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Reducing animal use in carcinogenicity testing

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Reducing animal use in carcinogenicity testing

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Editorial: Reducing animal use in carcinogenicity testing

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KEYWORDS

regulatory toxicology, carcinogenicity testing, 3Rs, Non-genotoxic risk assessment, alternative methods

Editorial on the Research Topic
Reducing animal use in carcinogenicity testing

Introduction

Internationally, new pharmaceuticals for human use are among the chemical substances for which regulatory authorities require the evaluation of carcinogenic potential with long-term studies in rats and mice. Large numbers of animals are used in these studies and in many cases, they endure prolonged distress. Therefore, reducing the number of animals used, as well as the duration of exposure, benefits animals. Reducing the time needed for preclinical development also benefits patients.

Although focusing mainly on studies in rats, the Addendum to the ICH S1B (R1) (International Council for Harmonisation, 2022) Guideline on testing for carcinogenicity of pharmaceuticals promises to decrease the number of long-term studies in both rats and mice. The greatest reduction in animal use will be achieved by assessing the Weight of Evidence (WoE) to determine whether a study in rats is likely to add value. Consistent with the original S1B Guideline, the Addendum also prioritizes the use of carcinogenicity studies in transgenic mice, which reduce animal use and exposure compared to studies in wild-type mice.

ICH-S1B related articles

Over 12 years, Bourcier et al. from the ICH Expert Working Group evaluated a dataset of 45 compounds for which a prediction of the outcome of the rat study was being tested. Bourcier et al. From industry partners in this process, Vahle et al. presents an in-depth discussion of the types of information sources that are available for the various factors in the WoE approach described in the Addendum. Bassan et al. contribute a similar commentary describing in detail the various steps in this WoE approach. Importantly, these authors emphasize that carefully planning the carcinogenicity evaluation process should start early in drug development.

Relevant approaches from other fields

Using TempO-Seq and microarray data, Ledbetter et al. including authors with the US Environmental Protection Agency) report the development of a 5-day rat study that identifies gene expression biomarkers linked to tumorigenic activation by liver carcinogens. While this approach uses animals, it has the potential to reduce animal use and exposure compared to carcinogenicity studies. Further, it could be combined with general toxicity studies to support the WoE assessment to determine whether a carcinogenicity study in rats adds value, as recommended in the Addendum, without increasing the overall number of animals used. Hopefully, it will also facilitate the development of human-based *in vitro* transcriptomic methods.

From the field of agrochemical safety assessment, Goetz et al. highlight a similar move away from the rodent cancer bioassay. As the pharmacological target is not defined in this group of chemicals, defining the biological target is more difficult than with pharmaceuticals. The article addresses this difficulty using case studies that include read-across approaches.

Specific cases

Both Keller et al. and Pillo et al. focus on specific compounds, the human pharmaceutical pregabalin (an antiepileptic also used as a mood-stabilizer) and the plasticizer bis (2-ethylhexyl) phthalate (DEHP), respectively. Intersecting with Ledbetter et al., Keller et al. describe *in silico* approaches in carcinogenic hazard assessment that emphasize toxicological modes-of-action that include oxidative stress, chronic inflammation, and cell proliferation.

Pillo et al. provide an overview of the carcinogenic activity and molecular mechanisms of DEHP, identifying multiple molecular signals that appear to be involved in its carcinogenicity. While some endpoints, such as PPARα-activation, are probably not relevant to human risk assessment, other mechanisms might also be involved. DEHP did not induce transformation in BALB/c-3T3 cells; however, the transcriptomic results demonstrate specific modulations of genes and cell-regulation signaling pathways. Such "transformics" assays show promise for minimizing the use of animal testing for carcinogenicity assessment.

Future use of databases

Finally, Karamertzanis et al. describe a database based upon the use of the pharmacotherapeutic criteria (ATC-code) and species/strain information in 520 carcinogenicity studies. As the full database also includes information from repeat-dose toxicity studies, it can be used to correlate histopathological findings with carcinogenicity, providing support for using WoE assessments to determine whether carcinogenicity studies are likely to add value.

Discussion and conclusion

These eight papers clearly fit into an important development in the toxicological world, i.e., the reduction of animal use in risk assessment. In addition to human pharmaceuticals, these contributions describe important approaches for agrochemicals and can be applied in other fields, such as industrial chemicals.

The Addendum to the ICH S1B (R1) International Council for Harmonisation (2022) indicates that "emerging technologies" might be used for additional investigations. The contributions to this Research Topic, such as Ledbetter et al., Goetz et al. and Keller et al., all illustrate the value of these emerging technologies.

