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The usefulness of *in vitro* mechanistic information in regulatory dossiers: the case of the CAR mode of action in carcinogenicity assessment

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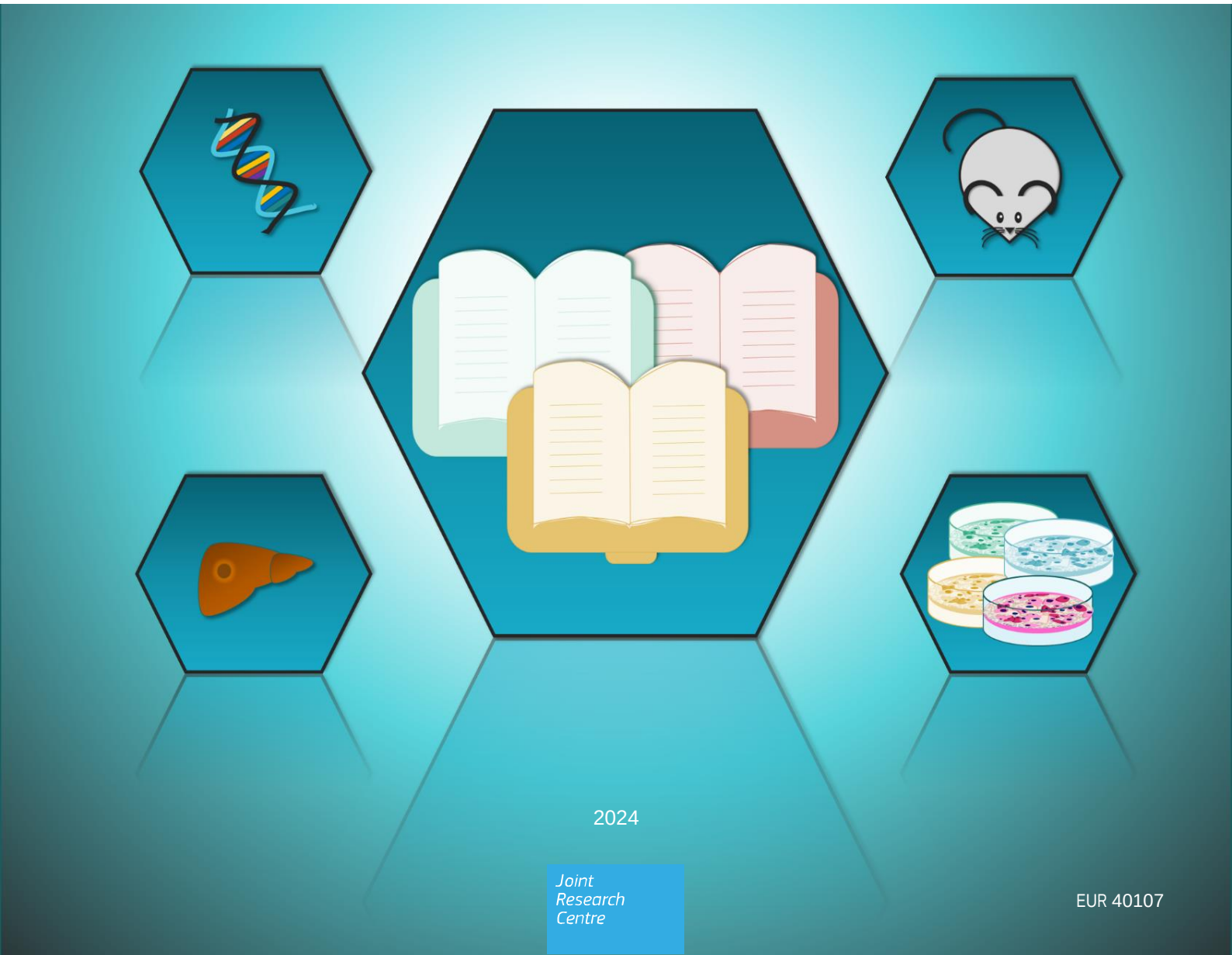

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Contents

Abstract

Data on mode of action (MoA) of rodent tumour formation have become an integral part of the information submitted in regulatory dossiers for some chemical substances registration or re-registration. Despite this, there is a lack of guidance or standardised approaches for addressing such results through evidence-based follow-up studies in dossiers within the agrochemical and chemical sectors. This instigates companies to proactively conduct extensive studies on vertebrates (including wild-type and transgenic mice and rats), which contradicts the principles of the 3Rs (Replacement, Reduction, and Refinement). Therefore, it is of general interest adhering to non-animal approaches and reducing reliance on animal studies, whenever possible. To better understand the role that *in vitro* mechanistic studies already can play in decision-making we conducted an analysis focusing on one of the most common MoAs involved in rodent liver carcinogenicity: the constitutive androstane receptor (CAR) MoA, which accounts for about 25% of tumours induced in rodents by agrochemicals. We collected information from 36 harmonised classification and labelling (CLH) dossiers on the actual use and weight of *in vitro* mechanistic data used to evaluate positive results from rodent cancer bioassays. The analysis showed that *in vitro* mechanistic studies play a relevant role in the weight of evidence evaluation to elucidate the human relevance of the CAR MoA in regulatory decision-making. Consequently, this information can give important hints on recommendations for *in vitro* mechanistic studies harmonisation in this specific regulatory field.

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1 Introduction

Long-term bioassays involving rats and mice, which are required by regulatory authorities for cancer risk assessment and marketing approval of agrochemicals, are limited in predicting the actual human relevance of detected tumours. Non-guideline short-term *in vivo* and/or *in vitro* mechanistic studies are conducted in an attempt to identify non-genotoxic modes of action (MoA) and to shed light into human relevance. However, due to the lack of regulatory guidance in methodological strategies for MoA assessment, a large amount of data is being generated without confidence in whether it will be considered by the regulatory authorities. Therefore, there is an urgent need to understand the reasons that might hinder the regulatory uptake of *in vitro* mechanistic data by exploring real cases when these data are submitted for regulatory assessments. Understanding the motivation for accepting or excluding data from non-animal approaches used in the harmonised classification and labelling (CLH) dossiers from agrochemicals, biocides and industrial chemicals, could contribute to recommendations for harmonisation and subsequently build confidence in the use of non-animal approaches in regulation.

In this study, we explored the European Chemicals Agency (ECHA) Risk Assessment Committee (RAC) approach and consistency when applying weight of evidence for scientific evidence-based studies (*in vivo* and/or *in vitro* studies), focusing on non-genotoxic MoA for rodent liver tumours. In parallel, we evaluated how the RAC is already using non-animal data to conclude on classification, including the human relevance of the findings. Finally, we investigated whether CAR MoA can be predicted in shorter-term mechanistic studies and how much weight *in vitro* mechanistic studies have in the overall assessment.

At regulatory level, long-term rodent studies are the golden standards to assess the potential carcinogenicity of chemical substances (Suarez-Torres et al., 2021). Liver is a common target organ for these substances, which makes liver tumour formation the most common in rodents (Thoolen et al., 2010). The human relevance of these tumours has been widely discussed and clear conclusions about interspecies and/or dose extrapolation after these expensive and time-consuming studies is not granted (Cohen et al., 2019). Mechanistic studies intended to clarify MoAs are used in regulatory dossiers to help verify that identified tumours in rodents, induced by non-genotoxic carcinogens, are not human relevant.

1.1 Guidelines and guidance overview for carcinogenicity and MoA evaluation

In the European Union (EU), data requirements for authorization and/or registration of substances are usually chemical sector specific (Table 1). However, the long-term *in vivo* bioassay for carcinogenicity evaluation is common to almost all legislations. The currently used test methods include the carcinogenicity OECD test guideline (TG) 451 and the combined chronic toxicity/carcinogenicity TG 453. Moreover, chronic toxicity TG 452 can in some cases also be used to detect early neoplastic lesions. The duration of these studies is usually between 12 to 24 months and they involve the use of a high number of animals per study (4-65/sex/group) (Madia et al., 2019). The fact that these long-term studies have been used for decades does not make them flawless. Long-term rodent bioassays are very costly and

time-consuming, involve testing and sacrificing a high number of animals, and are poorly reproducible (Paparella et al., 2017). Additionally, there is an increasing awareness that the results from rodents long-term bioassays are often not predictive of their actual human relevance (Goodman, 2018).

Nevertheless, no TG is available regarding methods for chemicals MoA identification in carcinogenicity, although understanding the MoA of chemicals is fundamental for their classification, especially in the context of agrochemicals and biocides. In this regard, ECHA Guidance on Classification Labelling and Packaging (CLP) (ECHA, 2024), on the Biocidal Products Regulation (ECHA, 2022f, ECHA, 2017b) and on information requirements and chemical safety assessment (ECHA, 2017a), highlight the importance of *in vivo* and *in vitro* mechanistic studies to elucidate MoA in the context of risk assessment and explain how to take them into account to evaluate human relevance. All of them advise to use as support the IPSC Conceptual Framework, which guides critical data collection and its respective organisation (Boobis et al., 2006, Sonich-Mullin et al., 2001, Meek et al., 2003). Yet, the selection of methods and models to produce that data or even the necessary proof of evidence to submit to the authorities as part of a chemical registration dossier extensively depends on the dossier submitter. Consequently, this results in a collection of not harmonised mechanistic studies making difficult the assessment of the MoA data, especially when the assessor is less familiar with the biological plausibility and the weight of evidence in this specific field. This also applies to the CAR MoA, despite two publications aimed to support the dossier submitters by providing a list of minimum data set and possible methods to generate this data based on the current state of the science at the time of the publication and taking into account alternative emerging technologies (Peffer et al., 2018, Elcombe et al., 2014).

Table 1 - EU regulatory data requirements for the carcinogenicity assessment.

Legislation	Chemical Sector	Data requirements	EU Agency responsible for Regulation implementation
EC 1107/2009 (EC 283/2013)	Plant Protection Products (PPP)	Standard 2-year bioassay or combined study in two species, always.	European Food Safety Agency (EFSA)
EU 528/2012	Biocidal Products (BPR)	Standard 2-year bioassay in two species, always. • Unless mutagen Cat 1A or 1B	European Chemical Agency (ECHA)
EC 1907/2006 *(76/769/EEC)*	Industrial chemicals	Standard 2-year bioassay in rodents, when: • Tonnage: > 1000 tns/year and; • Long-term exposition and; • Widespread dispersive use and; • If mutagens Category 2 or; • Hyperplasia or pre-neoplastic lesions from repeat-dose studies	ECHA
Directive 2001/83/EC	Human medicinal Products	Standard 2-year bioassay in rat and 18-month study in mouse or transgenic mouse models	European medicines agency (EMA)

Legislation	Chemical Sector	Data requirements	EU Agency responsible for Regulation implementation
		• Applies to medicinal products with a duration of treatment is continuous for at least 6 months or when is recurrent but for a chronic or recurrent condition. - Weight of evidence approach based on scientific reasoning factors such as target biology, secondary pharmacology, histopathology chronic studies, hormonal effects, genotoxicity and immune modulation.	
Regulation (EU) 2019/6	Veterinary Medicinal Products	Standard 2-year bioassay in rat and an 18-month study in mouse. • With appropriate scientific justification, carcinogenicity studies may be carried out in one rodent species, preferably the rat.	EMA

1.2 CLH Dossier evaluation and RAC opinion process

Adequate risk management of chemicals is performed on a case-by-case basis, throughout the EU by the CLH. The CLP Regulation ((EC) No 1272/2008) is based on the United Nations' Globally Harmonised System (GHS) and it was created to ensure a high level of protection of human health and the environment, as well as the free movement of substances, mixtures and articles.

In the case an active substance is placed on the market or there is evidence that some substances need to be re-evaluated due to emerging hazard concerns (e.g. availability of new information, new scientific or technical developments), manufacturers, importers or downstream users have to (self)classify and label hazardous substances and mixtures to ensure its safety use. Hazards of the highest concern are those with potential carcinogenicity, mutagenicity, reproductive toxicity (CMR), endocrine disruption and respiratory sensitisation. In principle, in the cases where active substances are used as plant protection products and biocidal products, they need to undergo harmonised classification for these hazard classes (Regulation 1107/(2009) and Regulation (EU) No 528/(2012)). The approval of these active substances initially takes place at the EU level. Subsequently, they are authorised at the Member State level. This process ensures a higher level of protection for human health and the environment (Bourguignon, 2017).

To date, a Member State competent authority (MSCA), or a manufacturer, importer and downstream user of a substance can submit CLH proposals to ECHA. This is done in situations when a substance is a CMR or a respiratory sensitiser, when it is justified that a classification for a substance at EU level is needed for other hazard classes, or to add one or more new hazard classes to an existing entry (according to the conditions above). It is worth to notice that only MSCAs may propose a revision of an existing harmonised entry, for any substance that is under the scope of the CLP Regulation (ECHA, 2024).

The CLH process includes several steps and it involves different parties with defined and distinct roles (Table 2 and Figure 1). The process begins from the moment ECHA receives an intention to prepare a CLH dossier. The party proposing classification and labelling (Dossier Submitter (DS)) provides pertinent information according to Part II of Annex VI to the CLP Regulation supporting the proposal and contributes for a comparison based on the classification criteria. The dossier should contain all relevant detailed study summaries of available information, and the DS has the obligation to consider REACH registration dossiers in their CLH proposals, or any relevant information produced for risk assessments of active substances in plant protection products (DAR) and/or biocidal products (CAR). Any planned studies are also reported to enable a decision whether results need to be considered for a final opinion on the CLH (ECHA, 2022e).

Following the substance identity check and ECHA's publication in the Registry of Intentions, the dossier is submitted and an accordance check is introduced. This step is crucial to ensure that the CLH dossier is prepared and formatted in agreement with the requirements described in the legal text as defined in the ECHA guidance. If the dossier is in compliance, ECHA will start the public consultation of the proposed CLH dossier as presented in the CLH report, on its website. During public consultation, which lasts around 60 days, parties concerned are invited to comment on the hazard classes included in the dossier based on the provided data. At this point, they can flag ongoing studies and if the results are available within reasonable time (e.g. a few months), it can be agreed that this data should be included. Comments are forwarded to the DS with the invitation to respond. Following that, the dossier is evaluated by RAC. The committee's primary role is to assess the proposal and adopt an opinion, considering all the information provided during the submission and the public consultation. After adoption, the opinion, the background document and the response to comments (RCOM) table are published in ECHA's website. In parallel, ECHA sends the RAC opinion together with its annexes to the European Commission for its final decision (ECHA, 2022d). The background document includes the CLH report and RAC evaluations, while the RCOM table contains the collected comments received during the consultation and the responses by the DS and RAC. In some cases, the harmonised classification proposed by the RAC can be different than the classification proposed by the DS. In special cases such as assessment of agrochemicals, ECHA will coordinate with EFSA, including on communication aspects, when the timelines for a substance which is in both the CLH and EFSA processes overlap (ECHA, 2022e).

