA/protein levels, but also functional enzymatic activity. Data evaluated in the literature search therefore included *in vitro* mRNA/protein quantity and enzyme activity data generated using human cells, as well as enzyme activity data generated in human liver microsomes (HLMs) and recombinant enzyme preparations (REPS). CYP1A1/1A2 gene expression is well understood and extensively documented to be induced by activation of the AhR (e.g. Bock 2014; Vogel, Van Winkle et al., 2020), and activation of CAR and PXR has been shown to induce CYP2B6 and CYP3A4 (Tolson and Wang 2010; Wang, Ong et al., 2012). Data showing activation of these receptors was taken to indicate likely mRNA/protein upregulation of the respective CYPs. Preliminary assessment as to whether a candidate chemical is likely to induce, inhibit, or have no effect on a specific CYP in the test method was made on the basis of enzyme activity data from human cell systems with innate CYP expression, when this data was available. Where this data was not available, estimations were made based on available mRNA/protein quantity data and enzyme activity data from non-cell preparations, when possible. In the latter case, estimations were considered to be of lower reliability as compared to cases where cell-based enzymatic activity data was available, and insufficient data were considered to be insufficient to estimate the effect of a chemical in the test method if overall the available good quality data was considered to be contradictory. In the design of validation experiments intended for applications beyond classification and prioritisation purposes, it is good practice to include proficiency chemicals that are expected to produce a potency range from negative to low, moderate, and strong effects, and to generate concentration-response information, as this is of greater efficiency and priority. Data sources that were considered to be of inadequate quality, due to lack of information regarding the successful establishment of the assay(s), or poor reproducibility (Taxvig 2020; Franzosa, Bonzo et al., 2021), were not utilised.

**Selection of the Validation Chemical Set**

From the candidate pool of chemicals, a proposed set of proficiency validation chemicals was selected to enable adequate coverage of structural diversity but also a representative selection of chemicals from relevant sectors (including industrial chemicals, pesticides, and food additives) that was practically possible, on the basis of publicly available scientific literature. Importantly, to be able to fully evaluate the functioning and reliability of the test method, the proposed proficiency chemicals were also chosen to try to ensure the inclusion of a sufficient number of negative chemicals (a minimum of 25% of total tested), with the range of positive chemicals that would adequately probe the ability of the test method to detect individual induction of each of the four measured CYPs (CYP1A1/1A2, CYP2B6, and CYP3A4).
TABLE 1 | Part A = Proposed Set of Additional Industrial, Pesticidal, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low (≤3 fold), moderate (>3 to 4.5 fold), and or strong (>4.5 fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.

| Chemical name | CAS number | Structure | Aus molecular weight (Dalton) | Predicted LogP* | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, - uncertain. Na = No or insufficient data | Supporting evidence from literature publications |
|---------------|------------|-----------|-----------------------------|----------------|-----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| Tebuconazole; | 107534-96-3| ![Structure](image1) | 307.818; 3.58 | Inducer (+) Inducer (+) Inducer (+) | CYP1A1: In HepaRG cells, 80 µM increased mRNA ≤4 (log2)x = ≤16.0x Abass and Pelkonen (2013), Lasch, Marx-Stoelting et al. (2021), and protein 3.25 (log2)x = 9.5x Schmidt, Lichtenstein et al. (2021). In Caco-2 cells, 8.1 µM weakly increased enzyme [ethoxyresorufin-O-deethylase (EROD)] activity (~7 pmol/min/mg protein vs 0 for the control) Sergent, Dupont et al. (2009). CYP1A2: In HepaRG cells, 1.25-40 µM increased mRNA ≤30x and enzyme activity ≤2.5x = ≤6.7x Knebel, Heise et al. (2019), Lasch, Marx-Stoelting et al. (2021), and 80 µM increased protein ≤2.75 (log2)x = 6.8x Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021). CYP2B6: In HepaRG cells, 5–40 µM increased mRNA and enzyme activity ≤2.5 × Knebel, Heise et al. (2019), Knebel, Neeb et al. (2018), Lasch, Marx-Stoelting et al. (2021), and 80 µM increased protein 1.72 (log2) = 3.3x Schmidt, Lichtenstein et al. (2021). CYP3A4: In HepaRG cells, 1.25-40 µM increased mRNA ≤6x, and enzyme activity ≤2.5 x Knebel, Heise et al. (2019), Knebel, Neeb et al. (2018), Lasch, Marx-Stoelting et al. (2021); and 80 µM increased protein ≤0.64 (log2)x = ≤1.6x Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021). In Caco-2 cells, 8.1 µM increased enzyme activity ~40% Sergent, Dupont et al. (2009). AhR, CAR, PXR: Activated AhR Knebel, Heise et al. (2019) and PXR Knebel, Neeb et al. (2018), and inhibited CARKnebel, Neeb et al. (2018). Human Exposure Data: Urine metabolite (hydroxy-tebuconazole) PK data from 6 volunteers after a single oral (1.5 mg) or dermal (2.5 mg) dose Oerlemans, Verscheijden et al. (2019), and urine PK data from 7 agricultural workers after occupational exposure Fustinoni, Mercadante et al. (2014). |
| Benfuracarb; 82560-54-1 | Carbamate insecticide; 410.528; 4.54 | ![Structure](image2) | Inducer (+) Inducer (+) Inducer (+) | CYP1A2: In HepaRG cells, 10/50 µM increased mRNA and enzyme activity ≤2 x Abass, Lämsä et al. (2012). CYP2B6: In HepaRG cells, 10/50 µM increased mRNA ≤5x and enzyme activity ≤2.5 x Abass, Lämsä et al. (2012). CYP3A4: In HepaRG cells, 10/50 µM increased mRNA ≤9x and enzyme activity ≤2.5 x Abass, Lämsä et al. (2012). In human liver microsomes (HLM), inhibited formation of 1-OH-midazolam and SO2-omeprazole with IC50s of 14.8 and 24.2 µM, respectively Abass, Reponen et al. (2014). CAR, PXR: Activated CAR but not PXR Abass, Lämsä et al. (2012). |
| Atrazine; 1912-24-9; | Triazine herbicide; 215.683; 2.63 | ![Structure](image3) | Inducer (+) Inducer (++) Inducer (+) | CYP1A2: In HepaRG cells, 10/50 µM increased mRNA and enzyme activity ≤3 x Abass, Lämsä et al. (2012). In HLM, 1–100 µM did not alter enzyme activity Abass, Lämsä et al. (2012). CYP2B6: In HepaRG cells, 10/50 µM increased mRNA ≤12x, and enzyme activity ≤4 x Abass, Lämsä et al. (2012). In HLM, weakly inhibited enzyme activity IC50 = 107 µM². CYP3A4: In HepaRG cells, 10/50 µM increased mRNA ≤10x, and enzyme activity ≤2.5 x Abass, Lämsä et al. (2012). |

(Continued on following page)
TABLE 1 (Continued) Part A = Proposed Set of Additional Industrial, Pesticidal, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low ($\leq 3$ fold), moderate ($> 3$ to $4.5$ fold), and or strong ($> 4.5$ fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.

| Chemical name               | CAS number | structure | Ause molecular weight (Dalton)* | Predicted LogP* | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, - uncertain. Na = No or insufficient data | Supporting evidence from literature publications |
|-----------------------------|------------|-----------|--------------------------------|-----------------|--------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| Cypermethrin; 52315-07-8; Pyrethroid insecticide; 416.297; 6.27 | No Effect | Inducer (+) | Inducer (+++) | HLM, inhibited formation of midazolam ($IC_{50} = 2.8$ µM) but not SO$_2$-omeprazole ($IC_{50} = 618$ µM) Abass and Pelkonen (2013). |
| Chlorpyrifos; 2921-88-2; Acetylcholinesterase (AChE) Inhibitor Organophosphate pesticide; 350; 4.77 |  |  |  | CAR, PXR: Activated PXR but not CAR Abass, Lämsä et al. (2012). |

HLM, inhibited formation of midazolam ($IC_{50} = 2.8$ µM) but not SO$_2$-omeprazole ($IC_{50} = 618$ µM) Abass and Pelkonen (2013).
CAR, PXR: Activated PXR but not CAR Abass, Lämsä et al. (2012).