More than 20 years ago, the use of transgenic mice was introduced as an additional option with various pro-oncogenic approaches, e.g., by introducing human ras-oncogene in rasH2-Tg mice. At that time, it was an emerging technology to enhance the detection of human relevant nongenotoxic compounds based upon a mechanistic principle. The original ICH S1B Guideline clearly indicates its usefulness as an alternative to life-time studies with wild type mice.

In this Research Topic, none of the papers on new methodologies refer to the use of rasH2-Tg mice, although the carcinogenic potentials of various compounds in the Prospective Evaluation Study reviewed by Bourcier et al. were determined based partially on a study with this strain. The question can be raised whether the added value of the use of rasH2-Tg mice can still be recognized.

The data from the Prospective Evaluation Study reviewed by Bourcier et al. indicate that by applying the WoE approach, even without data on recent emerging technologies, 27% of the studies could have been dismissed (unanimous decisions in 12/45 CAD's in Cat. 3A/3B), which is already an important result. The emerging technologies described in the other contributions to this Research Topic raise hope that the percentage of WoE assessments indicating there is "no-added value" in conducting a study in rats will increase in the near future.

Author contributions

JWL: Conceptualization, Writing–original draft, Writing–review and editing. JM: Conceptualization, Writing–original draft, Writing–review and editing.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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In silico approaches in carcinogenicity hazard assessment: case study of pregabalin, a nongenotoxic mouse carcinogen

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In silico toxicology protocols are meant to support computationally-based assessments using principles that ensure that results can be generated, recorded, communicated, archived, and then evaluated in a uniform, consistent, and reproducible manner. We investigated the availability of *in silico* models to predict the carcinogenic potential of pregabalin using the ten key characteristics of carcinogens as a framework for organizing mechanistic studies. Pregabalin is a single-species carcinogen producing only one type of tumor, hemangiosarcomas in mice via a nongenotoxic mechanism. The overall goal of this exercise is to test the ability of *in silico* models to predict nongenotoxic carcinogenicity with pregabalin as a case study. The established mode of action (MOA) of pregabalin is triggered by tissue hypoxia, leading to oxidative stress (KC5), chronic inflammation (KC6), and increased cell proliferation (KC10) of endothelial cells. Of these KCs, *in silico* models are available only for selected endpoints in KC5, limiting the usefulness of computational tools in prediction of pregabalin carcinogenicity. KC1 (electrophilicity), KC2 (genotoxicity), and KC8 (receptor-mediated effects), for which predictive *in silico* models exist, do not play a role in this mode of action. Confidence in the overall assessments is considered to be medium to high for KCs 1, 2, 5, 6, 7 (immune system effects), 8, and 10 (cell proliferation), largely due to the high-quality experimental data. In order to move

Abbreviations: bFGF, basic fibroblast growth factor; EC, Endothelial cell; GLP, Good laboratory practices; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; IST, In silico toxicology protocol; KC, Key characteristic; MOA, Mode of action; OECD, Organisation for Economic Cooperation and Development; PDGF, Platelet-derived growth factor (Q)SAR, Quantitative structure-activity relationship; ROS, Reactive oxygen species; RS, Reliability score; TD50, Toxic dose 50; TPO, Thrombopoietin; VDCC, voltage-dependent calcium channel; VEGF, Vascular endothelial growth factor.

away from dependence on animal data, development of reliable *in silico* models for prediction of oxidative stress, chronic inflammation, immunosuppression, and cell proliferation will be critical for the ability to predict nongenotoxic compound carcinogenicity.

KEYWORDS

In silico toxicology protocol, mode of action, pregabalin, non-genotoxic carcinogen, oxidative stress, chronic inflammation, cell proliferation

1 Introduction

Cancer is a multifaceted, multimodal disease. Whereas advances in cancer treatment over the last five decades have been remarkable (Arnold, et al., 2019; Kratzer, et al., 2022), many causes of cancer in the human population are still largely unknown. Given that there are tens of thousands of chemicals in commerce that have not had adequate carcinogenicity testing, there is a need for a swift and reliable assessment of the carcinogenic potential of chemicals. Over 50 years after coming into common use, the 2-year rodent carcinogenicity bioassay is still considered by many regulatory authorities, legislative bodies, industrial entities and other authoritative groups to be the gold standard for assessment of carcinogenicity. This bioassay has many flaws, including low sensitivity, dose levels that are often irrelevant to human exposure, expense, and high animal usage (Cohen, 2017; Goodman, 2018; Madia, et al., 2019). The environmental and agrochemical sectors generally require 2-year rat and mouse studies for carcinogenicity assessment of new chemical entities, with varying levels of acceptance of mechanistic data to modify risk assessment across regulatory bodies. The 6-month transgenic mouse assay has largely supplanted the 2-year mouse study in pharmaceutical development, and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) S1B(R1) guideline allows for a weight of evidence (WoE) assessment to determine the need for a 2-year rat study. However, replacement of the bioassay with alternative methods including *in vitro* or computational tools has not been well accepted as a definitive tool for risk assessment and regulatory purposes.