Figure 1 - RAC opinion process and CLH process adapted from "Framework for RAC opinion development on substances, for Harmonised Classification & Labelling, ECHA, 2022".

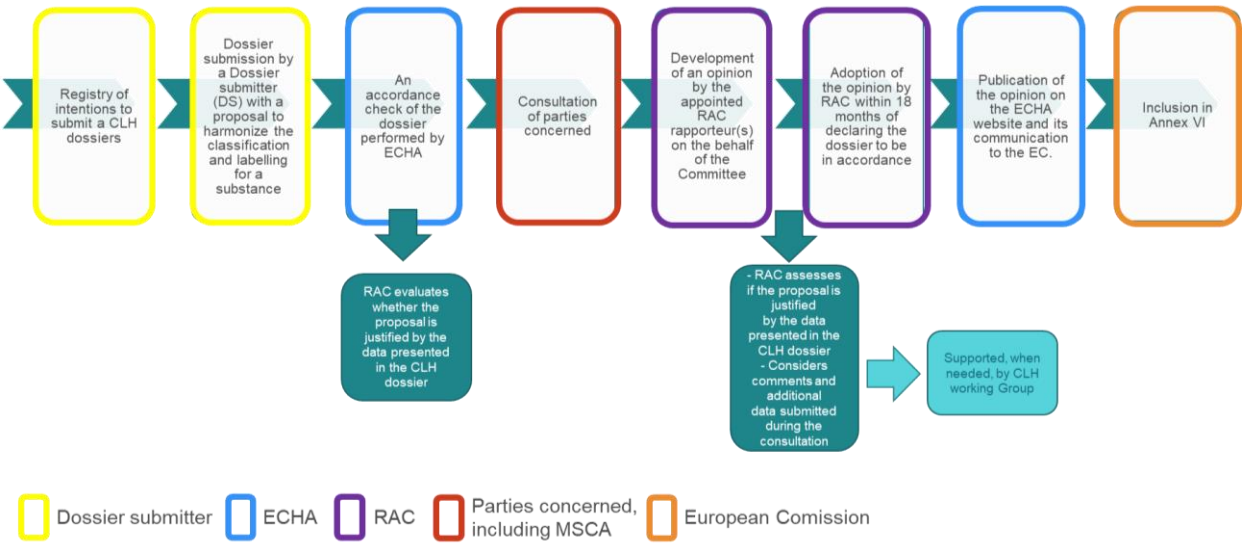


Table 2 – Roles and responsibilities of the different parties for RAC opinion development on substances for harmonised classification.

Party	Role	Institution
Dossier Submitter	- To ensure the compliance of the CLH dossier with the legal and scientific requirements; - Responsible for collecting and presenting the administrative, scientific and technical information for the proposed classification in the CLH dossier; - To respond to any comments received during the consultation.	MSCA, manufacturers, importers or downstream users of a substance
ECHA Secretariat	- To provide technical, scientific and administrative support for the Committees and ensure appropriate coordination between them. - To handle the dossiers.	ECHA
RAC	- To assess and adopt an opinion on the proposal; - To ensure that relevant information submitted by the DS or any party during the consultation is taken into account.	ECHA
RAC (co-)rapporteur	- The (co-)rapporteur is responsible for drafting, co-ordinating with the co-rapporteur and any members appointed by RAC in an ad hoc capacity to support the development of the opinion; - Required to present their opinion on the classification proposal to RAC, with the support of the ECHA Secretariat.	ECHA's committee member
Parties concerned	- To comment on any proposal for CLH, inclusive on data provided via the consultation; - Additional targeted consultation on a case-by-case basis.	Industry, Academia, NGOs, European Agencies, MSCAs, general public, etc

Party	Role	Institution
European Commission	- Entitled to attend RAC meeting as observers; - Decides whether the CLH of the substance concerned should be included in Annex VI of the CLP Regulation.	EC

1.3 MoA for liver tumours

The development of hepatic tumours in rodents after long-term exposure to chemical substances is a common adverse effect that has been markedly explored. Multiple MoAs have been reported for liver tumour formation, which can be related either to genotoxic or to non-genotoxic effects. In recent decades, frameworks for identifying well-established mechanisms of action by which certain chemicals can induce liver tumours have been proposed (Sonich-Mullin et al., 2001, Holsapple et al., 2006, Boobis et al., 2008, Meek et al., 2003, Meek et al., 2014).

Regarding non-genotoxic rodent liver carcinogens, the most commonly proposed MoAs include constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPARα) and aryl hydrocarbon receptor (AhR) activation, as well as cytotoxicity, hormonal perturbation, immunosuppression and porphyria (Boobis et al., 2008, Boobis et al., 2006, Holsapple et al., 2006, Klaunig et al., 2012, Meek et al., 2003). In liver tumours, activation of CAR, PXR, PPARα and AhR receptors actually enhance the transcription of xenobiotic-sensing transcriptional factors that induce hepatic microsomal P450 (CYP) enzymes, particularly CYP1A, CYP2B, CYP3A and CYP4A (Dickins, 2004, Omiecinski et al., 2011, Stanley et al., 2006, Yoshinari et al., 2008).

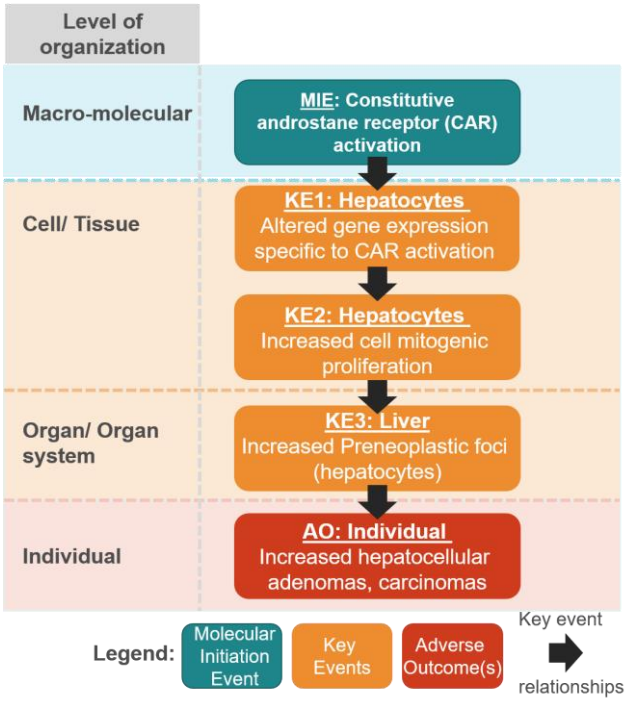
The activation of the CAR/PXR-mediated cascade is the most prevalent MoA among agrochemicals substances. This was demonstrated in an extensive analysis of data from regulatory dossiers, aiming at identifying the various mechanisms of action underlying the non-genotoxic carcinogenic potential of agrochemical active substances (Heusinkveld et al., 2020). This analysis reported that 112 out of 225 treatment-induced tumours, with identifiable MoA in rodents, were related to CAR/PXR activation, with more than half of these tumours occurring in the liver (58 tumours).

1.4 CAR MoA

The constitutive androstane receptor (CAR, NR1I3), part of the nuclear receptor superfamily, is primarily expressed in the liver and plays a crucial role in metabolising endogenous and xenobiotic substances by regulating target genes (Baes et al., 1994, Qatanani et al., 2005, Wei et al., 2000). CAR activation upregulates numerous xenobiotic metabolising enzymes, including phase I (e.g., CYP2B subfamily), phase II (e.g., glutathione S-transferases), and phase III transporters (e.g., MRP4) (Assem et al., 2004, Ueda et al., 2002). CAR activators include industrial chemicals, endogenous substances, insecticides, and therapeutic drugs, such as phenobarbital, which activates CAR by a ligand-independent mechanism (Maglich et al., 2003, Baes et al., 1994, Tzameli et al., 2000, Parkinson et al., 2006). Phenobarbital, used clinically since the early 20th century, is safe in humans but induces liver tumours in rodents

after prolonged treatment. It serves as a reference drug for CAR activation, inducing various CYP enzymes in rodent and human livers (Elcombe et al., 2014). In rodent models, CAR's role in liver tumour formation is confirmed using KO-CAR mice, where phenobarbital does not induce CYP activation or liver tumours (Martignoni et al., 2006, Pelkonen et al., 2008). Induction of liver tumours in rodents through CAR-mediated mechanisms, follow a pattern of key events (KE) that include CAR activation (KE 1), altered gene expression (KE 2), increased hepatocellular proliferation (KE3) clonal expansion (KE4), and liver adenomas/carcinomas (KE 5). Associative events, such as increased CYP enzyme expression (AE 1) and hepatocellular hypertrophy (AE 2), support these key events. (Elcombe et al., 2014). Emerging methods and minimum datasets for evaluating CAR mechanistic MoA are described in details by Peffer and colleagues (Peffer et al., 2018), which align with the Adverse Outcome Pathway for regulatory data on CAR liver tumour mechanisms, sponsored by OECD (Peffer, 2017))(Figure 2).

Figure 2 - CAR Adverse Outcome Pathway. Adapted from *https://aopwiki.org/aops/107*.



1.5 Human relevance of MoA

A large number of rat and mice studies have clearly demonstrated that long treatment with non-genotoxic CAR activators makes liver more susceptible to replicative DNA synthesis (RDS) induction, which plays a pivotal role in hepatocellular pre-neoplastic and neoplastic lesions (Yamada et al., 2021). For human risk assessment it is critical to evaluate whether CAR activators that induce rodent liver tumours can equally induce RDS in human hepatocytes (i.e., its human relevance). For phenobarbital, the non-relevance of CAR MoA for humans is actually supported by epidemiological data. Patients with epilepsy that received phenobarbital for several years achieved drug concentrations in the blood similar to those

detected in rodents, but without evidence of specific risk of human liver cancer (Stritzelberger et al., 2021, La Vecchia and Negri, 2014). Similar results were observed with another CAR activator, the benzodiazepine oxazepam, which produces liver tumours in rodents (Bucher et al., 1994) but did not show any clear association with liver tumour formation in humans (Friedman et al., 2009, Iqbal et al., 2015). Since direct testing of CAR activators in human liver is not possible, diverse experimental systems that either involve *in vitro* human primary hepatocytes, humanised hepatic nuclear receptors or even human hepatocyte chimeric livers rodent models, have been developed and used (Yamada et al., 2014, Huang et al., 2005, Strupp et al., 2020). Extensive data involving both *in vitro* human hepatocytes and PXR/CAR chimeric rodents models supported that CAR activators are mitogenic in mouse and rat liver but do not induce RDS in human hepatocytes (Yamada et al., 2021).

The International Life sciences Institute (ILSI) established a conceptual framework together with World Health Organization (WHO) - International Programme on Chemical Safety (WHO-IPCS) supported by the United States Environmental Protection agency (EU.EPA) and health Canada (Sonich-Mullin et al., 2001, Meek et al., 2003, Meek et al., 2014, Boobis et al., 2006, Boobis et al., 2008, Holsapple et al., 2006) to help assessing the MoA and human relevance for chemical associated carcinogenesis. During the past few years, this framework underwent some updates, but the underlying principles were unchanged. The overall weight of evidence for a MoA can be assessed by answering the following three questions:

1. Is the weight of evidence sufficient to establish a MoA in animals?
2. Can human relevance of the MoA be fairly excluded on the basis of fundamental, qualitative differences in key events between animals and humans?
3. Can human relevance of the MoA be reasonably excluded on the basis of quantitative differences in either kinetics or dynamic factors between animals and humans?

The first question is evaluated by taking into account dose concordance (e.g. escalating doses, maximum tolerated dose), robustness/consistency of certain effects across diverse studies, biological credibility and ponderation of alternative MoA. The information can be extracted from experiments (with or without animals) established to evaluate key or associate events, following the weight of evidence approach based on the modified Bradford Hill considerations (Meek et al., 2014). Regarding the second and third questions, if a robust MoA is identified, qualitative and quantitative relevance for humans will be considered. For CAR MoA, this is mainly based on the comparison of data obtained from a new test item and the extensive data from phenobarbital. CAR activators, like PB, will cause some early events in human liver (e.g. CYP induction and hypertrophy) but they will not produce late events, such as increased cell proliferation, increased altered *foci* or tumours, which seem to be unique for mice and rats (Elcombe et al., 2014). Consequently, a liver tumour derived by CAR activation that shows a phenobarbital like behaviour will not be of human relevance. It is worth to mention that when looking for human relevance, another question frequently asked is whether other MoAs can be excluded. Quite often a substance may exhibit more than one MoA, hence if alternative MoAs are supported, they need their own framework analysis (Boobis et al., 2006).

Additionally, the current evaluation of MoA is to filter positive results that are not relevant to humans. Therefore, at present regulators do not rely only on MoA for classification. Nevertheless, it is important to obtain as conclusive data as possible while avoiding unnecessary animal studies.

2 Analysis description

Thirty-six CLH dossiers including the background and RCOM documents were analysed (Table A1). They were selected based on the availability of CAR MoA evaluation and related RAC adopted opinions. Available legal acts and guidance, which could aid the process of characterising a proposed MoA for carcinogenicity and determining their human relevance, was assessed.

The information collected regarded long-term carcinogenic studies in rodents, short-term *in vivo* and *in vitro* mechanistic studies (including experimental methods) for liver tumours MoA evaluation and human relevance. The analysed dossiers included substances falling under different regulatory programmes assessed by RAC between 2011 and 2023. More than half (64%, 23 out of 36) of the assessed substances were under Plant Protection Product (PPP) Regulation only. From the remaining dossiers 14% (5 out of 36) were under Biocidal Products Regulation (BPR), 14% (5 out of 36) under REACH and 8% (3 out of 36) under both PPP and BPR.