CYP1A1: In HepG2 and HaCat cells, 100 µM had no effect on enzyme (EROD) activity Delescluse, Ledirac et al. (1998). In HLM, did not alter CYP1A1/1A2 enzyme activity Abass, Turpeinen et al. (2009).
CYP1A2: In HepaRG cells, 10/50 µM increased mRNA $\leq 3$x, but had no effect on enzyme activity Abass, Lämsä et al. (2012). In HLM, 1–100 µM did not alter enzyme activity Abass and Pelkonen (2013).
CYP2B6: In HepaRG cells, 10/50 µM increased mRNA $\leq 7$x and enzyme activity $\leq 3.5 \times$ Abass, Lämsä et al. (2012). In primary human hepatocytes (PHH), 10 µM increased protein $\sim 2 \times$ Lemaire, de Sousa et al. (2004). In HLM, 1–100 µM did not alter enzyme activity Abass and Pelkonen (2013).
CYP3A4: In HepaRG cells, 10/50 µM increased mRNA $\leq 35$x and enzyme activity $\leq 3.5 \times$ Abass, Lämsä et al. (2012). In PHH, 10 µM increased protein $\sim 2 \times$ Lemaire, de Sousa et al. (2004). In HLM, weakly inhibited formation of 1-OH-midazolam ($IC_{50} = 70$ µM) and SO$_2$-omeprazole ($IC_{50} = 249$ µM) Abass and Pelkonen (2013).

PXR: Activated PXR Abass, Lämsä et al. (2012), Abass and Pelkonen (2013).

Other CYP Effects: Inhibited CYP2A6 Abass and Pelkonen (2013), and weakly increased CYP1B1 mRNA Das et al. (2008a). Interspecies differences between rat and human are indicated for CYP2B6 inhibition D’Agostino, Zhang et al. (2015).

Human Exposure Data: Chlorpyrifos use is being phased-out due to it being associated with developmental neurotoxicity in human epidemiological studies at concentrations below the...
TABLE 1 | (Continued) Part A = Proposed Set of Additional Industrial, Pesticidal, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low (≤ 3-fold), moderate (>3 to 4.5 fold), and or strong (>4.5 fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.

| Chemical name | CAS number | structure | Ause molecular weight (Dalton) | Predicted LogP* | Supporting evidence from literature publications |
|---------------|------------|-----------|-------------------------------|----------------|-----------------------------------------------|
| **Perfluorooctanoic Acid (PFOA):** | 3825-26-1; | ![](image) | 414.068; 7.75 | Inhibitor | CYP1A1: 100 µM increased mRNA in HepG2 cells ~15x, but had no effect on mRNA or protein Niwa, Tsutsui et al. (2000). |}

| **Bisphenol A (BPA):** | 80-05-7; | ![](image) | 228.286; 3.43 | NA | CYP1A1: 100 µM increased mRNA in HepG2 cells ~15x, but had no effect on mRNA or protein Niwa, Tsutsui et al. (2000). |}

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|---------------|------------|-----------|----------------------------------|-----------------|---------------------------------------------------------------------------------|-------------------------------------------------|
| N,N-diethyl-m-toluamide (DEET)**: | 134-62-3; | ![Image](image1.png) | Insect repellent; | 191.270; 1.96 | CYP1A1: In PHH, 100 µM increased mRNA ≤8 × Das et al. (2008a), Lawrie, Mitchell et al. (2020). CYP1A2: In PHH, 100 µM increased mRNA ≤4 × Das et al. (2008a), Lawrie, Mitchell et al. (2020). In REPS, 50 µM had no effect on enzyme activity Usmani, Cho et al. (2006). CYP2B6: In PHH, 100 µM increased mRNA ≤8 × Das et al. (2008a), Lawrie, Mitchell et al. (2020). CYP3A4: In PHH, 100 µM increased mRNA ≤10x, protein ≤4x, and enzyme activity ~3 × Das et al. (2008a), Lawrie, Mitchell et al. (2020). In REPS, 50 µM produced a non-significant ~25% increase in enzyme activity Usmani, Cho et al. (2006). Other CYP Effects: Induced CYP2A6 Das et al. (2008a). | Human Exposure Data: Human biomonitoring data has shown 2 metabolites of DEET, 3-(diethylcarbamoyl)benzoic acid (DCBA) and 2,3-dihydroxy-4-methoxybenzaldehyde (DHMB), to be present in the urine in a sample of the US general population, with DCBA being detected at geometric mean concentrations ≤4.74 µg/g creatinine ATSDR (2017). Blood and urine PK data is also available for volunteers dermally dosed with [14C]-DEET Selim, Hartnagel et al. (1995). CYP1A1: In HepaRG cells, 5 µM increased mRNA ≤350 × Vlach, Quesnot et al. (2019), and protein 5.1 (log2)x [= 34.3x] Schmidt, Lichtenstein et al. (2021), and 10 µM increased mRNA 2.2 (log2)x [= 6.8x] Jennen, Magkoufopoulou et al. (2010). In Caco-2 cells, 0.4 µM strongly increased enzyme (EROD) activity (~40 pmol/min/mg protein vs. 0 for the control) Sergent, Dupont et al. (2009). In REPS, inhibited enzyme activity with an IC50 of 0.35 µM Shimada and Guengerich (2006). CYP1A2: In HepaRG cells, 5 µM increased protein ≤5.3 (log2)x [=<39.4x] Schmidt, Lichtenstein et al. (2021). In REPS, inhibited enzyme activity with an IC50 of 0.14 µM Shimada and Guengerich (2008). CYP2B6: In HepaRG cells, 5/10 µM had no effect on mRNA Goedtke, John et al. (2021), Vlach, Quesnot et al. (2019), and increased protein by 1.7 (log2)x [= 3.2x] Schmidt, Lichtenstein et al. (2021), and 10 µM decreased mRNA Jennen, Magkoufopoulou et al. (2010). CYP3A4: In HepaRG cells, 5 µM increased mRNA ~2.5 × Vlach, Quesnot et al. (2019) and had no effect on protein Schmidt, Lichtenstein et al. (2021). In REPS, inhibited enzyme activity with an IC50 of >10 µM Wanchana, Yamashita et al. (2003). AhR, CAR, PXR: Activated AhR Goedtke, John et al. (2021), weakly activated CAR Goedtke, John et al. (2021), and activated PXR as measured by CYP3A4 promoter activity Luckert, Ehlers et al. (2013). |
| Benzo-[a]-pyrene (B[a]P): | 50-32-8; | ![Image](image2.png) | Polycyclic aromatic hydrocarbon found in combustion products, for example, in chargrilled food, cigarette smoke, and car exhaust fumes; | 252.309; 6.4 | CYP1A1: Inducer (+++) NA NA | Human Exposure Data: Human biomonitoring data has shown 2 metabolites of DEET, 3-(diethylcarbamoyl)benzoic acid (DCBA) and 2,3-dihydroxy-4-methoxybenzaldehyde (DHMB), to be present in the urine in a sample of the US general population, with DCBA being detected at geometric mean concentrations ≤4.74 µg/g creatinine ATSDR (2017). Blood and urine PK data is also available for volunteers dermally dosed with [14C]-DEET Selim, Hartnagel et al. (1995). CYP1A1: In HepaRG cells, 5 µM increased mRNA ≤350 × Vlach, Quesnot et al. (2019), and protein 5.1 (log2)x [= 34.3x] Schmidt, Lichtenstein et al. (2021), and 10 µM increased mRNA 2.2 (log2)x [= 6.8x] Jennen, Magkoufopoulou et al. (2010). In Caco-2 cells, 0.4 µM strongly increased enzyme (EROD) activity (~40 pmol/min/mg protein vs. 0 for the control) Sergent, Dupont et al. (2009). In REPS, inhibited enzyme activity with an IC50 of 0.35 µM Shimada and Guengerich (2006). CYP1A2: In HepaRG cells, 5 µM increased protein ≤5.3 (log2)x [=<39.4x] Schmidt, Lichtenstein et al. (2021). In REPS, inhibited enzyme activity with an IC50 of 0.14 µM Shimada and Guengerich (2008). CYP2B6: In HepaRG cells, 5/10 µM had no effect on mRNA Goedtke, John et al. (2021), Vlach, Quesnot et al. (2019), and increased protein by 1.7 (log2)x [= 3.2x] Schmidt, Lichtenstein et al. (2021), and 10 µM decreased mRNA Jennen, Magkoufopoulou et al. (2010). CYP3A4: In HepaRG cells, 5 µM increased mRNA ~2.5 × Vlach, Quesnot et al. (2019) and had no effect on protein Schmidt, Lichtenstein et al. (2021). In REPS, inhibited enzyme activity with an IC50 of >10 µM Wanchana, Yamashita et al. (2003). AhR, CAR, PXR: Activated AhR Goedtke, John et al. (2021), weakly activated CAR Goedtke, John et al. (2021), and activated PXR as measured by CYP3A4 promoter activity Luckert, Ehlers et al. (2013). |
| Fludioxonil; | 131341-86-1; | ![Image](image3.png) | Insect repellent; | 191.270; 1.96 | CYP1A1: Inducer (+++) Inducer (~) Inhibitor | CYP1A1: In HepaRG cells, 50 µM increased mRNA strongly and enzyme activity >10 × Lasch, Marx-Stoelting et al. (2021), and 250 µM increased mRNA ≤8x and protein ≤8x (log2) [= (Continued on following page) |
TABLE 1 (Continued) Part A = Proposed Set of Additional Industrial, Pesticidal, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low ($\leq 3$ fold), moderate ($>3$ to $4.5$ fold), and strong ($>4.5$ fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.