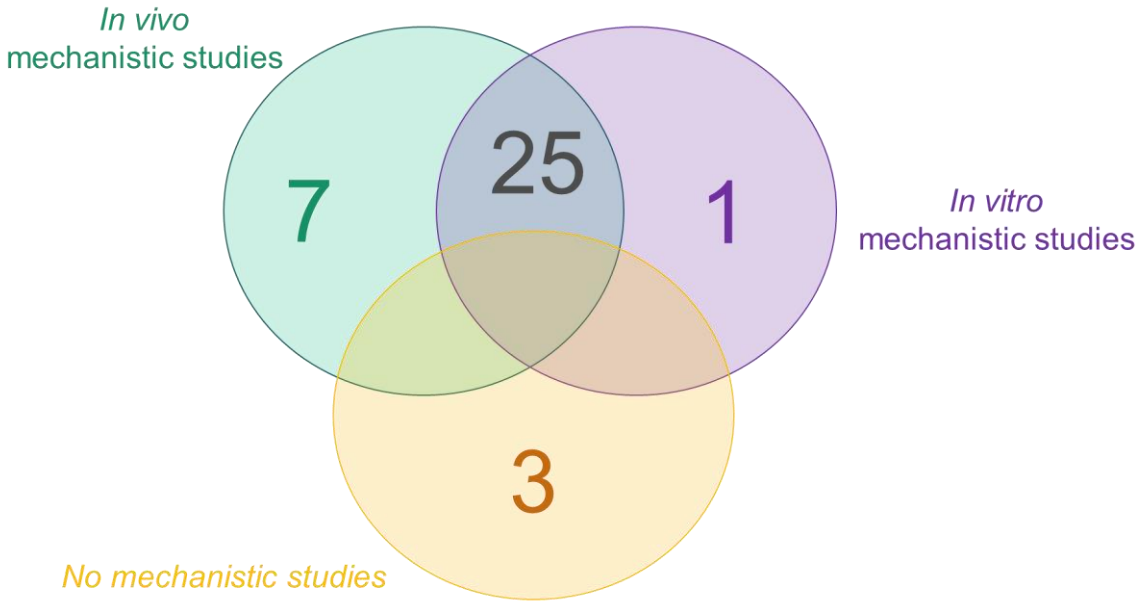
Collected data was summarised and grouped according to presented experimental approaches to evaluate CAR MoA key and associative events (*in vivo* or *in vitro*), the species of concern (mouse, rat or human), the exclusion of other MoA and its human relevance.

3 Results

Long-term carcinogenicity rat studies were present in all the 36 dossiers, while the two-year mouse studies were reported in 33 dossiers. Regarding mechanistic studies, 7 out of 36 dossiers presented only animal studies, only one dossier presented data exclusively from *in vitro* studies, the remaining 25 dossiers included both *in vivo* and *in vitro* studies, while 3 studies did not report any mechanistic data. Five dossiers included CAR data provided during public consultation, of which 2 included *in vivo* and *in vitro* data, 2 only *in vivo* and 1 only *in vitro* (Figure 3). Human epidemiological data for carcinogenicity evaluation was included in only one dossier.

Transgenic animal models have been considered strongly relevant for CAR MoA evaluation (Elcombe et al., 2014), however just 7 dossiers showed data from CAR/PXR-KO animal models and 4 dossiers from humanised-CAR animal models. Interestingly, data from cultured hepatocytes derived from transgenic mice or rats was reported in 7 cases (PPARα- and CAR/PXR-KO hepatocytes).

Figure 3 – Number of dossiers including *in vivo* and *in vitro* mechanistic studies.



3.1 *In vivo* short-term *versus in vitro* mechanistic studies

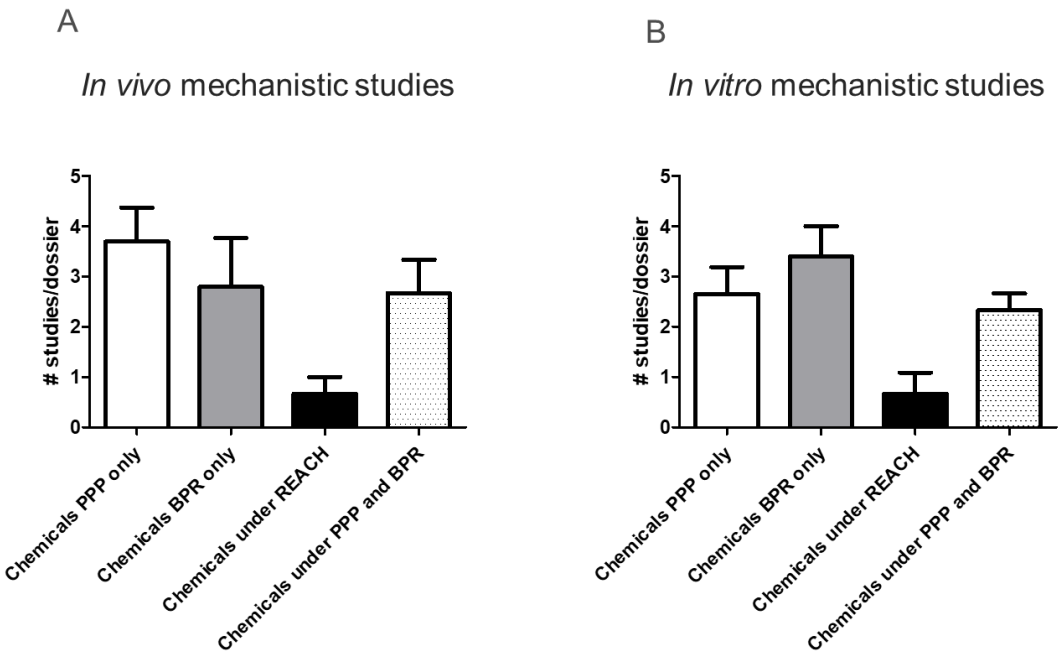
Despite the absence of regulatory requirements for MoA evaluation, the dossiers included a considerable number of *in vivo* and *in vitro* mechanistic studies.

Short-term *in vivo* studies (e.g. 1–28 days duration) can actually provide data to establish a CAR-dependent MoA for rodent liver tumour formation (Cohen, 2010, Elcombe et al., 2014, Peffer et al., 2018). For compounds that have demonstrated to be non-genotoxic and have been evaluated in long-term bioassays, short-term MoA studies should be performed at carcinogenic and non-carcinogenic dose levels. Essential experimental endpoints should include liver hypertrophy (both liver weight and histology), hepatocellular proliferation (RDS),

and induction of CYP2B enzymes (both enzyme activity and/or mRNA levels) evaluation as CYP2B induction is an indirect marker for CAR receptor activation. Finally, to exclude the potential role in CYP induction of either AhR, PXR, or PPARα receptors, both enzyme activity and/or mRNA levels of CYP1A, CYP3A, and CYP4A subfamily enzymes should be assessed. Additionally, in cases where other MoAs are suspected, such as cytotoxicity and metal overload, available data from longer studies (e.g. 90 days studies) can be used to exclude other potential MoA.

A significant difference in the number of *in vivo* mechanistic studies performed per dossier was observed between active substances under PPP and chemicals registered under REACH. Dossiers including active substances under both PPP and BPR had more studies than the latter (Figure 4A). Similar results were observed regarding *in vitro* mechanistic studies, but this difference was only significant between active substances under BPR and chemicals under REACH (Figure 4B). No significant differences were seen between the PPP and BRP for both *in vivo* and *in vitro* mechanistic studies (Figure 4). In fact, the number of *in vivo* and *in vitro* studies are very similar for active substances classified under both PPP and BPR regulations. This may suggest that the PPP and BPR registrants hold more resources for data generation relevant for a proper regulatory assessment.

Figure 4 - Representation of the number of *in vivo* and *in vitro* studies performed per dossiers according to the chemical sector.



Short-term *in vivo* mechanistic studies had an average duration of 29.4 days per study. The shortest studies included 1, 3 and 4 days and the longest ones from 28 to 90 days. The most common duration for short-term mechanistic studies was between 3 and 7 days (Table 3).

Table 3: *In vivo* short-term studies duration.

In vivo short-term studies duration (days)	Samples collection (days)	# Number of Studies
3	3 and 28	1
4	1,3 and 4	1
5	1 and 5	1
7	1 and 7	2
7	3 and 7	8
7	7 and 28	2
14	1, 3, 7 and 14	1
14	2, 7 and 14	1
14	3, 7 and 14	2
14	7 and 14	7
14	3 and 14	1
21	1, 3, 7 and 21	1
21	4 and 21	1
28	1, 2, 3, 4, 7, 14, 21 and 28	1
28	1, 2, 7, 14 and 28	1
28	2, 4, 7, 14 and 28	1
28	2, 7 and 28	1
42	14, 21 and 42	2
56	14, 28 and 56	1
60	1, 2, 3, 4, 7, 14, 21, 28 and 60	1
60	1, 2, 3, 4, 7, 14, 28 and 60	1
60	3, 4, 7, 14, 28 and 60	1
90	14 and 90	2
90	28 and 90	3
90	28 and 90	1
90	7, 28 and 90	2

Interestingly, despite the number of *in vivo* mechanistic studies surpassing that of *in vitro* mechanistic studies, the latter still accounted for a significant proportion (111 *versus* 89).

This indicates that *in vitro* methods are already being used to support regulatory decision-making related to chemical safety (Table 4).

Table 4: *In vivo* and *in vitro* mechanistic studies considered per dossier.

Compound/dossier	*In vivo* mechanistic studies	*In vitro* mechanistic studies	RAC conclusion on MoA and human relevance
Propiconazole	4	3	Conclusive
Imazalil	14	2	Inconclusive
Piperonyl Butoxide	2	2	Conclusive
Phenylphenol 2	2	2	Inconclusive
Pethoxamide	2	2	Partly conclusive
Metolachlor	4	7	Inconclusive
Penflufen	2	2	Partly conclusive
Amisulbrom	10	0	Inconclusive
Carbetamide	1	0	Inconclusive
Epsilon-metofluthrin	3	4	Inconclusive
Fluopyram	1	2	Conclusive
Isoflucypram	2	0	Inconclusive
Sedaxane	4	4	Conclusive
Cyproconazole	6	2	Conclusive
Transfluthrin	0	4	Inconclusive
Trimethylolpropane triacrylate	1	0	Inconclusive
Isoproturon**	6	2	-
Benfluralin	1	10	Partly conclusive
Momfluorothrin	3	5	Conclusive

Compound/dossier	*In vivo* mechanistic studies	*In vitro* mechanistic studies	RAC conclusion on MoA and human relevance
Metazachlor	2	0	Inconclusive
4-methylpentan-2-one	2	2	Inconclusive
Ammonium pentadecafluorooctanoate	0	0	Inconclusive
Benthiavalicarb-isopropyl	5	4	Partly conclusive
Clofentezine	4	0	Inconclusive
Cumene	1	2	Inconclusive
Difenoconazole	4	5	Inconclusive
Fluopicolide	3	3	Conclusive
Fluxapyroxad	4	5	Conclusive
Valifenalate	3	1	Inconclusive
Pydiflumetofen	1	4	Inconclusive
Pentadecafluorooctanoic acid (PFOA)	0	0	Inconclusive
Pyriofenone	7	4	Inconclusive
Isopyrazam	1	2	Inconclusive
Silthiofam	1	4	Conclusive
Sulfoxaflor	5	0	Conclusive
Thiophanate-methyl	0	0	Inconclusive
Total number of mechanist studies	111	89	

** The information regarding carcinogenicity for this compound was collected from additional mechanistc studies submitted with the dossiers. Available in "Summary table 3" tab, from excel file "CAR MoA Dossier Analysis Database".

3.2 *In vitro* based experimental data used to identify the main events for CAR MoA evaluation

To better understand how many *in vitro* methods are actually used in the regulatory assessment and decision making to elucidate CAR MoA, we identified the main events considered relevant by RAC.

Data information from the diverse studies was collected in different tables, and presented in the Annex 3. Table A2 indicates, under "Methodologies performed", the main strategies followed to determine the KE and AE per compound. The cases where these strategies were tested, and the models used (*in vivo versus in vitro*; performed in mice (M), rats (R), or humans (H)), are indicated in colour orange. The table also shows the cases where these strategies were not tested (not determined (ND)). It can be observed from this table that the strategies are well distributed *in vivo* and *in vitro*. Additionally, Table A3 indicates whether the overall results of the presented *in vitro* and *in vivo* studies were positive, negative, or inconclusive regarding each KE and AE. These results show that these studies are mostly conclusive and used by RAC in their assessment as weight of evidence. The outcome in the column "Determinant Events for Accepting CAR MoA" from Table A4 was based on the positive and well-accepted data by RAC, which was then used to identify the *in vitro* approaches that contributed to those conclusions. This information was the base for building Table 5.

Table 5 enumerates cases where *in vitro* methods were used to evaluate each of the main KE or AE. Interestingly, from the eight events listed, six can be evaluated using *in vitro* approaches. It is also worth to notice that *in vitro* hepatocellular proliferation was actually assessed in more than half of the analysed dossiers (61% for rodents and humans).

Table 5*:* CAR MoA main determinants and *in vitro* based experimental data.

Main events for CAR MoA decision	*In vitro* approaches used	# dossiers	Total %
i) Treatment induced CAR activation in liver	CAR3 Transactivation assay with mouse, rat and human CAR	6	17%
	Cultured hepatocytes transfected with siRNA (CAR) and siRNA (control) for CAR activation evaluation	2	6%
ii) Altered CYP gene expression in the liver	*In vitro* human hepatocytes experiments: *CYP2B6* and *CYP3A4* gene expression	12	33%
	In vitro rodent hepatocytes experiments: *CYP2B10* and *CYP3A11* gene expression	13	36%
iii) Induction of CYP enzymes	*In vitro* human hepatocytes experiments: CYP activity	14	39%
	In vitro rodent hepatocytes experiments: CYP activity	16	44%
iv) Hepatocellular proliferation in rodents	*In vitro* rodent hepatocytes experiments: cell proliferation	22	61%
v) Increased liver weight and hepatocellular hypertrophy	-	-	-
vi) Liver tumours	-	-	-
vii) Differential hepatocellular cell proliferation in mice and humans	*In vitro* human hepatocytes experiments: cell proliferation	22	61%
viii) Exclusion of other modes of action	*In vitro* PXR and AhR transactivation experiments: luciferase reporter	6	17%

In order to comprehend the weight of these *in vitro* studies in RAC opinions for CAR MoA decision, we considered in a second analysis only the cases where CAR MoA was identified. In the majority of the dossiers (25 out of 36, 69%), the data reported was not sufficient to conclude for a specific MoA, while one compound (Phenylphenol-2) plausibly seemed to induce liver tumours in rodents due to PPARα activation. The remaining 11 compounds (31%) were considered CAR activators according to the data evaluated by RAC (Table 6). Interestingly, *in vitro* proliferation studies with human hepatocytes were present in most of the dossiers (10 of 11, 91%), demonstrating their significance for the evaluation of CAR MoA and the associated lack of human relevance.

Table 6: CAR MoA main events and in vitro based experimental data underlying CAR activation properties of chemicals assessed

Main events for CAR MoA decision	*In vitro* approaches used	# dossiers	% of dossiers with CAR MoA identification
i) Treatment induced CAR activation in liver	CAR3 Transactivation assay with mouse, rat and human CAR	2	18%
	Cultured hepatocytes transfected with siRNA (CAR) and siRNA (control) for CAR activation evaluation	2	18%
ii) Altered CYP gene expression in the liver	*In vitro* human hepatocytes experiments: *CYP2B6* and *CYP3A4* gene expression	8	73%
	In vitro rodent hepatocytes experiments: *CYP2B10* and *CYP3A11* gene expression	5	45%
iii) Induction of CYP enzymes	*In vitro* human hepatocytes experiments: CYP activity	2	18%
	In vitro rodent hepatocytes experiments: CYP activity	2	18%
iv) Hepatocellular proliferation in rodents	*In vitro* rodent hepatocytes experiments: cell proliferation	6	55%
v) Increased liver weight and hepatocellular hypertrophy	-	-	-
vi) Liver tumours	-	-	-
vii) Differential hepatocellular cell proliferation in mice and humans	*In vitro* human hepatocytes experiments: cell proliferation	10	91%
viii) Exclusion of other modes of action	*In vitro* PXR and AhR transactivation experiments: luciferase reporter	1	9%

3.3 The variety of techniques used to produce experimental data presented in the dossiers

The ability of compounds to directly activate CAR was determined in various ways from dossier to dossier (Table 7):

1) Transfecting cultured hepatocytes with small interfering RNA (siRNA) to silence the expression of CAR (2/36);
2) Transfecting C3A cells with an expression plasmid containing hCAR, along with a vector containing the promoter region of its target gene (*CYP2B6*) with a downstream luciferase reporter (1/36); and
3) Transfecting COS-1 cells with cDNA expression vectors for CAR3 variants of mouse, rat, and human to investigate the relative ability of a compound to activate CAR from different species (4/36).

Results for PXR and/or CAR activity in high throughput assays (TOXCAST) were also reported in one dossier.

Indirect CAR activation was determined in 13 dossiers by CYP induction gene expression in cultured mouse, rat and human hepatocytes, upon treatment with the test compounds, by measuring *CYP2B10* and *CYP3A11* mRNA levels by quantitative real time-polymerase chain reaction (PCR). It was also demonstrated in cultured rat, mouse and human hepatocytes by CYP protein expression (1 dossier) and through assessing CYP enzyme activity by evaluating BROD and PROD activities (15 dossiers).

Additional *in vitro* experiments performed to exclude other MoA included transfection of different type of cells with luciferase reporters for PXR and AhR gene expression (6/36). Oxidative stress was assessed using cultured rat hepatocytes by measuring glutathione (GSH) production levels (1/36) and cytotoxicity was evaluated in cultured mouse, rat and/or human hepatocytes by measuring Adenosine Triphosphate (ATP) production (14/36). Finally, a microarray using mouse liver samples was performed to explore differentially expressed genes between treated and control samples (1/36).

Hepatocellular proliferation evaluation using BrdU incorporation was considered in 23 dossiers, and it was evaluated in cultured rat, mouse and human hepatocytes.

Table 7*:* List of *in vitro* assays used to generate data for CAR MoA evaluation.

Mechanistic Endpoint	Techniques	Models	# Dossiers
CAR activation	Cell transfection with small interfering RNA (siRNA) for CAR	Cultured rat hepatocytes	2/36
CAR activation	Luciferase reporter for hCAR	C3A cells	1/36
In vitro CAR3 transactivation assay	cDNA expression vectors for CAR3 variants of human, mouse and rat	COS-1	4/36
CAR and PXR activation	High-throughput assay using data for methylpentan and its metabolites screened via TOXCAST7 database (EPA, 2018) with special focus on CAR and PXR	Data derived from *in vitro* data	1/36

Mechanistic Endpoint	Techniques	Models	# Dossiers
CYP induction gene expression (*CYP2B10* and *CYP3A11*; *CYP2B6* and *CYP3A4*)	Quantitative real time-PCR	Cultured mouse/ rat /human hepatocytes	13/36
CYP induction protein expression (CYP2B10 and CYP3A11)	Western blot	Cultured mouse and human hepatocytes	1/36
CYP isoenzymes activity (e.g BROD and PROD)	Not indicated in the dossier	Cultured mouse/ rat /human hepatocytes	15/36
Reduction/oxidation	Glutathione (GSH) production	Cultured rat hepatocytes	1/36
PXR transactivation assay	Cell transfection using pSG5-hPXR or pSG5-mPXR plasmids with luciferase reporter	COS-7 simian kidney cells	4/36
hPXR and hAhR activation	Luciferase reporter for PXR and AhR gene assay	C3A cells	1/36
hAhR activation	Luciferase reporter for AhR gene assay	Cultured mouse hepatoma Hepa1c1c7 cells	1/36
Agilent Whole Mouse Genome Oligo Microarray	A set of differentially expressed genes (DEGs) was obtained by comparisons of treated and control microarray results	Liver samples from mouse*	1/36
Cytoxicity	ATP production	Cultured mouse/ rat /human hepatocytes	14/36
Cell Proliferation	DNA synthesis evaluation by BrdU incorporation	Cultured mouse/ rat /human hepatocytes	23/36

* This assay includes the use of mouse organs.

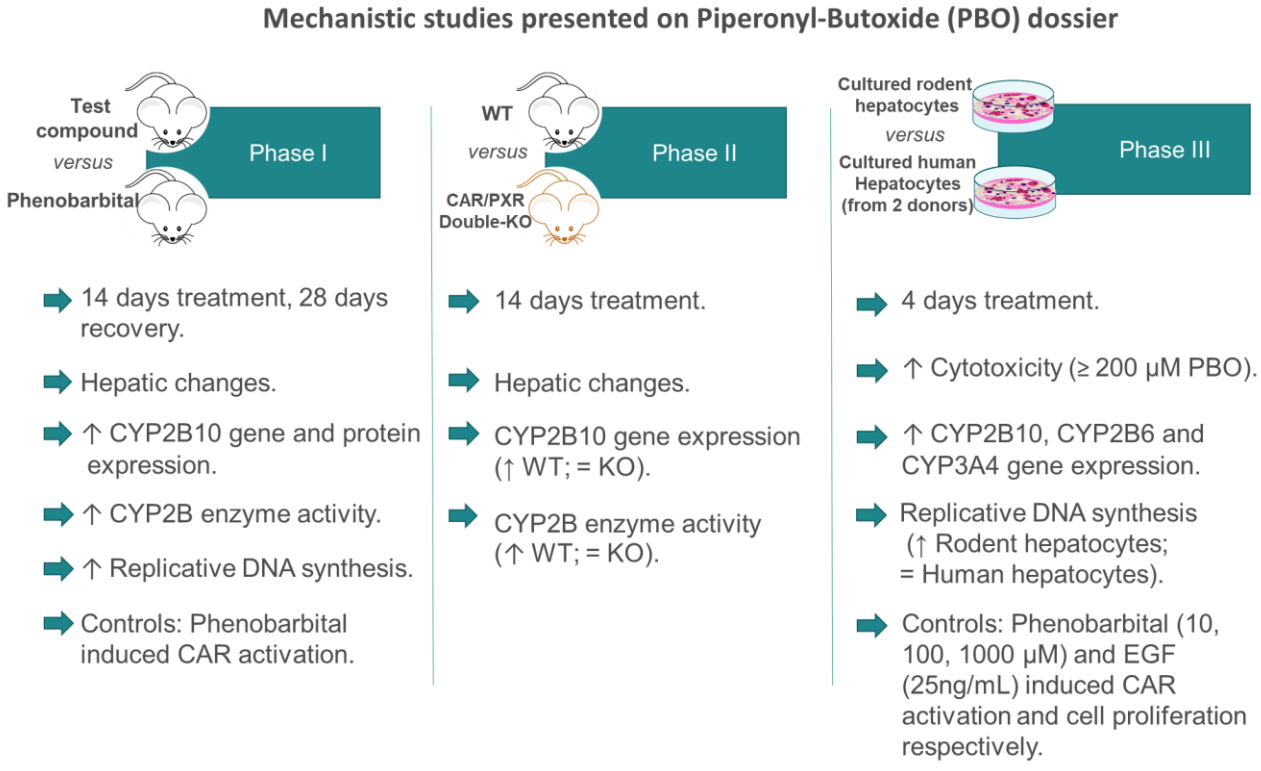
4 Case examples to illustrate difference in dossiers

Clear dossier: In terms of clarity and organisation of the studies presented in order to identify the main events for CAR MoA, a good example is the dossier from Piperonyl-Butoxide. Piperonyl-Butoxide is a Biocidal Product, assessed by RAC in 2020. It demonstrated to promote liver tumours in mice, but not in rat upon the 2 years carcinogenicity bioassay. Regarding the mechanistic studies, industry provided a test strategy to clarify the presumed CAR MoA. This strategy was divided in three phases.

- Phase I: since the MoA proposed is the same as for phenobarbital, hepatic effects of Piperonyl-Butoxide and sodium phenobarbital (NaPB) were compared in CD-1 mice;
- Phase II: hepatic effects of Piperonyl-Butoxide administration were compared in wild type and CAR/PXR double knockout (KO) mice to investigate whether effects are mediated through CAR;
- Phase III: an *in vitro* comparison of Piperonyl-Butoxide - and sodium Phenobarbital-induced effects was conducted in human donors and male CD-1 mouse hepatocytes concerning CYP activation and induction of replicative DNA synthesis.

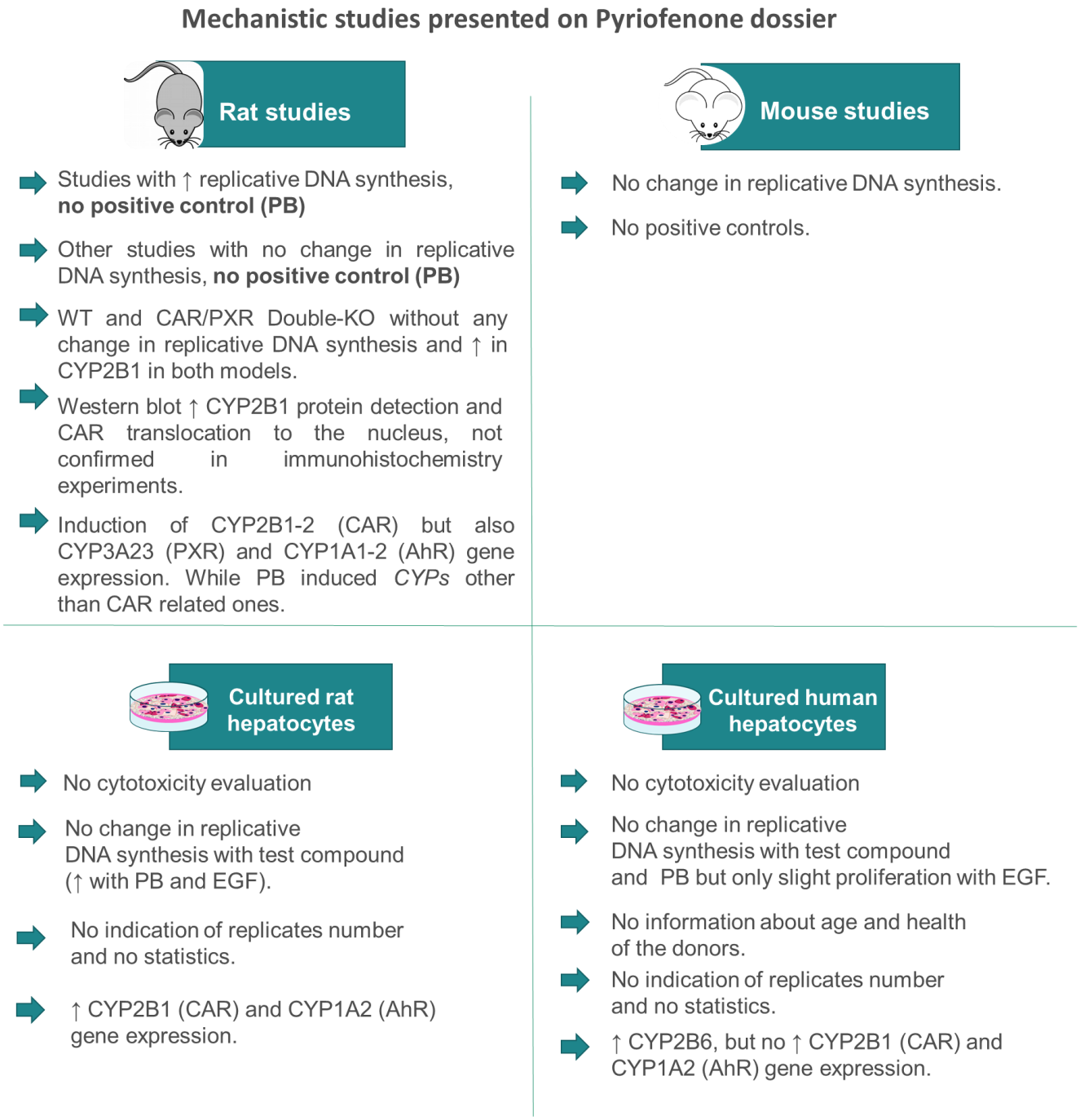
The way the different studies were presented to demonstrate the KE and AE for CAR MoA, made the dossier to be easily assessed by RAC. However, some gaps can be identified: the *in vivo* models used for the KO-CAR/PXR (C57BL/6J mice) are not the same as in the main study (CD-1 mice) and no PB was used as positive control for the phase II study.