| Chemical name | CAS number | structure | Ause molecular weight (Dalton) | Predicted LogP | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: | Supporting evidence from literature publications |
|---------------|------------|-----------|-------------------------------|---------------|-------------------------------------------------|--------------------------------------------------|
| Non-systemic phenoxyprrole class fungicide; | 248.185; 3.67 | | | | No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, ~ uncertain. Na = No or insufficient data | |
| Malathion; 121-75-5 | | | | | No Effect | No Effect or Inhibitor | NA |
| Organophosphate insecticide (AChE inhibitor); 330.3582.92 | | | | | | CYP1A2: In HepaRG cells, 10/50 µM increased mRNA $\leq 4$x, and had no effect on enzyme activity in one publication Abass, Lämsä et al. (2012), while in another, it had no effect on mRNA or enzyme activity Josse, Sharanek et al. (2014). In HLM, inhibited enzyme activity with an IC$_{50}$ of 19 µM Abass and Pelkonen (2013).
| | | | | | CYP2B6: In HepaRG cells, 10–50 µM increased mRNA $\leq 3$x, and decreased enzyme activity $\sim 95$% in one publication Abass, Lämsä et al. (2012) while in another, it had no effect on mRNA or enzyme activity Josse, Sharanek et al. (2014). In HLM, inhibited enzyme activity with an IC$_{50}$ of 69 µM Abass and Pelkonen (2013).
| | | | | | CYP3A4: In HepaRG cells, 10–50 µM increased mRNA $\leq 4$x and enzyme activity $\leq 2$x in one publication Abass, Lämsä et al. (2012), while in another, it had no effect on mRNA or enzyme activity Josse, Sharanek et al. (2014). In HLM, inhibited enzyme activity with an IC$_{50}$ of 57 µM Abass and Pelkonen (2013).
| | | | | | CAR, PXR: Activated CAR but not PXR Abass, Lämsä et al. (2012). |
| Triclosan; 3380-34-5; Antibacterial and antifungal biocide; 289.542; 5.17 | | | | | NA | NA | Inducer (-) |
| Caffeine | 58-08-2; | | | | Inhibitor | NA | NA |

(Continued on following page)
| Chemical name | CAS number | Structure | Ause molecular weight (Dalton) | Predicted LogP | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, - uncertain. Na = No or insufficient data | Supporting evidence from literature publications |
|---------------|------------|-----------|-------------------------------|---------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| CYP1A1 | 120068-37-3; | ![Structure](image) | 194,191; −0.13 | | | Stimulant found naturally in coffee and tea that is added to drinks and medicines; CYP1A2: In PHH, 20–200 µM had no effect on mRNA, and a high 400 µM increased mRNA 2.3 x Vaynshteyn and Jeong (2012). Inhibited enzyme activity in REPS (inhibition of ~15% at 0.1 mM and ~70% at 1 mM) Tassaneeyakul, Birkett et al. (1993) and in a clinical study (600 mg increased the AUC of the CYP1A2 substrate, melatonin by 120%) Hartter, Nordmark et al. (2003). AhR: Did not activate AhR Vaynshteyn and Jeong (2012). Additional Info: Metabolised by CYP1A2 (>95% of its primary metabolism), CYP2C8/9, CYP2A6, CYP2E1, and CYP3A4 Kot and Daniel (2008b), OECD (2020b), The Danish Centre on Endocrine Disrupters (2020), Thorn, Aklillu et al. (2012). In humans, caffeine metabolism has been used as a marker of CYP1A2 and CYP2A6 metabolism, and gender did not affect CYP1A2 or CYP2A6 mediated metabolism of caffeine Begas, Kouvaras et al. (2007). There are species differences between rat and human metabolism, but in both species, metabolism is mainly mediated by CYP1A2 Kot and Daniel (2008a). Human Exposure Data Available: Urine concentrations of caffeine and its metabolites in volunteers dosed orally with caffeine are available Begas, Kouvaras et al. (2007), Kim, Choi et al. (2019). |
| CYP2B6 | | | | | | |
| CYP3A4 | | | | | | 