Figure 5: Details regarding MoA mechanistic studies presented on the Piperonyl-Butoxide dossier.



Less clear dossier: Pyriofenone is a Plant Protection Product assessed by RAC in 2019 and classified as Carcinogen category 2 - H351 (CLP). It produced a weak carcinogenic response in liver of male mice and rat in the 2 years carcinogenicity bioassay. Despite the amount (11) of *in vivo* and *in vitro* mechanistic studies available, most of the studies were not in agreement with each other (e.g. incoherence between rat and mice hepatocytes replicative DNA synthesis studies). Besides, of missing positive controls (Phenobarbital) in some *in vivo* studies, the results in several mechanistic studies were contradictory or non-supportive of the CAR MoA. In crucial experiments regarding specificity of CAR activation, no increase in cell proliferation in either WT or CAR-KO rats was observed while in another study cell proliferation was observed in WT animals upon Pyriofenone treatment. Additionally, *in vitro* studies involving both rat and human hepatocytes were not reliable since EGF positive control response for the DNA replicative synthesis investigations was too weak and did not enable any conclusion regarding the key event of cell proliferation in human liver cells (Figure 5). Therefore, Pyriofenone dossier was considered unclear mainly due to the lack of data quality from the studies presented for CAR MoA evaluation.

Figure 6: Details regarding MoA mechanistic studies presented on the Pyriofenone dossier.



5 Discussion

RAC approach and consistency during weight of evidence:

During this analysis, we observed that each chemical was assessed on a case-by-case basis. RAC made conclusions taking into consideration rigorous analysis of all available data from the CLP dossier and data received during public consultation. In fact, in certain cases data submitted during the public consultation were key to decide on the final classification. An example is Fluopyram where the additional submitted *in vitro* studies regarding human hepatocytes were essential to decide on rodents liver tumours human relevance.

In line with ECHA guidance for carcinogenicity assessment (ECHA, 2017a) mechanistic data for MoA evaluation is not mandatory, but it is recommended. The lack of specific guidance regarding these mechanistic studies results in a notable heterogeneity in the duration of the *in vivo* short-term studies and models used, as well as in the type of *in vitro* methods used. Nevertheless, it was noted that in the majority of the dossiers the weight of evidence for CAR MoA and its human relevance were assessed in detail and in agreement with the IPSC Conceptual Framework, with the exception of Isoflucypram, Transfluthrin, TBBPA, Phenylphenol-2 and Metalochlor.

It was very interesting to notice that dossiers including a considerable amount of mechanistic studies were more often those with more inconsistencies related to *in vivo* and *in vitro* data, as it has been observed that *in vivo* and *in vitro* experimental data does not always agree across different studies. These inconsistencies were especially noticed and taken into consideration by RAC. The dossiers and type of studies that presented these inconsistencies are indicated with an "I" and in blue colour in the Table A3, from the Annex 3. There are at least six cases (Imazalil, Penflufen, Transfluthrin, Metazachlor, Benthiavalicarb-isopopyl and Pyriofeone) where RAC could not conclude on the MoA due to inconsistencies among the various studies. One good example is Pyriofeone, described above as a less clear dossier (Figure 6).

How is RAC already using non-animal data to conclude on classification, including the human relevance of the findings?

According to Elcombe *et al*., 2014, transgenic animals expressing human CAR [humanized CAR models (animals expressing hCAR/hPXR receptors in murine hepatocytes) or chimeric models (animals engrafted with human hepatocytes)] and primary human hepatocytes can be used to demonstrate the different hepatocellular cell proliferation in mice and humans, as well as alterations of CAR activation upon treatment with a test compound. However, transgenic animal models are expensive and raise some uncertainties. While humanised transgenic models, hCAR/hPXR models are seen as reference experiments for CAR activation and human relevance, their reliability is not higher than a well designed study using cultured human hepatocytes (Yamada, 2021). This is especially true when the hCAR/hPXR rodent models carry a murine liver with human receptors functioning in a murine environment, with the risk of triggering similar results to the ones in the wild-type mice.

In practice, experiments with primary human hepatocytes seem to be a suitable alternative to hCAR/hPXR models as they are more straightforward. In fact, data involving the use of human cultured hepatocytes was available and used in 91% of the dossiers to confirm non-

human relevance of the liver tumours induced by the CAR MoA. This shows that, regardless the fact that human hepatocytes-based methods are not included in any internationally recognised guideline (e.g. OECD TG), regulatory assessors are already considering these data in their decision-making process.

Actually, as long as the presented experiments were performed with the proper controls, and concentrations that do not exceed cytotoxicity and using the correct models (e.g. hepatocyte *in vitro* culture from the same rodent model that demonstrated liver tumours in long term assays), RAC considered these experiments with confidence in its weight of evidence evaluation. In fact, RAC did not accept *in vitro* data when the quality of the experiments was questioned. Per example, RAC considered the experiments performed to investigate the effect of Pyriofenone on DNA replication unreliable and insufficient for hazard assessment since the EGF positive control response for the DNA replicative synthesis investigations was weak, and consequently not reliable.

Additionally, there was one isolated case (Pethoxamide) where RAC questioned the use of frozen primary human hepatocytes for the evaluation of hepatocellular proliferation. In this dossier, RAC argued that the process of isolation, preservation and culturing of primary human hepatocytes was complex, their quality was highly donor-dependent, and their functionality could be compromised. In the same dossier, RAC added that the use of EGF as positive control for cell proliferation assays was also debatable, as it is not known how similar this growth factor and the liver carcinogen in question is in terms of their MoA with respect to e.g., receptor activation and triggering subsequent cell proliferation. In another dossier (Metolachlor), RAC commented that the results from human hepatocytes were questionable since one of the donors was under chemotherapy just four days before the hepatocytes were collected for cell culture. The number of human hepatocyte donors and the replicates per study can also be a hindering factor (i.e. quality of *in vitro* data) for the acceptance of the *in vitro* data.

This analysis clearly indicates that data generated with non-guideline *in vitro* mechanistic methods are already being considered in regulatory assessments. It would be interesting to extend such an analysis to other areas of interest to get a deeper insight into the methods that are already in use and to identify gaps needing prioritisation for further work. Moreover, building such an awareness may encourage the scientific and regulatory communities to make further use of non-animal approaches and thus foster the development of methods that are ready to be used in the regulatory environment. In this regard and looking ahead, efforts to enhance *in vitro* methods should prioritise the use of human-cell based methods (e.g., human hepatocytes).

It is important to keep in mind that, while predicting certain tumours such as the CAR-mediated one in rodent liver is crucial, the overall benefit remains uncertain if other cancer types cannot be excluded or identified. In fact, CAR-mediated liver tumours not relevant to humans were identified in several occasions along with other potentially human-relevant tumours not CAR-related. This highlights the challenge in comprehensively predicting different tumour types, which was explored by Heusinkveld and colleagues (Heusinkveld et al., 2020), in an attempt to identify the most relevant MoAs triggered by agrochemicals. To develop such a MoA-driven approach, EPAA is finalising an in depth analysis of the available and up-to-date information regarding unknown tumour related MoAs induced by agrochemicals in all types of organs.

Can CAR MoA be predicted in shorter-term mechanistic studies and how much weight do in vitro mechanistic studies have in the overall assessment?

Primary hepatocytes isolated from KO-CAR/PXR transgenic rodents and primary cultured human hepatocytes are *in vitro* models used to evaluate CAR activation and human relevance, respectively, of a certain active substance. In the dossiers from the eleven compounds considered as CAR activators, ten included only data from *in vitro* human hepatocytes. The single dossier that included only data from a transgenic humanized mouse model was Sufoxaflor. Regarding the transgenic models used to evaluate CAR activation, seven of the eleven dossiers presented data from KO-CAR/PXR *in vivo* models while three of them presented data from KO-CAR/PXR rodent hepatocytes. Another method that enables to evaluate CAR activation *in vitro* involves the use of luciferase specific CAR reporters. Although less used, this method was the only one included in four out of the eleven dossiers to evaluate direct CAR activation (KE 1). These observations suggest that it is possible to address some of the key determinants for CAR MoA exclusively with *in vitro* methods, especially without using *in vivo* transgenic models. Cyp induction evaluation through gene expression, protein expression or Cyp activity was always assessed *in vivo*, although in some cases also *in vitro* tests were performed. The animal studies lasted in average 18,9 days and used 10,63 animals per group/condition.

The idea of considering using only *in vitro* methods to evaluate six of the eight main events for CAR MoA could be seen as an alternative for short-term *in vivo* studies. However, certain chemical characteristics of the active substance such as low solubility or *in vivo/in vitro* metabolic discrepancies should be taken into consideration when planning the mechanistic experiments (Ooka et al., 2020). To draw a concrete opinion about this topic, data analysis from industry in house data could give more insights of its feasibility. These characteristics are actually relevant for the acceptance of the data from the regulatory bodies. Per example, *in vitro* studies with Benthiavalicarb-isopropyl were not taken in consideration by EFSA and member states during an open discussion, which rather would have preferred to see data from transgenic mice models, arguing that the experiments *in vitro* with this compound can hardly mirror *in vivo* conditions since Benthiavalicarb-isopropyl is extensively metabolised.

6 Conclusions

The mechanistic studies for carcinogenicity MoA evaluation currently lack detailed regulatory guidance. The absence of clear guidance leads to notable heterogeneity in how experimental data are presented across different dossiers. Many of these dossiers include considerable *in vivo* data, but the quality of this data is often compromised by e.g., missing controls and discrepancies in animal sex and strains, which do not align with OECD Test Guidelines (TG).

Despite these gaps, a significant number of *in vitro* studies within the dossiers have been considered relevant by RAC to conclude on carcinogenicity classification. However, the acceptance of experimental data varies from dossier to dossier. This inconsistency highlights the need for additional work in terms of standardisation or validation to ensure uniformity and reliability in the evaluation of positive results from carcinogenicity studies.

Based on the analysis presented here, the use of mechanistic knowledge from *in vitro* studies in this specific regulatory field would benefit from standardisation, ideally developing some guidance or a guideline. This would improve the confidence on *in vitro* data during evaluation of CLH proposals, redirect resources (e.g. animals, money, time) and facilitate the work of RAC during the dossier assessment, as well as guide the generation of data by the submitter and provide confidence that these data will be considered in the assessment.

The engagement with other stakeholders such as the dossier submitters would be of value to complement this analysis and better understand their in-house practices, their needs and related issues.

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List of abbreviations and definitions

3Rs	Replacement, Reduction, and Refinement
AhR	Aryl hydrocarbon Receptor
AOP	Adverse Outcome Pathway
ATP	Adenosine Triphosphate
BPR	Biocidal Products
BrdU	Bromodeoxyuridine
BROD	Benzyloxyresorufin O-dealkylase
CAR	Constitutive Androstane Receptor
cDNA	Complementary DNA
CLH	Harmonised Classification and Labelling
CLP	Classification for Labelling and Packaging
CMR	Reproductive Toxicity
CYP	Cytochrome P450 enzyme
DNA	Deoxyribonucleic Acid
DS	Dossier Submitter
EC	European Commission
ECHA	European Chemical Agency
EFSA	European Food Safety Authority
EMA	European medicines agency
EU	European Union
GHS	United Nations' Globally Harmonised System
GSH	Glutathione
ICH	International Council on Harmonization of Technical Requirements of Pharmaceuticals for Human Use
ILSI	International Life sciences Institute
IPSC	International Programme on Chemical Safety
KO	Knock-out
MoA	Mode of Action
mRNA	messenger Ribonucleic Acid
MRP4	Multidrug Resistance-associated Protein 4
MSCA	Member State Competent Authority
NaPB	Sodium phenobarbital
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
PPARα	Peroxisome Proliferator-Activated Receptor alpha
PPP	Plant Protection Products
PROD	7-penthoxyresorufin
PXR	Pregnane X Receptor
RAC	Risk Assessment Committee of ECHA
RCOM	Response to comments
RDS	Replicative DNA Synthesis
REACH	Registration, Evaluation, Authorization and restriction of Chemicals
siRNA	small interfering RNA
TG	Test Guidelines
WHO	World Health Organization

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Annexes

Annex 1. Supplementary CAR MoA information

The constitutive androstane receptor (CAR, *NR1I3*), is a part of the nuclear receptor superfamily, and it is mainly expressed in the liver, playing an important role in the metabolism of endogenous and xenobiotic substances by regulating its target genes (Baes et al., 1994, Qatanani et al., 2005, Wei et al., 2000). CAR activation can upregulate expression of numerous xenobiotic metabolising enzymes including phase I (e.g. mostly CYP2B subfamily) and phase II (e.g. glutathione S-transferases) enzymes, as well as phase III transporters (e.g. MRP4) (Assem et al., 2004, Ueda et al., 2002). In some cases, a considerable crosstalk between CAR and PXR receptors can occur when chemical compounds work as activators of both nuclear receptors (Omiecinski et al., 2011, Daujat-Chavanieu and Gerbal-Chaloin, 2020). There are a variety of CAR activators, which include industrial chemicals [e.g., 6-(4-chlorophenyl)-imidazo[2,1-b]thiazole-5-carbaldehyde (CITCO) and 1,4-Bis [2-(3,5-Dichloropyridyloxy)] benzene (TCPOBOP)], endogenous substances (e.g. steroids), insecticides [e.g., 1,1′-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene (DDT)], and therapeutic drugs [e.g., oxazepam, phenobarbital] (Maglich et al., 2003, Baes et al., 1994, Tzameli et al., 2000, Parkinson et al., 2006). Phenobarbital and sodium phenobarbital are non-genotoxic compounds, known to activate CAR by a ligand-independent mechanism (Mutoh et al., 2013). Phenobarbital is used in clinics since the beginning of the twentieth century (Methaneethorn and Leelakanok, 2021) and although its safety in humans has been reported in a number of epidemiological studies, the induction of liver tumour formation in rodents when administered in prolonged treatments has been demonstrated in significant amount of *in vivo* studies (Elcombe et al., 2014). Phenobarbital is used as the reference drug for CAR activation, since it induces CYP2B, CYP2C and CYP3A in rodent liver and mainly CYP2B6, CYP4A4 and CYP2A6 in human liver (Martignoni et al., 2006, Pelkonen et al., 2008). In humans, this Phenobarbital induced CYP activation does not appear to increase the risk for liver tumour (Friedman et al., 2009, La Vecchia and Negri, 2014, IARC, 2001). In the case of rodent liver tumours, the role of CAR had been confirmed by using KO-CAR mice transgenic models. In these models, Phenobarbital does not induce CYP activation, hepatocellular proliferation nor liver tumour formation (Scheer et al., 2008).