**Part B. Candidate Selection Pool Chemicals Evaluated but not Selected**

| Chemical name | CAS number | Structure | Ause molecular weight (Dalton) | Predicted LogP | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, - uncertain. Na = No or insufficient data | Supporting evidence from literature publications |
|---------------|------------|-----------|-------------------------------|---------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| Fipronil | 120068-37-3; | ![Structure](image) | Broad-spectrum phenylpyrazole class insecticide | | | CYP1A1: In HepaRG cells, 50 µM decreased mRNA and had no effect on protein Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021), whereas, in PHH, 0.1–25 µM increased mRNA ≤50x and enzyme activity ≤2.5x (bell-shaped concentration response) Das, Cao et al. (2006), Mitchell, Dhammi et al. (2016), CYP1A2: In PHH, 0.1-25 µM had no effect on mRNA Das, Cao et al. (2006), Mitchell, Dhammi et al. (2016). In HepaRG cells, 50 µM had no effect on protein Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021). In REPS, 50 µM non-significantly increased enzyme activity ~15% Usmani, Cho et al. (2006), CYP2B6: In PHH, 0.1-25 µM increased mRNA ≤3.5 x Das, Cao et al. (2006), Lawrie, Mitchell et al. (2020), Mitchell, Dhammi et al. (2016), In HepaRG cells, 50 µM had no effect on protein Schmidt, Lichtenstein et al. (2021), CYP3A4: In HepaRG cells, 50 µM increased protein 0.4 (log2)x (=1.34) Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021). In PHH, 0.1-25 µM increased mRNA ≤28x and protein and enzyme activity ≤4x (bell-shaped concentration response) Das, Cao et al. (2006), Hodgson and Rose (2007), Lawrie, Mitchell et al. (2020), Mitchell, Dhammi et al. (2016), In REPS, 50 µM had no effect on enzyme activity Usmani, Cho et al. (2006), PXR: Activated PXR Lemaire, Mnif et al. (2006). CYP1A1: In HepG2 cells, 1–50 µM increased mRNA ≤350x, protein by an undescribed amount, and enzyme activity ≤4x, whereas, in PHH, 1–50 µM increased mRNA ≤10x, and had no (Continued on following page) |
| Ketoconazole | 65277-42-1; | ![Structure](image) | | | | |

**TABLE 1** (Continued) Part A = Proposed Set of Additional Industrial, Pesticidial, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low (≤3 fold), moderate (>3 to 4.5 fold), and or strong (>4.5 fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.
TABLE 1 (Continued) Part A = Proposed Set of Additional Industrial, Pesticidal, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low (≤3 fold), moderate (>3 to 4.5 fold), and or strong (>4.5 fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.

| Chemical name | CAS number | structure | Ause molecular weight (Dalton) | Predicted Log P* | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, ~ uncertain. Na = No or insufficient data | Supporting evidence from literature publications |
|---------------|------------|-----------|-------------------------------|-----------------|-------------------------------------------------------------------------------------------------|

**Fungicide (pharmaceutical); 437.148; 4.76**

- **CYP1A1/1A2**: No Effect
- **CYP2B6**: No Effect
- **CYP3A4**: NA

Effect on enzyme activity: Novotna et al. (2014a). In Caco-2 cells, 4.7 µM moderately increased enzyme (EROD) activity (~10 pmol/min/mg protein vs. 0 for the control) Sergent, Dupont et al. (2009). In HepG2 cells, 1–50 µM increased mRNA ≥10x, protein ≥4x and enzyme (EROD) activity ≤3 x Korashy, Shayeganpour et al. (2007).

**CYP1A2**: In PHH, 1–50 µM increased mRNA ≤10x, and protein by an undefined amount Novotna et al. (2014a). In REPS, produced no Yim, Kim et al. (2020) or weak inhibition (~20% at 40 µM and ~50% at 120 µM) Emoto, Murase et al. (2003) of enzyme activity.

**CYP2B6**: In REPs, inhibited enzyme activity with an IC50 of 3.18 µM Walsky, Astuccio et al. (2006).

**CYP3A4**: In HepG2 cells and PHH, 1–50 µM increased mRNA ≤5x and protein by an undefined amount, but in HLM, potently inhibited enzyme activity with K5s of 0.27 and 2.28 µM Novotna et al. (2014b). In Caco-2 cells, 4.7 µM produced a non-significant ~20% decrease in enzyme activity Sergent, Dupont et al. (2009).

**AhR**: Activated and inhibited AhR Novotna et al. (2014a).

**Other CYP Effects:** Inhibited CYP2C19, 11B1, 11B2, 11A1, and 17 Hu and Hartmann (2014). Potential enantiospecific effects were observed on microsomal CYP3A4 enzyme inhibition, but not on PXR agonism or CYP3A4 enzyme induction in the HepG2 cell bioassay Novotna et al. (2014b).

**Permethrin**: 52645-53-1;

- **CYP1A2**: No Effect
- **CYP2B6**: No Effect
- **CYP3A4**: NA

Effect on enzyme activity: Novotna et al. (2014a), in Caco-2 cells, 4.7 µM moderately increased enzyme (EROD) activity (~10 pmol/min/mg protein vs. 0 for the control) Sergent, Dupont et al. (2009), in HepG2 cells, 1–50 µM increased mRNA ≥10x, protein ≥4x and enzyme (EROD) activity ≤3 x Korashy, Shayeganpour et al. (2007).

**CYP1A2**: In PHH, 1–50 µM increased mRNA ≤10x, and protein by an undefined amount Novotna et al. (2014a). In REPS, produced no Yim, Kim et al. (2020) or weak inhibition (~20% at 40 µM and ~50% at 120 µM) Emoto, Murase et al. (2003) of enzyme activity.

**CYP2B6**: In PHH, 100 µM had no effect on mRNA Das et al. (2008b). In REPS, 50 µM had no effect on enzyme activity Usmani, Cho et al. (2006).

**CYP3A4**: In PHH, 100 µM had no effect on mRNA Das et al. (2008b). In REPS, 50 µM inhibited enzyme activity ≤37% Usmani, Cho et al. (2006).

**PXR**: Activated PXR Das et al. (2008b), Yang, Wang et al. (2009).

**Human Exposure Data:** Plasma and urine PK data from volunteers orally dosed with 0.1 mg/kg permethrin Rateles, Côté et al. (2015), urine PK data from volunteers after dermal application National Research Council (1994), and urine PK data from agricultural workers after occupational exposure Ferland, Côté et al. (2015).

**Parathion**: 56-38-2;

- **CYP1A1**: Inducer (~)
- **CYP1A2**: No Effect
- **CYP2B6**: No Effect

Effect on enzyme activity: Novotna et al. (2014a), in HepG2 cells, 100/1000 µM increased mRNA ≤160 x Vrzal, Zenata et al. (2015).

**CYP1A2**: In PHH, 100 µM had no effect on mRNA Das et al. (2008b). In REPS, 50 µM had no effect on enzyme activity Usmani, Cho et al. (2006).

**CYP2B6**: In PHH, 100 µM had no effect on mRNA Das et al. (2008b).

**CYP3A4**: In PHH, in one study, 100 µM had no effect on mRNA Das et al. (2008b), while in another, 10 µM increased mRNA ≤2 x Yang, Wang et al. (2009). In REPS, 50 µM inhibited enzyme activity ≤37% Usmani, Cho et al. (2006).

**PXR**: Activated PXR Das et al. (2008b), Yang, Wang et al. (2009).

**Human Exposure Data:** Plasma and urine PK data from volunteers orally dosed with 0.1 mg/kg permethrin Rateles, Côté et al. (2015), urine PK data from volunteers after dermal application National Research Council (1994), and urine PK data from agricultural workers after occupational exposure Ferland, Côté et al. (2015).

**Parathion**;

- **CYP1A1**: Inducer (~)
- **CYP1A2**: No Effect
- **CYP2B6**: No Effect

Effect on enzyme activity: Novotna et al. (2014a), in HepG2 cells, 100/1000 µM increased mRNA ≤160 x Vrzal, Zenata et al. (2015).

**CYP1A2**: In PHH, 100/1000 µM increased mRNA ≤20 x Vrzal, Zenata et al. (2015). In REPS, inhibited enzyme activity with an IC50 of 0.8 µM Di Consiglio, Meneguz et al. (2005).

**CYP2B6**: In PHH, 100/1000 µM increased mRNA ≤10 x Vrzal, Zenata et al. (2015).

**CYP3A4**: In PHH, 100/1000 µM increased mRNA ≤40x and strongly increased protein Vrzal, Zenata et al. (2015). In REPS, inhibited enzyme activity with an IC50 of 5 µM Di Consiglio, Meneguz et al. (2005).
### TABLE 1 (Continued)

| Chemical name               | CAS number | Structure | Ause molecular weight (Dalton) | Predicted LogP | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, ~ uncertain. Na = No or insufficient data | Supporting evidence from literature publications |
|-----------------------------|------------|-----------|-------------------------------|----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Pyrimethanil;              | 53112-28-0 | ![Pyrimethanil](image) | 199.252; 2.84                 | NA             | Inducer (~) NA NA | AhR, PXR: Activated the AhR and PXR with bell shaped concentration responses Vrzal, Zenata et al. (2015). Human Exposure Data: Urine PK data from volunteers dosed with 0.17 mg/kg/day via the oral and dermal route, from an environmentally exposed general population cohort, and from an occupationally exposed horticulturist cohort Faniband, Ekman et al. (2019), |
| Propropamphos;             | 31218-83-4 | ![Propropamphos](image) | 281.309; 1.61                  | NA             | NA NA NA | Human Exposure Data: Blood and urine PK data from orally and dermally dosed volunteers Garritt, Jones et al. (2002). |
| Tetrabrominated BPA (TBBPA); | 79-94-7 | ![Tetrabrominated BPA](image) | 543.871; 7.29                  | NA             | NA Inducer (~) CYP3A4: In HepG2 cells, 10 µM increased mRNA –3 x Gramec Skledar, Tomasic et al. (2016), AhR, PXR: Activated PXR, and had no effect on AhR Gramec Skledar, Tomasic et al. (2016), Other CYP Effects: After in vivo dosing to rats, there was no significant effect on CYP3A1/3A3, CYP1A1/1A2 and CYP2B mRNA levels and enzyme activities Gerner Piersma et al. (2006), Human Exposure Data: After oral dosing, TBBPA has very low systemic bioavailability in humans and rats Schauer et al. (2006), |
| Prochloraz;                | 67747-09-5 | ![Prochloraz](image) | 376.665; 3.98                  | NA             | No Effect Inducer (~) CYP1A1: In HepaRG cells, 80 µM increased mRNA ~1.6x, and had no effect on protein Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021), CYP1A2: In HepaRG cells, 80 µM reduced protein –0.5 (log2)x [ = ~1.4x] Schmidt, Lichtenstein et al. (2021), CYP2B6: In HepaRG cells, 80 µM had no effect on protein Schmidt, Lichtenstein et al. (2021), CYP3A4: In HepaRG cells, 80 µM increased protein ≤1.6 (log2)x [ ≤3.0x] Braeuning, Mentz et al. (2020), Schmidt, Lichtenstein et al. (2021), Other CYP Effects: Is a potent phenobarbital-type inducer of CYP enzyme activity in rats and mice EFSA (2011), Additional Info: It is an aromatase inhibitor, and has anti-androgenic and anti-estrogenic activity The Danish Centre on Endocrine Disrupters (2020), Its antiandrogenic action is produced by a dual mode: androgen receptor blocking and fetal steroidogenesis inhibition Vinggaard, Haas et al. (2006), |
| Rotenone;                  | 83-79-4   | ![Rotenone](image) | 361.203; 2.79                  | NA             | NA NA NA | Other CYP Effects: Metabolised via CYP3A4 and CYP2C19, but not via CYP2A6, 2C9, 2D6, 2E1 Caboni, Sherer et al. (2004), OECD (2020a), (Continued on following page) |
TABLE 1 | Continued | Part A = Proposed Set of Additional Industrial, Pesticidal, and Food Additive Proficiency Chemicals to Use in Further CYP Induction Validation Experiments. Part B = Initial Candidate Selection Pool Chemicals that were Evaluated but Not Selected. The magnitude of any observed CYP enzyme induction was categorized as low (<3 fold), moderate (>3 to 4.5 fold), and or strong (>4.5 fold), when data from cell-based enzymatic activity assays was available. When cell-based enzymatic data was absent (not tested), the magnitude of an expected effect was categorized as uncertain. Data on induction of mRNA or protein was not used to estimate magnitudes of effect.

| Chemical name | CAS number | structure A | weight (Dalton)* | Predicted LogP* | Expected overall net effect on human CYP enzymatic activity in vitro in HepaRG cells: | Supporting evidence from literature publications |
|---------------|------------|-------------|-----------------|----------------|---------------------------------------------|------------------------------------------------|
| Chlorpyrifos-methyl | 5598-13-0 | ![Chemical Structure](image) | 394.417 | 4.65 | No effect/Inducer/Inhibitor. For Induction Magnitude = + Weak, ++ Moderate, +++ Strong, ~ uncertain. Na = No or insufficient data | Human Exposure Data: Concentrations in biological samples from a fatally poisoned girl De Wilde, Heyndrickx et al. (1986). |
| *Source Chemspider, [www.chemspider.com]. Accessed 14 April 2022. |

**RESULTS AND DISCUSSION**

**Proposed Proficiency Chemical Set to Use in Further Validation Experiments**

Using a targeted literature search and the selection criteria detailed in the methodology section, an initial pool of potential proficiency candidates consisting of a total of 23 chemicals were identified, and a tabular listing of these chemicals which includes summaries of available CYP activity data and chemical structure images are provided in Table 1, Parts A and B. From these candidates, on the basis of the review exercise, the following 13 chemicals were selected to augment the current proficiency chemical list, consisting of pharmaceuticals and to be proposed as additional proficiency chemicals for further validation experiments: tebuconazole, benfuracarb, atrazine, cypermethrin, chlorpyrifos, perfluorooctanoic acid (PFOA), bisphenol A (BPA), N,N-diethyl-m-toluamide (DEET), benzo-[a]-pyrene (B[a]P), fludioxonil, malathion, triclosan and caffeine (See Table 1 Part A). These 13 chemicals have a diverse range of structures and molecular weights and include representative examples of industrial chemicals, pesticides, and food and cosmetics additives. Excepting caffeine, all the chemicals display a degree of lipophilicity (experimental log P ranging from 1.97 to 7.75). Any potential solubility issues will be evaluated and addressed by solubility and cytotoxicity assessment that are part of the planned validation augmentation study design. These data will be reported following completion of the planned experiments.

The selected set contains the following number of expected enzyme inducers: 7 x CYP1A1/1A2 (four low, two strong, one uncertain), 6 x CYP2B6 (two low, two moderate, two uncertain), and 8 x CYP3A4 (four low, two moderate, one strong, one uncertain). These will allow for a sufficient evaluation of the ability of the assay to accurately measure the activity of all four CYP isoforms that are covered by this assay. Additionally, the selected set also contains two expected inhibitors for CYP1A1, CYP2B6, and CYP3A4, which will allow also for an evaluation of the performance of the test method in detecting CYP enzyme inhibitors. For validation experiments, whilst it is considered good practice to include a proficiency chemical set that contains at least 25% of negative chemicals, unfortunately it was not possible to fully meet this criterion for this augmented chemical set, as sufficient data regarding no CYP activity was only available for two of the 23 candidate pool chemicals. Therefore, the augmentation chemical set contains two chemicals that are expected to have no effect on CYP1A1/1A2 activity, but no additional non pharmaceutical chemicals that are expected to have no effect on CYP2B6 or CYP3A4 activity. Following the additional chemical augmentation validation confirmatory testing, the relative potencies of the chemicals in Table 2 will be consolidated.