In addition to phenobarbital, many other substances have been reported to induce liver tumours in rodents through CAR MoA (Yamada et al., 2021). The amount of data from literature allowed the establishment of key and associative events involved in CAR-mediated MoA for phenobarbital-induced rodent liver tumours (Andersen et al., 2014, Elcombe et al., 2014). The key events include:

KE1 - CAR activation: Some substances, like phenobarbital, can activate CAR in an indirect way, mainly by interacting with the EGF receptor and inducing CAR dephosphorylation at its Threonine[38] (Mutoh et al., 2013). However, most of the substances will activate CAR by directly binding its ligand binding domain (Omiecinski et al., 2011). In both cases, activated CAR is translocated to the nucleus where it dimerizes with the Retinol X Receptor (RXRα) and induce the transcription of specific CAR-responsive genes (Mutoh et al., 2013).

KE2 – Altered gene expression secondary to CAR activation: a significant number of genes involved in phase I and II xenobiotics metabolizing enzymes (such as *cyp2b* and *cyp3a*), cell proliferation, apoptosis and energy metabolism (Kobayashi et al., 2015, Elcombe et al., 2014) altered their expression after CAR activation.

KE3 – Increased hepatocellular proliferation: CAR activators induce liver cell proliferation, which can be observed in whole liver surface, by histopathology, in the first 1-3 weeks of treatment. However, while cell labelling index returns to control level with continuous treatment, overall hepatocytes proliferation is still augmented due to the increase in the total number of hepatocytes per animal. Additionally, enhanced cell proliferation within altered foci had been demonstrated in rodent livers upon long-term treatment with CAR activators (Kolaja et al., 1996, Klaunig, 1993, Bursch et al., 2005, Jones et al., 2009, Tinwell et al., 2014).

KE4 - Clonal expansion leading to altered hepatocyte foci: The chronic administration of phenobarbital results in the development of altered hepatic foci in rodents (Deguchi et al., 2009, Jones et al., 2009). Spontaneously mutated hepatocytes form upon CAR activators long-term treatment and these cells replicate via clonal expansion leading to precursor's lesions of subsequent tumour formation. Altered foci show differential staining properties being mostly eosinophilic. This key event can be missed in certain studies due to the intrinsic characteristic of the chemical or the timing of the sacrifice (Peffer et al., 2018).

KE5 - Liver adenomas/carcinomas: Ultimately, chronic CAR activation, leads to expansion of (pre-)neoplastic lesions that form hepatocellular adenomas and carcinomas in rodent livers (Elcombe et al., 2014).

Associative events (AE) are markers for key events, and although not considered as direct evidence of causality of CAR mediated MoA, they are part of the overall CAR MoA. The first associative event is directly interrelated to KE2. An increased CYP metabolizing enzymes gene expression usually leads to an augment of CYP2B and CYP3A protein levels and/or enzyme activities in the hepatocytes (AE1). In its turn, the induction of CYP protein expression will account for an increase in cell size (hepatocellular hypertrophy (AE2) and consequently an increase in liver weight (AE3). Hepatocellular hypertrophy and liver weight changes also occur upon PPARα and AhR activation, but with different CYP induction patterns (Budinsky et al., 2014, Corton et al., 2014). A review made by Elcombe and colleagues indicate additional associative events, such as decreased apoptosis, inhibition of gap junctions or cytotoxicity. Even though these AEs can be involved in tumour formation, they are not specific to CAR activation, and hence not required to establish a CAR-depended MoA for rodent liver tumour formation (Elcombe et al., 2014, Peffer et al., 2018).

Other alternatives and emerging methods on how to evaluate CAR MoA were highlighted as minimum datasets for CAR MoA (Peffer et al., 2018). These datasets are in agreement with the Adverse Outcome Pathway (AOP) created for stablishing a minimum regulatory data for CAR liver tumour MoA, an initiative sponsored by OECD (Peffer, 2017) (Figure 2).

Annex 2. References from the consulted documents, organised by compound

Table A1. References from the CLH dossiers, consulted for each compound.

Compounds	CLH report	Background document	RCOM (Comments received)
Propiconazole	(ECHA, 2015d)	(ECHA, 2016d)	(ECHA, 2016h)
Imazalil	(ECHA, 2012a)	(ECHA, 2013a)	(ECHA, 2013d)
Piperonyl Butoxide	(ECHA, 2019f)	(ECHA, 2020g)	(ECHA, 2020q)
Biphenyl-2-ol	(ECHA, 2021d)	(ECHA, 2022b)	(ECHA, 2022i)
Pethoxamid	(ECHA, 2022g)	(ECHA, 2023a)	(ECHA, 2023b)
S-metolachlor	(ECHA, 2021e)	(ECHA, 2022c)	(ECHA, 2022j)
Penflufen	(ECHA, 2017d)	(ECHA, 2018b)	(ECHA, 2018l)
Amisulbrom	(ECHA, 2014b)	(ECHA, 2016b)	(ECHA, 2016f)
Carbetamide	(ECHA, 2014d)	(ECHA, 2015b)	(ECHA, 2015h)
Epsilon-metofluthrin	(ECHA, 2015e)	(ECHA, 2016a)	(ECHA, 2016e)
Fluopyram	(ECHA, 2013c)	(ECHA, 2014a)	(ECHA, 2014f)
Isoflucypram	(ECHA, 2018f)	(ECHA, 2020f)	(ECHA, 2020j)
Sedaxane	(ECHA, 2018i)	(ECHA, 2019c)	(ECHA, 2019q)
Cyproconazole	(ECHA, 2014e)	(ECHA, 2015c)	(ECHA, 2015i)
Transfluthrin	(ECHA, 2019k)	(ECHA, 2021c)	(ECHA, 2021i)
Trimethylolpropane triacrylate	(ECHA, 2019j)	(ECHA, 2020a)	(ECHA, 2020k)
Isoproturon	(ECHA, 2015f)	(ECHA, 2016c)	(ECHA, 2016g)
Benfluralin	(ECHA, 2019h)	(ECHA, 2021a)	(ECHA, 2021g)
Momfluorothrin	(ECHA, 2014c)	(ECHA, 2015a)	(ECHA, 2015g)
Metazachlor	(ECHA, 2009)	(ECHA, 2011b)	(ECHA, 2011e)
4-methylpentan-2-one	(ECHA, 2018h)	(ECHA, 2019a)	(ECHA, 2019o)
Ammonium pentadecafluorooctanoate	(ECHA, 2010a)	(ECHA, 2011a)	(ECHA, 2011d)
Benthiavalicarb-isopropyl	(ECHA, 2021f)	(ECHA, 2022a)	(ECHA, 2022h)
Clofentezine	(ECHA, 2019l)	(ECHA, 2020b)	(ECHA, 2020m)
Cumene	(ECHA, 2019m)	(ECHA, 2020c)	(ECHA, 2020n)
Difenoconazole	(ECHA, 2020i)	(ECHA, 2021b)	(ECHA, 2021h)
Fluopicolide	(ECHA, 2019n)	(ECHA, 2020d)	(ECHA, 2020o)
Fluxapyroxad	(ECHA, 2017c)	(ECHA, 2018a)	(ECHA, 2018k)
Valifenalate	(ECHA, 2019i)	(ECHA, 2020e)	(ECHA, 2020p)
Pydiflumetofen	(ECHA, 2018d)	(ECHA, 2019d)	(ECHA, 2019r)
Pentadecafluorooctanoic acid	(ECHA, 2010b)	(ECHA, 2011c)	(ECHA, 2011f)
Pyriofenone	(ECHA, 2018g)	(ECHA, 2019b)	(ECHA, 2019p)
Isopyrazam	(ECHA, 2019g)	(ECHA, 2020h)	(ECHA, 2020l)
Silthiofam	(ECHA, 2018j)	(ECHA, 2018c)	(ECHA, 2018m)

Compounds	CLH report	Background document	RCOM (Comments received)
Sulfoxaflor	(ECHA, 2012b)	(ECHA, 2013b)	(ECHA, 2013e)
Thiophanate-methyl	(ECHA, 2018e)	(ECHA, 2019e)	(ECHA, 2019s)

Annex 3. List of methodologies performed to evaluate carcinogenicity as presented in the dossiers

Table A2. This table summarises the different methodologies, carried out in different species, using *in vivo* and *in vitro* models, that provided information about the different key and associative events, through the different mechanistic studies presented in the different dossiers (except KE 5, AE 2 and AE 3, where the information was collected, mainly, from the 2-year assay studies).

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
Propiconazole	M	ND		ND		ND								ND	ND	ND	ND
	R	ND		ND	ND	ND	ND		ND	a)	ND	ND	ND	ND	ND	ND	ND
	H	ND		ND		ND		ND	ND	ND		ND		ND	ND	ND	ND
Imazalil	M	ND	ND		ND	ND	ND					ND		ND	ND	b)	ND
	R	ND	ND		ND	ND	ND		ND		ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND
Piperonyl Butoxide	M	ND	ND				ND	ND	ND			ND			ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND		ND		ND	ND	ND	ND
Phenylphenol 2	M	ND		ND		ND	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pethoxamide	M	ND	ND			ND	ND		ND			ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND		ND		ND	ND	ND	ND
Metolachlor	M	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND		ND	ND		ND					ND		ND	ND	ND	ND
	H	ND		ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND
Penflufen	M	ND	ND		ND	ND	ND		ND		ND	ND	ND	ND	ND	ND	ND
	R	ND	ND		ND	ND	ND					ND		ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND
Amisulbrom	M	ND	ND		ND	ND	ND		ND		ND		ND	ND	ND		ND
	R	ND	ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND	ND
	H	ND	ND	ND	c)	ND	ND	ND	c)	ND	ND	ND	ND	ND	ND	ND	ND
Carbetamide	M	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
Epsilon-metofluthrin	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND					ND		ND			ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND		ND		ND	ND	ND	ND	ND	ND
Fluopyram	M	ND	ND	ND	ND	ND	ND		ND		ND	ND	ND		ND	ND	ND
	R	ND	ND		ND	ND	ND	ND		ND		ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND		ND	ND	ND		ND	ND	ND	ND
Isoflucypram	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sedaxane	M	ND			ND		ND		ND		ND	ND	ND	ND	ND	ND	ND
	R	ND		ND		ND	ND	ND				ND	ND	ND	ND	ND	ND
	H	ND		ND	ND	ND	ND	ND		ND		ND	ND	ND	ND	ND	ND
Cyproconazole	M	ND	ND				ND		ND			ND			ND	ND	ND
	R	ND	ND	ND	ND	ND	ND		ND		ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND
Transfluthrin	M	ND	ND	ND		ND	ND	ND		ND		ND		ND	ND	ND	ND

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND
Trimethylopropane triacrylate	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isoproturon	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND			ND		ND	ND	ND		ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND
Benfluralin	M	ND	ND	ND	ND	ND	ND	ND	ND	ND		ND		ND	ND	ND	ND
	R	ND	ND		ND	ND	ND		ND					ND		ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND		ND		ND	ND	ND	ND
Momfluorothrin	M	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND				ND	ND		ND			ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND
Metazachlor	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND		ND	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Methylpentan	M	ND	ND		ND	ND	ND		ND		ND	ND	ND		ND	ND	ND
	R	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ammonium pentadecafluorooctanoate	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benthiavalicarb-isopropyl	M	ND	ND				ND					ND		ND		ND	ND
	R	ND	ND	ND	ND		ND	ND	ND		ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND		ND		ND	ND	ND	ND
Clofentezine	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cumene	M	ND	ND	ND	ND	ND	ND		ND		ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Difenoconazole	M	ND					ND			ND		ND			ND		ND

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
	R	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND		ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND
Fluopicolide	M	ND	ND	ND	ND	ND	ND					ND		ND		ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND
Fluxapyroxad	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND		ND		ND	ND					ND		ND		ND	ND
	H	ND	ND	ND		ND	ND	ND		ND		ND		ND	ND	ND	ND
Valifenalate	M	ND	ND			ND	ND					ND		ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pydiflumetofen	M	ND		ND	ND	ND	ND					ND		ND	ND	ND	ND
	R	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND		ND	ND	ND	ND	ND		ND		ND		ND	ND	ND	ND
Pentadecafluorooctanoic acid	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Compound	Species	Methodologies performed															
		CAR activation (luciferase reporter assays, siRNA)		CYP gene expression (mRNA levels by Q-PCR)		CYP protein expression (western blot/ ELISA)		CYP activity induction (BROD, EROD, PROD)		Cell proliferation (PCNA/BrdU)		Cytotoxicity		CAR/PXR KO		hPXR/hCAR	
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
Pyriofenone	M	ND	ND	ND	ND	ND	ND		ND		ND	ND	ND	ND	ND	ND	ND
	R	ND	ND				ND		ND			ND	ND		ND	ND	ND
	H	ND	ND	ND		ND	ND	ND	ND	ND		ND		ND	ND	ND	ND
Isopyrazam	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND					ND		ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Silthiofam	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND				ND					ND		ND		ND	ND
	H	ND	ND	ND		ND	ND	ND		ND		ND		ND	ND	ND	ND
Sulfoxaflor	M	ND	ND		ND		ND		ND		ND	ND	ND		ND		ND
	R	ND	ND		ND	ND	ND		ND		ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND
Thiophanate-methyl	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

"M": Mouse; "R": Rat; "H": Human; "KE": key events; "AE": Associative events; "ND": Not determined; "Cyp": Cytochromes P450; "a)": Electron microscopy of histological slides; "b)": Reacted the same way as WT mice; "c)": Results from humanised mice model.

Table A3. Overall studies performed to identify key and associated events for each dossier.