Validation data for the test method has previously been generated for the 12 pharmaceutical proficiency chemicals shown in Table 2, and in silico chemical space evaluations are reported to demonstrate that the structures of the latter pharmaceuticals are representative of...
### TABLE 2 | Set of Proficiency Pharmaceuticals that have been Evaluated in Experiments Carried out by Bernasconi, Pelkonen et al., 2019 to Validate the In Vitro CYP Induction Assay in Primary Human Hepatocytes and HepaRG cells.

| Chemical name | CAS number | Molecular weight (Dalton)** | Predicted LogP** | Structure | Pharmaceutical class | Evidence for the presence (yes) or lack (no) of human CYP induction activity in vitro or in vivo in clinical studies. Na = No data found/Available | CYP1A1/1A2 | CYP2B6 | CYP3A4 |
|---------------|-------------|----------------------------|-----------------|-----------|---------------------|--------------------------------------------------------------------------------|------------|--------|--------|
|                |             |                            |                 |           |                     | **In Vitro** | **In Vivo** | **In Vitro** | **In Vivo** | **In Vitro** | **In Vivo** | **In Vitro** | **In Vivo** |
| Omeprazole    | 73590-58-6  | 345.416                    | 2.17            | ![Structure](image) | Proton-pump inhibitor | Yes Bernasconi, Pelkonen et al. (2019) | Yes Rost, Brosicke et al. (1994); Rost, Fuhr et al. (1999); Sarich, Kalhorn et al. (1997); Andersson, Rohss et al. (2001); Bernasconi, Pelkonen et al. (2019) | Yes Bernasconi, Pelkonen et al. (2019) | NA | No Bernasconi, Pelkonen et al. (2019) | No Rost, Brosicke et al. (1994); Andersson, Rohss et al. (2001); Zhou, Pant et al. (2018) |
| Carbamazepine | 298-46-4    | 236.269                    | 2.67            | ![Structure](image) | Anticonvulsant | Yes Bernasconi, Pelkonen et al. (2019) | Yes Parker, Pritchard et al. (1998); Lucas, Giffian et al. (1998); Oscarson, Zanger et al. (2006) | Yes Bernasconi, Pelkonen et al. (2019) | Yes Ketter, Jenkins et al. (1995); Ji, Damile et al. (2008) | Yes Bernasconi, Pelkonen et al. (2019) | Yes Bernasconi, Pelkonen et al. (2019) | Yes Moreland, Park et al. (1982); Crawford, Chadwick et al. (1993); Herman, Locatelli et al. (2009); Perucca, Hedges et al. (2004) |
| Phenytoin     | 630-93-3    | 274.250                    | 2.29            | ![Structure](image) | Anticonvulsant | Yes Bernasconi, Pelkonen et al. (2019) | Yes Weitholtz, Zyset al. (1989); Miller, Coggriff et al. (1984) | Yes Bernasconi, Pelkonen et al. (2019) | Yes Slattery, Kalhorn et al. (1996); Williams, Wainer et al. (1999) | Yes Bernasconi, Pelkonen et al. (2019) | Yes Bernasconi, Pelkonen et al. (2019) | Yes Werk, Macgeo et al. (1964); Crawford, Chadwick et al. (1990) |
| Penicillin G sodium | 69-57-8 | 356.372                     | 1.67            | ![Structure](image) | Antibiotic | No Bernasconi, Pelkonen et al. (2019) | NA | No Bernasconi, Pelkonen et al. (2019) | No Bernasconi, Pelkonen et al. (2019) | NA | NA |
| Rifabutin     | 72559-06-9  | 847                        | 3.45            | ![Structure](image) | Antimicrobial | Yes Bernasconi, Pelkonen et al. (2019) | No Gillum, Seiler et al. (1996) | Yes Bernasconi, Pelkonen et al. (2019) | NA | Yes Bernasconi, Pelkonen et al. (2019), Horita and Doi (2014) | Yes Barditch-Crovo, Trapnell et al. (1999) |
| Sulfinpyrazone| 57-96-5     | 404.482                    | 2.3             | ![Structure](image) | Uricosuric | Yes Bernasconi, Pelkonen et al. (2019) | Yes Birkett, Miners et al. (1983) | Yes Bernasconi, Pelkonen et al. (2019) | NA | Yes Birkett, Miners et al. (1983); Wing, Miners et al. (1985); Walter, Staiger et al. (1982); Staiger, Schlicht et al. (1983) |
| Bosentan hydrate | 157212-55-0 | 569.629                    | 1.15           | ![Structure](image) | Endothelin Receptor Antagonist | Yes Bernasconi, Pelkonen et al. (2019) | NA | Yes Bernasconi, Pelkonen et al. (2019) | NA | Yes Bernasconi, Pelkonen et al. (2019) | Yes Weber et al. (1999a); Weber et al. (1999b); van Giersbergen et al. (2002a) |

(Continued on following page)
EU REACH (https://echa.europa.eu/regulations/reach/legislation), Drugbank, and Tox21 listed chemicals (Bernasconi, Pelkonen et al., 2019). Overall, the total set of 25 proficiency chemicals (including the 12 pharmaceuticals and the proposed 13 additional industrial, pesticidal, and food additive chemicals) is considered to be sufficiently diverse and representative of OECD TG Programme relevant chemical classes to facilitate draft TG approval at the OECD.

### TABLE 2 (Continued) Set of Proficiency Pharmaceutical Chemicals that have been Evaluated in Experiments Carried out by Bernasconi, Pelkonen et al., 2019 to Validate the In Vitro CYP Induction Assay in Primary Human Hepatocytes and HepaRG cells.

| Chemical name | CAS number | Structure | Pharmaceutical class | Evidence for the presence (yes) or lack (no) of human CYP induction activity in vitro or in vivo in clinical studies. Na = No data found/Available | CYP1A1/1A2 | CYP2B6 | CYP3A4 |
|---------------|------------|-----------|----------------------|-----------------------------------------------------------------------------------------------------------------------------------|-----------|--------|--------|
|               |            |           |                      | In Vitro In Vivo In Vitro In Vivo In Vitro In Vivo In Vitro In Vivo In Vitro In Vivo |
| Artemisinin   | 63968-64-9 | Anti-malarial | No | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes/No<sup>10,h</sup> |
|               | 282.332    |           |                     | NA                                                                  | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) |
| Efavirenz     | 154598-52-4A | Anti-retroviral | No | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) |
|               | 315.675    |           |                     | NA                                                                  | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) |
| Rifampicin    | 13292-48-1 | Antibiotic | Yes | Bernasconi, Pelkonen et al. (2019)<sup>b</sup> | Yes | Robson, Miners et al. (1984); Wietholtz, Zysset et al. (1995); Backman, Granfors et al. (2006) | Yes | Lopez-Cortes, Ruiz-Valdenas et al. (2002); Loboz, Gross et al. (2006) | Yes | Bernasconi, Pelkonen et al. (2019) |
|               | 822.940    |           |                     | Bernasconi, Pelkonen et al. (2019)<sup>b</sup> | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) | Yes | Bernasconi, Pelkonen et al. (2019) |
| Metoprolol    | 51394-51-1 | β<sub>1</sub> receptor blocker | No | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> | No | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> | No | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> | No | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> |
|               | 267.364    |           |                     | NA                                                                  | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> | NA | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> | NA | Bernasconi, Pelkonen et al. (2019)<sup>e</sup> |
| Sotalol       | 959-24-0   | Non-selective β<sub>1</sub> receptor blocker | No | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> | No | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> | No | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> | No | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> |
|               | 272.364    |           |                     | NA<sup>1</sup>                                                        | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> | NA<sup>1</sup> | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> | NA<sup>1</sup> | Bernasconi, Pelkonen et al. (2019)<sup>f</sup> |

<sup>a</sup>Source Chemspider, (www.chemspider.com). Accessed 14 April 2022.