Compound	Species	Key events according to IPCS framework																CAR MoA
		KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
Propiconazole	M	Y	Y	ND	Y	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	YES
	R	Y	Y	ND	ND	ND	ND	Y	ND	NO	ND	Y	ND	ND	ND	Y	ND	
	H	ND	Y	ND	Y f)	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Imazalil	M	Y	ND	Y	ND	I	Y	Y	ND	Y	ND	Y	Y	NO	ND	Y	ND	NO
	R	I	ND	NO	ND	NO	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	Y c)	NO d)	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	
Piperonyl Butoxide	M	Y	Y	Y	Y	Y	Y	NO	ND	Y	ND	Y	Y	Y	ND	Y	ND	YES
	R	ND	ND	ND	ND	ND	ND	Y	ND	NO	ND	ND	ND	NO	ND	Y	ND	
	H	ND	Y	ND	Y	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Phenylphenol 2	M	ND	NO	ND	NO	ND	NO	Y	ND	Y e)	ND	ND	ND	ND	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	NO	ND	NO	ND	ND	ND	ND	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Pethoxamide	M	Y	Y	Y	Y	Y	Y	NO	ND	Y	ND	Y	ND	Y	ND	Y	ND	NO

Compound	Species	Key events according to IPCS framework																CAR MoA
		KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
	R	ND	ND	Y	ND	ND	ND	NO	ND	NO	ND	ND	ND	ND	ND	ND	ND	
	H	ND	Y	ND	Y	ND	NO d)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Metolachlor	M	ND	ND	ND	ND	ND	ND	NO	ND	NO	ND	ND	ND	ND	ND	Y	ND	NO
	R	Y	Y	ND	ND	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	ND	ND	
	H	ND	Y	ND	ND	ND	NO	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	
Penflufen	M	Y	ND	Y	ND	Y	ND	NO	ND	Y	ND	Y	Y	Y	ND	Y	ND	NO
	R	Y	Y	Y	ND	Y	Y	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	ND	I	ND	ND	ND	I	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	
Amisulbrom	M	Y	ND	Y	ND	Y	ND	ND	ND	Y	ND	Y	ND	NO	ND	Y	ND	NO
	R	Y	ND	ND	ND	Y	ND	ND	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	Y c)	ND	Y c)	ND	NO c)	ND	ND	ND	ND	ND	Y c)	ND	ND	ND	ND	ND	
Carbetamide	M	ND	ND	ND	ND	ND	ND	ND	ND	Y	ND	Y	ND	ND	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Epsilon-metofluthrin	M	ND	ND	ND	ND	ND	ND	NO	ND	NO	ND	ND	ND	Y	ND	ND	ND	YES
	R	Y	Y	ND	Y	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	

Compound	Key events according to IPCS framework																
	Species	KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		CAR MoA
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
	H	ND	ND	ND	Y	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Fluopyram	M	ND	ND	ND	ND	ND	ND	NO	ND	NO	ND	Y	ND	Y	ND	Y	ND	YES
	R	Y	Y	Y	ND	ND	Y	Y	ND	Y	ND	ND	Y	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Isoflucypram	M	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	ND	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Sedaxane	M	ND	Y	ND	ND	Y	ND	NO	ND	Y	ND	ND	ND	NO	ND	Y	ND	YES
	R	ND	Y	ND	Y	Y	Y	Y	ND	Y	ND	ND	Y	Y	ND	Y	ND	
	H	ND	Y	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Cyproconazole	M	Y	ND	Y	Y	Y	Y	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	YES
	R	ND	ND	ND	ND	NO	ND	NO	ND	NO	ND	Y	ND	Y	ND	Y	ND	
	H	ND	Y	ND	Y	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Transfluthrin	M	ND	ND	ND	I	ND	I	Y	ND	Y	ND	ND	NO	Y	ND	ND	ND	NO
	R	ND	ND	ND	ND	ND	ND	NO	ND	Y	ND	ND	ND	ND	ND	ND	ND	
	H	ND	ND	ND	I	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

Compound	Species	Key events according to IPCS framework																CAR MoA
		KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
Trimethylopropane triacrylate	M	ND	ND	ND	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	ND	ND	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	ND	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Isoproturon	M	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	ND	ND	NO
	R	Y	ND	ND	ND	ND	Y	Y	ND	Y	ND	Y	Y	ND	ND	Y	ND	
	H	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Benfluralin	M	ND	ND	ND	ND	ND	Y	Y	ND	Y	ND	ND	ND	ND	ND	Y	ND	NO
	R	ND	Y	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Momfluorothrin	M	Y	ND	Y	ND	Y	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	YES
	R	Y	Y	Y	Y	Y	Y	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Metazachlor	M	ND	ND	ND	ND	ND	ND	NO	ND	I	ND	ND	ND	ND	ND	Y	ND	NO
	R	Y	ND	Y	ND	ND	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Methylpentan	M	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	NO

Compound	Species	Key events according to IPCS framework																CAR MoA
		KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Ammonium pentadecafluorooctanoate	M	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	ND	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	Y	ND	ND	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Benthiavalicarb-isopropyl	M	Y	ND	Y	Y	I	Y	ND	ND	Y	ND	Y	Y	Y	ND	Y	ND	NO
	R	Y	ND	ND	ND	Y	ND	ND	ND	Y	ND	Y	ND	ND	ND	Y	ND	
	H	ND	ND	ND	Y	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Clofentezine	M	ND	ND	ND	ND	ND	ND	Y	ND	Y	ND	ND	ND	Y	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Cumene	M	ND	ND	ND	ND	Y	ND	Y	ND	ND	ND	Y	ND	ND	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Difenoconazole	M	Y	Y	Y	Y	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	NO
	R	ND	Y	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	

Compound	Species	Key events according to IPCS framework																CAR MoA
		KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
	H	ND	NO	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Fluopicolide	M	Y	Y	ND	ND	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	YES
	R	ND	ND	ND	ND	ND	ND	Y	ND	NO	ND	ND	ND	Y	ND	Y	ND	
	H	ND	NO	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Fluxapyroxad	M	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	YES
	R	Y	Y	ND	Y	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	
	H	ND	NO	ND	Y	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Valifenalate	M	Y	ND	Y	Y	Y	NO	ND	ND	Y	ND	Y	Y	NO	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Pydiflumetofen	M	Y	Y	ND	ND	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	NO
	R	ND	Y	ND	ND	ND	ND	Y	ND	NO	ND	ND	ND	Y	ND	Y	ND	
	H	ND	Y	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Pentadecafluorooctanoic acid	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	NO
	R	ND	ND	ND	ND	ND	ND	Y	ND	Y	ND	ND	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

Compound	Species	Key events according to IPCS framework																CAR MoA
		KE 1: CAR activation		KE 2: altered gene expression specific to CAR activation		KE 3: Increased proliferation		KE 4: Clonal expansion leading to altered foci		KE 5: Liver adenomas/ carcinomas		AE 1: Increased Cyp2b /CYP2B6 enzyme activity and/or protein		AE 2: Increased liver hypertrophy		AE 3: Increase liver weight		
		In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	
Pyriofenone	M	ND	ND	ND	ND	NO	ND	ND	ND	ND	ND	NO	ND	Y	ND	Y	ND	NO
	R	I	ND	Y	Y	I	Y	NO	ND	NO	ND	Y	ND	Y	ND	Y	ND	
	H	ND	ND	ND	Y	ND	NO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Isopyrazam	M	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	NO
	R	Y	Y	ND	ND	ND	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	
	H	ND	Y	ND	ND	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Silthiofam	M	ND	ND	ND	ND	ND	ND	Y	ND	Y	ND	ND	ND	Y	ND	Y	ND	YES
	R	Y	Y	Y	Y	Y	Y	Y	ND	Y	ND	Y	Y	Y	ND	Y	ND	
	H	ND	Y	ND	Y	ND	NO	ND	ND	ND	ND	ND	Y	ND	ND	ND	ND	
Sulfoxaflor	M	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	Y	ND	YES
	R	Y	ND	Y	ND	Y	ND	ND	ND	Y	ND	Y	ND	Y	ND	Y	ND	
	H	Y c)	ND	Y c)	ND	NO c)	ND	ND	ND	ND	ND	Y c)	ND	NO c)	ND	Y c)	ND	
Thiophanate-methyl	M	ND	ND	ND	ND	ND	ND	ND	ND	Y	ND	ND	ND	Y	ND	Y	ND	NO
	R	ND	ND	ND	ND	ND	ND	ND	ND	NO	ND	ND	ND	Y	ND	Y	ND	
	H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

"IPCS": International Programme on Chemical Safety; "Y": the overall result of the studies demonstrated a positive outcome; "NO": the overall result of the studies demonstrated a negative outcome; "I": the overall of the studies demonstrated inconclusive results; "ND": Not Determined; "M": Mouse; "R": Rat; "H": Human; "KE": key events;

"AE": associative events; "a": electron microscopy of histological slides, "f)" : much weaker that mouse or rat, "b)" : reacted the same way as a WT mice, "d)" : cryopreserved hepatocytes; "e)" : And also hepatoblastoma; "c)" : results from humanized mice model; "MoA": Mode of Action.

Table A4. Summary of the main determinants of CAR MoA for each dossier, *in vitro* assays used for KE and AE evaluation, and RAC opinion: Gaps and observations.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
Propiconazole	YES	I) All the KE and AE of CAR-mediated induction of liver tumors were demonstrated for mice, as seen for PB. II) The activation of human CAR is around 20 times lower than the activation of mouse CAR III) Cell proliferation could not be detected through replicative DNA synthesis in human hepatocytes, while did in mouse hepatocytes.	1) KE 1- CAR activation: CAR3 Transactivation assay with mouse, rat and human CAR 2) KE 2, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, CYP2B6 and CYP3A4 gene expression, cell proliferation).	- No information regarding independent human hepatocyte cultures number. -Absence of data with CAR-KO mouse.
Imazalil	NO	-	-	- hPXR/CAR showed similar results to the WT mice. - No reliable results to take conclusions since there was no consistency among studies.
Piperonyl Butoxide	YES	I) 14-d feeding mouse study, with the oral administration of Piperonyl Butoxide produced about the same induction of CYP enzymes as administration of the CAR activator NaPB, this effect was most pronounced for Cyp2b. II) The comparison of C57BL/6J wild type with CAR/PXR double knockout mice, demonstrated that KO mice lacked the induction of	1) KE 2, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, CYP2B6 and CYP3A4 gene expression, cell proliferation).	- Different strains of mice for mechanistic studies (hampers comparisons); - Only one male and female donor for the human hepatocytes studies; - Only male hepatocytes used for the mouse studies; - Precipitation of the substances *in vitro* human studies but not in *in vitro* mouse studies.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
		Cyp2b when treated with PBO for 14 days via the diet. III) Cultured human hepatocytes did not react with replicative DNA synthesis to treatment with Piperonyl Butoxide, while male mouse hepatocytes did. Cyp2b induction in male human hepatocytes for both NaPB and Piperonyl Butoxide.		
Phenylphenol 2	NO	-	-	- Presented activation studies showed that PPARa was the only receptor activated.
Pethoxamide	NO	-	-	- Primary human hepatocytes not suited for the evaluation of hepatocellular proliferation; - No *in vivo* studies with CAR/PXR-KO animals or hCAR animals to confirm CAR MoA.
Metolachlor	NO	-	-	- Lack of experiments to exclude other MoA (no CAR knockout hepatocyte or humanised-CAR data; - Lack of PROD activity in human hepatocytes, missing positive control in some studies and effects not always comparable to + control).
Penflufen	NO	-	-	- Limited evidence: cells from a single donor only, no studies with animals containing humanised CAR/PXR; - Uncertainty regarding alternative MoA exclusion and human relevance.
Amisulbrom	NO	-	-	- Key events and AE missing (Inconclusive results from cell proliferation, altered foci and liver hypertrophy)