<sup>b</sup>For CYP1A2, carbamazepine, sulfonpyrazone, bosentan, and rifampicin were positive in HepaRG, cells and negative in PHH, cells.

<sup>c</sup>For CYP2B6, omeprazole was positive in PHH, cells, and negative in HepaRG, cells.

<sup>d</sup>For CYP3A4, artemisinin was positive in PHH, cells, and negative in HepaRG, cells.

<sup>e</sup>A small fraction of metoprolol is metabolised in vitro by CYP3A4, CYP2B6 and CYP2C9 (Berger, Bachmann et al., 2018), but there is no evidence for CYP, induction.

<sup>f</sup>Sotalol is considered unlikely to induce CYP, enzymes, due to its PK characteristics (Yanreudeewong, DeBisschop et al., 2003).

<sup>g</sup>Omeprazole induced CYP1A2 in humans, but only at high non-clinically relevant doses.

<sup>h</sup>For artemisinin, CYP3A4 induction was detected in Asimus, Elsnerbiny et al., 2007; while in Svensson, Ashton et al., 1998 no induction was observed.
Applications of In Vitro Metabolism Data Including CYP Induction Data in Chemical Hazard Assessment: Meeting the outstanding needs to achieve OECD test method adoption

OECD Human in vitro Metabolism Test Method Development Needs

PK, including absorption, distribution, metabolism, and excretion (ADME) play a key role in determining in vivo exposure to a parent chemical and its metabolites after dosing, and PK data is used extensively in the design and interpretation of toxicological assessments of test chemicals. Currently, toxicokinetic ADME data for single and repeated dose in vivo studies (OECD 2010) is commonly generated and used in Europe for high tonnage industrial chemicals and for pesticides and worldwide for pharmaceuticals (ICH S3A, 1994). Relevant data from several in vitro ADME assays, including, for example, Caco-2 cell permeability assays measuring absorption potential; transporter protein substrate/inhibition assays which provide data on distribution, excretion, and PK interactions; S9 and microsome addition and in vitro metabolism assay data can contribute on a weight of evidence basis to the toxicological hazard assessment. However, when the data is generated according to an accepted OECD TG as part of the Mutual Acceptance of Data agreement, this data can be submitted without additional testing to many OECD regulatory jurisdictions. The process of validation is intended to establish the relevance, reproducibility and reliability of a test method for a specific regulatory purpose (OECD 2005) and gives much greater confidence in the reliability of the test data generated.

That there is an urgent need to develop new OECD TGs for these in vitro test method is well established at the OECD (Jacobs, Janssens et al., 2008; OECD 2008; Jacobs, Laws et al., 2013; Bernasconi, Pelkonen et al., 2019), as data from these assays could be used alongside standard in vivo PK data in a complimentary fashion to aid toxicological assessments. Importantly, several quantitative in vitro to in vivo extrapolation (QIVIVE) and in silico physiology-based pharmacokinetic (PBPK) models have been shown to be capable of accurately predicting in vivo PK parameters from in vitro data (OECD, 2021; Tsaioun, Blaauwbe, et al., 2016), and it is hoped that in the future QIVIVE and PBPK approaches could replace certain types of in vivo PK data, which would allow for a significant reduction in animal use. With regards to metabolism, the availability of additional validated in vitro assays capable of generating human relevant metabolic profiling and CYP induction/inhibition data would be particularly valuable. Animal relevant metabolic profile data from non-human in vivo studies is often available. However, to date, there are no adopted TGs available for in vitro test method that produce human metabolic profile data, which hampers efforts focused on determining the relevance of animal metabolite data to humans. Moreover, for results from in vitro toxicity assays to accurately predict potential in vivo toxicity, it is essential that the concentrations of parent chemical/metabolites that are tested in vitro are representative of in vivo levels, and, for this reason, metabolic transformation steps are included in many in vitro toxicity assays, including all of the OECD TG in vitro genotoxicity test method. At the moment, there are, however, a number of in vitro OECD TGs that lack appropriate (pre-) incubation steps to account for in vivo metabolism, including, for example, all of the Level 2 in vitro mechanistic human cell based test method specified in the current OECD Endocrine Disruptor Guidance Document 150 (OECD 2018). Moreover, for many chemicals, there are significant differences in metabolism between rats and humans, and only rat S9 microsomes are used to produce metabolic transformation in the OECD genotoxicity TGs. In a recent European Food Safety Authority (EFSA) scientific panel opinion paper relating to the toxicological testing of pesticides, the use of in vitro human metabolite data is recommended to identify any potential human relevant metabolites that had not been adequately tested in non-human toxicological studies (EFSA 2021).

Applications of in vitro Human CYP Induction Data in Chemical Hazard Assessment

CYP induction data can indicate whether and to what extent a chemical is likely to undergo CYP-mediated metabolism, and results showing significant CYP induction could be used as an indicator that (pre-) incubation steps to account for in vivo metabolism should be included in any toxicity assays lacking metabolic competence. In relevant situations, CYP induction and inhibition data could facilitate the selection of optimal in vivo test chemical doses for human and other animal studies, and also indicate the possible involvement of CYP metabolism/metabolites in adverse or PK effects. Perturbations in the levels of endogenous chemicals that are metabolised by CYPs are associated with several adverse effects, and CYP induction data could also be used to support the contribution of CYP-mediated mechanisms in adverse outcome pathways and IATAs as shown in Figure 1B. Furthermore, the performance of a number of QIVIVE/PBPK models and PK databases such as MetaPath (Kolaczzyk, Schmieder et al., 2012) would be substantially expanded by the incorporation of available CYP induction data. It would be particularly beneficial to generate CYP data for food chemical classes for example, as in Europe, little mammalian in vivo data is available for these.

Moreover, CYP induction and inhibition data are currently commonly used to predict potential CYP mediated clinical PK drug interactions for pharmaceuticals (EMA 2012; US FDA, 2020) and the availability and use of a validated and adopted OECD CYP induction test method, that will fall under the Mutual Acceptance of Data agreement, will therefore also be valuable for drug discovery and regulatory application for pharmaceutical CYP drug interaction evaluations.

Examples of Applications of in vitro Human CYP Induction Data for IATAs

Relevant in vitro metabolism data would greatly benefit several specific IATAs, including the developmental...
FIGURE 1 | Selected illustrations of regulatory applications of in vitro metabolism test method/systems falling within the OECD TG Programme. panel (A) Introduction of metabolism in vitro testing at Level 2 of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals (EDCs).

FIGURE 1 | panel (B) Metabolic activation or deactivation of a chemical, generation of and positioning of CYP induction data in an Integrated Approach for Testing and Assessment of Non Genotoxic Carcinogens.
neurotoxic carcinogenicity (Jacobs, Colacci et al., 2020) and non-genotoxic carcinogenicity (Jacobs, Colacci et al., 2020) IATAs currently under development, and the OECD Conceptual Framework for Endocrine Disruptors (updated OECD 2018). With respect to the latter, as proposed in 2013 (Jacobs, Laws et al., 2013), in vitro metabolism test method can be added at level 2 of the Endocrine Disruptor Conceptual Framework as shown in Figure 1A,B below, but will also have great utility in informing all levels of the Conceptual Framework. This will begin to accommodate the additional in vitro assay flexibility needs for the regulatory identification of endocrine disruptors (Solecki, Kortenkamp et al., 2017), by filling the metabolism translational gap between in vitro level 2 assays and the WHO definition of endocrine disruptor, as “an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub) populations”. Quoting from the consensus paper ‘a) Alterations of the function of the endocrine system may arise from interaction with hormone receptors, changes in circulating levels of the hormone, and from the impact of chemical(s) on hormone synthesis, transport, metabolism and other factors’. c) The term “intact organism” is understood to mean that the effect would occur in vivo, either observable in a test animal system, epidemiologically or clinically. However, it does not necessarily mean that the adverse effect has to be demonstrated in an intact test animal, but may be shown in adequately validated alternative test systems predictive of adverse effects in humans and/or wildlife” (Solecki, Kortenkamp et al., 2017).