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
Carbetamide	NO	-	-	- Lack of evidence for CAR MoA: i) activation of the CAR nuclear receptor; ii) hepatocyte proliferation, and; iii) evidence that other modes of action are not operative.
Epsilon-metofluthrin	YES	I) Epsilon-metofluthrin is a CAR activator, inducing CYP 2B1/2 and hepatocellular proliferation, *in vivo*, in rats II) Human hepatocytes were found to be completely unresponsive to mitogenic stimulation with epsilon-metofluthrin and phenobarbitone in two separate investigations using male and female donors. In contrast, rat hepatocytes responded with increased replicative DNA synthesis. Both human and rat hepatocytes responded to growth factor stimulated mitogenesis, demonstrating that they were responsive preparations.	1) KE 1- CAR activation: Cultured hepatocytes transfected with siRNA (CAR) and siRNA (control) 2) KE 2, AE 1, KE 3: *In vitro* rat hepatocytes experiments (cytotoxicity, CYP2B10 and CYP3A11 gene expression, CYP enzyme activity, cell proliferation) 3) KE 2, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, CYP2B6 and CYP3A4 gene expression, cell proliferation).	- Uncertainties raised on the immaturity of human cell transfected, specificities associated with this mice strain.
Fluopyram	YES	I) Possible contribution of others MoA sufficiently excluded II) Activation of the CAR shown in knockout mice III) Specific CYP enzyme induction (CYP 2B family) including hypertrophy of liver IV) Increased hepatocellular proliferation in the rat V) Lack of hepatocellular	1) AE 1, KE 3, Human relevance: *In vitro* human hepatocytes experiments. (cytotoxicity, CYP enzyme activity, cell proliferation).	- Based on the data provided RAC considers that it has been demonstrated that a CAR mediated MoA contributes to the formation of liver tumors.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
		proliferation (S-phase) in human hepatocytes VI) Reversibility of effects		
Isoflucypram	NO	-	-	- Long-term rat study does not satisfy the MTD requirement; - Inconclusive data.
Sedaxane	YES	I) CAR and/or PXR activation in the liver. II) Altered expression of CAR-responsive genes that promoted a pro-proliferative and anti-apoptotic environment in the liver and an early transient increase in hepatocellular proliferation. III) Increased hepatocellular foci, as a result of clonal expansion of spontaneously mutated cells in the mouse and rat resulted in slight increases in liver tumor incidences compared to concurrent controls. IV) Associative events including: increased expression of genes encoding cytochrome P450s, increased microsomal (endoplasmic reticulum) proliferation and hepatocellular hypertrophy and increased liver weight. V) Similar to phenobarbital, sedaxane did not induce DNA replication (prerequisite for tumor formation) in human hepatocytes following induction of	1) KE 1 - CAR activation: CAR3 Transactivation assay with mouse, rat and human CAR 2) KE 2, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (CYP2B6 and CYP3A4 gene expression, CYP enzyme activity, cell proliferation).	- Studies including CAR-KO-mice and convincing data on cell proliferation and more human hepatocyte donors would have increased RAC´s confidence in the assessment.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
		human CAR, in contrast to rat. Due to this qualitative difference, the liver tumors as a result of CAR-activation by sedaxane were considered to be of little relevance to humans		
Cyproconazole	YES	I) CAR activation was demonstrated by the observed increase in Cyp2b transcription levels and with the associative event of enzyme expression and activation upon cyproconazole treatment, *in vivo* (mice) and *in vitro* (mice and human hepatocytes). Supportive associative events included increased liver weight and microscopic hepatocellular hypertrophy II) Increase in hepatocellular proliferation in mice and rats, and not in CAR-knockout mice (similar to PB) III) Similiar to PB, cyproconazole does not induce DNA replication in human hepatocytes, in contrast to mice.	1) KE 2, KE 3: *In vitro* mouse hepatocytes experiments (cytotoxicity, CYP2B10 and CYP3A11 gene expression, cell proliferation) 2) KE 2, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, CYP2B6 and CYP3A4 gene expression, cell proliferation).	- Mechanistic studies demonstrated a behavior similar to phenobarbital and so, not relevant for humans.
Transfluthrin	NO	-	-	- No study using hCAR/PXR or KO mice to demonstrate specificity for the CAR mechanism of action; - Problems of solubility; - Most of *in vitro* studies with mouse and human hepatocytes did not allow a clear conclusion for the MoA in liver tumor.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
Trimethylopropane triacrylate	NO	-	-	- Tumour types observed on carcinogenicity studies are rare but given a lack of mechanistic data, their relevance for humans cannot be excluded.
Isoproturon	NO		-	- No information available from the dossier on RAC opinion.
Benfluralin	NO	-	-	- No CAR/PXR KO animal studies; - Inconclusive *in vitro* studies with PB as + control; - Not enough evidence to presume the CAR MoA is plausible in this case, significant uncertainties and a lack of in vivo investigations promotes caution.
Momfluorothrin	YES	I) Others MoA are excluded II) Evidence was present for the key events and some of the associative events in this MoA, also with respect to dose-response relation and temporal association. III) The *in vitro* study with rat hepatocytes in which the CAR gene was knocked down, showed that CAR activation is involved in the induction of CYP2B1/2 mRNA by 1R-trans-Zmomfluorothrin (key event 1). IV) CYP2B induction *in vivo* and *in vitro*: *in vivo* and *in vitro* in rat hepatocyte increased CYP2B1/2 mRNA expression (key event 2) and CYP2B activity (associative event). V) Increased liver weights and increased hepatocellular hypertrophy (associative event)	1) KE 1 - CAR activation: Transfection of primary rat hepatocytes with CAR siRNA (short interfering RNA specific to CAR) 2) KE 2, KE 3: *In vitro* rat hepatocytes experiments (CYP2B10 and CYP3A11 gene expression, cell proliferation) 3) KE 2, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (CYP2B6 and CYP3A4 gene expression, cell proliferation).	- RAC agrees with the DS that CAR activation is the most plausible mechanism behind the liver tumor formation in the rat

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
		were observed in all toxicity (short- and long-term) and MoA studies in rats and mice. Evidence for increased cell proliferation (key event 3) was provided in the rat and mouse MoA studies and in an in vitro study with rat hepatocytes. VI) Increased incidences of eosinophilic foci (key event 4) and liver tumours (key event 5) in rats. VII) As to the relevance to humans of this MoA, the in vitro study with human hepatocytes has shown that CAR activation is also possible in humans however, without replicative DNA synthesis in human hepatocytes induction, in contrast to rat hepatocytes.		
Metazachlor	NO	-	-	- Metazachlor appears to have potential to activate CYP2B enzymes, is capable to activate CAR and stimulates proliferation of rat liver cells. It is found that there are some similarities to a phenobarbital-like response. However there are also some inconsistencies (lack of tumour response in mice, indications on cytotoxicity) and data are not yet sufficient to conclude that CYP mediated CAR activation is the only critical key event; - A mode of action was not unanimously identified for the liver tumours and in conclusion the observed induction of liver tumours could not be ruled out as of no relevance for humans.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
Methylpentan	NO	-	-	- The proposed MoA is plausible in mice. Nevertheless, the MoA is not sufficiently investigated. Some limitations were noted: no + controls, increase in liver weight and hypertrophy in CAR KO mice; no CAR activation in high throughput assay data.
Ammonium pentadecafluorooctanoate	NO	-	-	- Beyond the question on whether biological responses related to activation of PPAR are of relevance for humans, there is still some degree of uncertainties with the significance of other nuclear receptor activation on tumour growth and RAC follows argumentation of the dossier submitter that other MoA can not fully be excluded.
Benthiavalicarb-isopropyl	NO	-	-	- Remaining uncertainties with regard to alternative MoAs (AhR, no *in vivo* knock-out study, Wnt/ß-Catenin signalling not fully excluded). In addition, cytotoxicity is assumed to contribute to tumour formation; - Human relevance cannot be excluded.
Clofentezine	NO	-	-	- There is no clear evidence that CAR receptor activation is involved in the tumourigenic action of clofentezine in the liver of CD-1 mice. The absolute certainty on CAR involvement could have been confirmed with a CAR-knock-out mouse study and the evidence for cell proliferation could have been strengthened with an *in vitro* comparative cell proliferation study (mouse, rat, human). Further enzyme induction studies might also have been done.
Cumene	NO	-	-	- RAC concludes that the carcinogenic signal in female mice is not very strong and the proposed CAR/PXR mediated MoA is plausible. However, not all mechanistic studies required to demonstrate this MoA are available and some findings in the

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
				newly submitted study do not support the proposed MoA. Importantly, human relevance has not been investigated. In conclusion, the relevance of the observed tumours for humans cannot be completely dismissed.
Difenoconazole	NO	-	-	- RAC concludes that the carcinogenic signal in female mice is not very strong and the proposed CAR/PXR mediated MoA is plausible. However, not all mechanistic studies required to demonstrate this MoA are available and some findings in the newly submitted study do not support the proposed MoA. Importantly, human relevance has not been investigated. In conclusion, the relevance of the observed tumours for humans cannot be completely dismissed.
Fluopicolide	YES	1) Treatment resulted in the activation of CAR and weak activation of the PXR in the liver. This led to altered expression of CAR-responsive genes that promoted a pro proliferative and anti-apoptotic environment in the liver and an early, transient, increase in hepatocellular proliferation. 2) Increased hepatocellular foci because of clonal expansion of spontaneously mutated cells in the mouse resulted in slight increases in liver adenomas incidence compared to concurrent controls. This MoA was supported by a series of associative events including: increased expression of genes	1) AE 1, KE 3: *In vitro* mouse hepatocytes experiments (cytotoxicity, CYP enzyme activity, cell proliferation) 2) AE 3, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, CYP enzyme activity, cell proliferation)	- RAC agrees with the dossier submitter that the available data provide enough evidence to support the postulated MoA (CAR activation) to be the underlying MoA of liver adenomas observed in mice. Similar to phenobarbital (a known CAR inducer), fluopicolide did not induce DNA replication (prerequisite for tumour formation) in human hepatocytes nor in CAR/PXR KO mouse hepatocytes following induction of human CAR, in contrast to rats. Due to this qualitative difference, the liver adenomas because of CAR-activation by fluopicolide were considered to be of little relevance to humans.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
		encoding CYPs, particularly CYP2B and (to a lesser extent) CYP3A isoforms, increased proliferation and hepatocellular hypertrophy and increased liver weight. 3) No cell proliferation was observed in CAR/PXR KO mouse hepatocytes or human hepatocytes (from 3 donors) after treatment		
Fluxapyroxad	YES	1) Key mechanistic events demonstrated CAR-activation in the wild-type (WT) Sprague-Dawley and Wistar rats but not in CAR-KO SD rats. 2) Studies in primary human hepatocytes demonstrated that the initial key events of the proposed CAR mediated mechanism, i.e. CAR activation and alteration of gene expression specific to CAR can also occur in human hepatocytes. However, proliferation (essential for subsequent tumour formation) is not observed in primary human hepatocytes.	1) KE 3: *In vitro* experiments with rat microsomes (CYP enzyme activity) 2) KE 2, AE 1, KE 3: *In vitro* rat hepatocytes experiments (cytotoxicity, CYP enzyme activity, CYP2B10 and CYP3A11 gene expression, cell proliferation) 3) KE 2, AE 1, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, CYP enzyme activity, CYP2B6 and CYP3A4 gene expression, cell proliferation)	- The available experimental data for fluxapyroxad indicate that the CAR-mediated MoA is the most likely mechanism for induction of rat liver tumours.
Valifenalate	NO	-	-	- Role of AhR in the mechanism of action cannot be totally ruled out. - Inconsistencies detected in the study with PPAR-α mice, where, moreover, lack of positive control was detected; - Lack of data with CAR/PXR knock-out mice; - Lack of data with human hepatocytes; - Fails in the valifenalate to induce *in vitro* changes in

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
				biochemistry of hepatocytes without evidences that hepatocytes were not metabolically competent.
Pydiflumetofen	NO	-	-	- There is positive data regarding CAR/PXR receptor activation the mode of action, however weight of evidence is missing: - No study was presented involving CAR/PXR-KO or hCAR/PXR models The level of increase in DNA replicative synthesis in mouse cells does not seem to follow a convincing dose response relationship. - Only one donor was used regarding the human hepatocytes study - Also noted is the greater sensitivity of the human hepatocytes to cytotoxicity with pydiflumetofen treatment. - It was also observed toxicity on mouse hepatocytes, but just at higher concentrations. Based on such limited test samples it is difficult to conclude on a qualitative difference in the established CAR activation MoA. - Data to investigate alternative modes of action is also limited. - An explanation for the differential sensitivity between male and female mice with respect to the development of liver tumours is also lacking. Overall RAC considers insufficient evidence has been presented to indicate no concern for human health; there is insufficient data to conclude on other alternative modes of action; and that whether the sole MoA for liver tumours in mice were secondary to hepatocellular proliferation induced by activation of the CAR/PXR nuclear receptors has not been adequately addressed.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
Pentadecafluorooctanoic acid	NO	-	-	- Much of the response to APFO can be attributed to PPARa and induction of PPARa regulated genes. The impact of activation of PPARγ-regulated genes that are proposed to interfere with mitochondrial DNA transcription biogenesis and with lipid and glucose metabolism on tumour growth is not known to the rapporteurs. Beyond the question on whether biological responses related to activation of PPARa are of relevance for humans, there is still some degree of uncertainties with the significance of other nuclear receptor activation on tumour growth and RAC follows argumentation of the dossier submitter that other mode of actions can not fully be excluded.
Pyriofenone	NO	-	-	- CAR mode of action appears to be a plausible explanation for the increase in liver tumours observed in male rats treated with pyriofenone. However, a number of uncertainties remain.
Isopyrazam	NO	-	-	- RAC notes that the liver carcinogenicity induced by isopyrazam in rats is consistent with mode of action based on the CAR activation that is not relevant to humans. However, RAC notes that other possible mechanisms of action have not been sufficiently ruled out and therefore the relevance of the isopyrazam-induced hepatocarcinogenicity for humans has to be considered. Uncertainties: 1 - Lack of data with CAR-knock out animals 2- Data with human hepatocytes have been generated with a single donor 3- The AOP 41 (Sustained AhR Activation leading to Rodent Liver Tumours) has not been fully ruled out 4- The AOP 32 (Inhibition of iNOS, hepatotoxicity, and regenerative proliferation leading to liver tumours) has not been verified 5- The AOP 46 (AFB1: Mutagenic Mode-of-Action leading to

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
				Hepatocellular Carcinoma) has not been fully verified. 6- Isopyrazam is a succinate dehydrogenase inhibitor
Silthiofam	YES	1) Activation of CAR and PXR nuclear receptors 2) Increased hepatocellular proliferation. 3) Exclusion of alternative MOA 4) Weaker CAR activation and no cell proliferation in primary human hepatocytes.	1) KE 1- CAR activation: *In vitro* rat CAR-KO/PXR-KO hepatocytes experiments (cytotoxicity, CYP enzyme activity, CYP2B10 and CYP3A11 gene expression, cell proliferation) 2) KE 2, AE 1, KE 3: *In vitro* WT rat hepatocytes experiments (cytotoxicity, CYP enzyme activity, CYP2B10 and CYP3A11 gene expression, cell proliferation) 3) KE 2, AE 1, KE 3 and Human relevance: *In vitro* human hepatocytes experiments (cytotoxicity, enzyme activity, CYP2B6 and CYP3A4 gene expression, cell proliferation)	- RAC agrees that the proposed MoA could be plausible in male rats. Nevertheless, the following uncertainties remained: - Absence of dose-response data for CAR/PXR activation (only a single dose tested); - No decrease in apoptosis as a consequence of alterations in gene expression was noted; - No hypertrophy was observed in rats in the carcinogenicity study; this finding would also have been expected since it was seen after 14 days; - No *in vivo* studies using CAR/PXR knock out animals were performed to confirm the *in vitro* results; - No exclusion of AhR activation; - Sex differences in tumour induction have not been investigated. Indeed, no mechanistic data in female rats (*in vitro* and *in vivo*) have been provided.
Sulfoxaflor	YES	1) Robust data showing the MoA for sulfoxaflor-mediated liver effects to be PB-like 2) Absence of hepatocellular proliferation in sulfoxaflor-treated humanized (and knockout) PXR/CAR mice shows no human relevance of rodent liver tumors	ND	- It was concluded that the key events are consistent with a CAR-mediated, PB-like MoA, for which there is a high level of confidence.

Compound	CAR MoA	Determinant events for accepting CAR MoA	*In vitro* assays used for KE and AE evaluation	RAC opinion: Gaps and observations
Thiophanate-methyl	NO	-	-	- The study on mice reported hepatocellular adenomas below the maximum tolerated dose; - The hepatocellular carcinomas observed in mice were not clearly dose-dependent. It was noted that the mode of action is not clear.

"RAC": Risk Assessment Committee; "KE": key events; "AE": associative events; "ND": Not Determined; "CAR": Constitutive androstane receptor; "PPAR": Peroxisome proliferator-activated receptor; "AhR": Aryl hydrocarbon receptor; "PXR": Pregnane X receptor; "MoA": Mode of Action; "AOP": Adverse Outcome Pathway; "PB": phenobarbital.

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