In addition to mediating detoxification, CAR, PXR and AhR have been implicated in the regulation of a broader range of physiological functions (Kretschmer and Baldwin 2005; Wang and Tompkins 2008; Yi, Fashe et al., 2020), where dysregulation can lead to adverse effects (Hakkola, Bernasconi et al., 2018) and receptor and CYP induction have well documented roles for instance in inflammation (Christmas, 2015; Rubin, Janefeldt et al., 2015), cholesterol, steatosis (Gomez-Lechon, Jover et al., 2009), hepatotoxicity (Woolbright and Gomez-Lechon, Jover et al., 2009), by in vitro assay flexibility needs for the regulatory identification of endocrine disruptors (Solecki, Kortenkamp et al., 2017), by filling the metabolism translational gap between in vitro level 2 assays and the WHO definition of endocrine disruptor, as “an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub) populations”. Quoting from the consensus paper ‘a) Alterations of the function of the endocrine system may arise from interaction with hormone receptors, changes in circulating levels of the hormone, and from the impact of chemical(s) on hormone synthesis, transport, metabolism and other factors’. c) The term “intact organism” is understood to mean that the effect would occur in vivo, either observable in a test animal system, epidemiologically or clinically. However, it does not necessarily mean that the adverse effect has to be demonstrated in an intact test animal, but may be shown in adequately validated alternative test systems predictive of adverse effects in humans and/or wildlife” (Solecki, Kortenkamp et al., 2017).

Potential of the HepaRG CYP System to Be Further Developed for Additional Metabolism Relevant Endpoints

Following successful adoption and application of the HepaRG CYP enzyme induction test method, it would be useful to develop complementary components of in vitro human metabolism systems into OECD TGs, including induction/inhibition assays for additional CYP isoforms, and assays for Phase II metabolism, metabolite profiling, and metabolic transformation pre-incubation steps (as discussed above). The use of human microsomes and/or S9 mixes, or cryopreserved primary human hepatocytes (PHH) have utility in research and drug discovery, but for chemical hazard assessment purposes, cryopreserved PHH with very wide ranging variability in responses, had reproducibility issues in a validation exercise (Bernasconi, Pelkonen et al., 2019), and following OECD peer review, were considered too

Further Developed for Additional Metabolism Relevant Endpoints

Following successful adoption and application of the HepaRG CYP enzyme induction test method, it would be useful to develop complementary components of in vitro human metabolism systems into OECD TGs, including induction/inhibition assays for additional CYP isoforms, and assays for Phase II metabolism, metabolite profiling, and metabolic transformation pre-incubation steps (as discussed above). The use of human microsomes and/or S9 mixes, or cryopreserved primary human hepatocytes (PHH) have utility in research and drug discovery, but for chemical hazard assessment purposes, cryopreserved PHH with very wide ranging variability in responses, had reproducibility issues in a validation exercise (Bernasconi, Pelkonen et al., 2019), and following OECD peer review, were considered too
variable for TG development. There are also (unknown) viral transmission concerns with the use of primary human tissues in routine chemical testing.

The HepaRG CYP enzyme induction test method was agreed by the OECD member countries to be the best (longer term) option to take forward for regulatory use in 2008 (Jacobs, Janssens et al., 2008; OECD 2008; Jacobs, Laws et al., 2013), and following successful validation with pharmaceuticals (Bernasconi, Pelkonen et al., 2019) it is the most ready and reliable system available, and superior in validation performance to cryopreserved hepatocytes. A variety of additional non validated in vitro human liver on a chip and 3D liver organoid systems are currently in development, but the complexity, between batch variability, and lack of validation data for these models (Telles-Silva, Pacheco et al., 2022) mean that they are not ready for test guideline development at present. However available literature evidence indicates that HepaRG cells are well suited to all of these applications, as mRNA for many key proteins involved in xenobiatic metabolism are expressed in differentiated HepaRG cells, including xenobiotic sensing nuclear receptors (AhR, PXR, CAR, and PPAR-α), CYPs (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7), other Phase I metabolic enzymes [including various isoforms of alcohol dehydrogenase, aldehyde dehydrogenase, and flavin-containing monoxygenase], and Phase II metabolic enzymes [including various isoforms of glutathione S-transferase, UDP-glucuronosyltransferase, N-acetyltransferase, and sulfortransferase] (Aninat, Piton et al., 2006; Guillouzo, Corlu et al., 2007; Josse, Aninat et al., 2008; Antherieu, Chesne et al., 2010; Hart, Li et al., 2010). Enzymatic activity for CYP1A1/1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 has also been confirmed to be present (Aninat, Piton et al., 2006; Josse, Aninat et al., 2008; Antherieu, Chesne et al., 2010; Lubberstedt, Muller-Vieira et al., 2011). Furthermore, chemical-induced induction and inhibition of CYP1A1/1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 has been demonstrated in HepaRG cells (Aninat, Piton et al., 2006; Josse, Aninat et al., 2008; Kanebratt and Andersson 2008; Turpeinen, Tolonen et al., 2009; Antherieu, Chesne et al., 2010; Yajima, Uno et al., 2014). The metabolic profile data generated for several chemicals in HepaRG cells have also been shown to be equivalent to that produced using PHH, including aflatoxin B1 and acetaminophen (Aninat, Piton et al., 2006) but without the inherent biological variability observed with different batches of PHH. Intrinsic clearance values generated for a large number of reference drugs in HepaRG cells have also been shown to be equivalent to the values generated in PHH (Lubberstedt, Muller-Vieira et al., 2011; Zanelli, Caradonna et al., 2012). In the future a potentially promising approach to explore would therefore be to develop the HepaRG cell system into an all-in-one system/assay providing all the desired abovementioned in vitro metabolic functionalities.

For now however, it is really important to first address the outstanding steps required to enable the successful adoption of the HepaRG CYP enzyme induction test method as a TG, and the work described herein provides the essential concrete chemical selection and applications step required to enable international TG progress within an immediate timeframe. Following the planned additional validation experiments with this candidate chemical selection list there will be an evidence basis upon which to refine the chemical list for use as additional proficiency chemicals for the HepaRG CYP enzyme induction test method, and to do any further analyses that may be warranted.

4 CONCLUSION

Overall, it is apparent that the availability of CYP induction data would significantly aid the toxicological assessment of chemicals, and our ongoing work to augment the chemical applicability domain as an extension to the validation of the CYP enzyme induction HepaRG test method is a requirement for the (near) future approval of this test method, at the OECD.

Here we have also given some examples of immediate applications of the assay for the OECD Conceptual Framework for Endocrine Disruptors, and for the OECD IATA for non-genotoxic carcinogens. There will also be necessary applications to other complex human health IATAs, including that for thyroid disruption (OECD, 2014), and metabolic disruption (Legler, Zalko et al., 2020).

AUTHOR CONTRIBUTIONS

MNJ conceived, instigated and developed the project, discussion and acquired funding. BK expanded the original database, which was then supplemented by EB, both of whom also contributed to the discussion. All authors contributed to manuscript drafting, revision, and read and approved the submitted version.

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