There are many theories about the origins of cancer and multiple attempts have been made to categorize chemicals into classes of carcinogens for the purposes of hazard or risk assessment (Doll and Peto, 1982; Wolf et al., 2019). Classification systems such as those from the International Agency for Research on Cancer (IARC) and the US National Toxicology Program (NTP) are generally hazard classification systems, with little account for exposure to assess risk. Other classification systems focus mode of action (MOA) (Boobis et al., 2006; Cohen et al., 2019) and also account for exposure where key characteristics are altered to give an estimate of risk. Smith et al. (2016, Smith et al., 2020) describe the ten key characteristics (KC) of carcinogens as an approach to evaluate mechanistic evidence in cancer hazard identification. Many of the KCs described by Smith et al. are also part of the assessment system proposed by Cohen et al. (2019) but are described in more detail and with proposed experimental methods of assessment in the Smith papers. The KCs are a method that can be used to organize data relevant to the MOA of a carcinogen, and to provide a systematic evaluation of cancer hazards.

Tice, et al. (2021) extended these concepts and described the current status and future needs for *in silico* carcinogenicity assessment based on the attributes of the KCs of carcinogens (Smith, et al., 2016; Smith, et al., 2020). In this context, *in silico* (computational) approaches refer to different methodologies that aim at predicting adverse effects from the structure of molecules. These approaches are based on structure activity relationships (SARs) between structural information and biological activity; SARs may be either qualitative or quantitative in nature and are commonly referred to as (Q)SARs. Tice, et al. (2021) make clear that additional *in silico* models are needed to describe many of the KCs for carcinogens in order to expedite the analysis of the potential carcinogenicity of the many thousands of chemicals in commerce (Tice, et al., 2021). Moreover, the ultimate goal is the integration of such *in silico* approaches in a hazard assessment framework of carcinogens in a transparent, consistent, and defendable manner.

This view follows the *in silico* toxicology (IST) protocol initiative for the development of standardized approaches for the prediction of toxicity from a chemical structure (Myatt, et al., 2018; Myatt, et al., 2022). Similar to the published test guidelines for *in vivo* or *in vitro* test methods, the IST protocols are meant to support *in silico* assessments using principles that ensure that results can be generated, recorded, communicated, archived, and then evaluated in a uniform, consistent, and reproducible manner. The protocols define the effects and/or mechanisms to be predicted by the *in silico* methods as part of the assessment of interest. They describe how the data are combined to assess one or more endpoints including creation of an overall confidence score based on a weight of evidence. To further illustrate the needs for *in silico* model development in support of carcinogenicity assessment as well as to gain knowledge for the development of a corresponding IST protocol, an international consortium has undertaken a series of case studies of chemicals and drugs with varying carcinogenicity bioassay outcomes. This consortium workgroup builds upon the efforts that were undertaken to evaluate the extent to which *in silico* models exist for each of the 10 KCs (Tice, et al., 2021).

Here, we report on pregabalin, a compound that is carcinogenic in mice via a nongenotoxic mechanism (Pegg, et al., 2012). The overall goal of this exercise is to test the ability of *in silico* models to predict nongenotoxic carcinogenicity with pregabalin as a case study. Available experimental data and computational model predictions are organized in terms of the KC framework, and gaps in the availability of models are discussed. The 10 KCs conceptual framework (Smith, et al., 2016; Smith, et al., 2020) offers a construct that supports the expert review of available evidence, with a focus on the ability of *in silico* tools to predict the outcome of carcinogenicity studies. Smith, et al. (2020) also present a summary of *in vitro* assays and *in vivo* biomarkers that can be used to investigate certain aspects of modes of action (MOAs) and the KCs.

Insights from Adverse Outcome Pathways are also integrated in such a process. Nongenotoxic rodent carcinogenicity is often considered to be non-relevant to human health (Silva Lima and van der Laan, 2000), but significant experimental research is needed to substantiate that claim. The use of *in vitro* and *in silico* tools to support this process could increase the speed of research and decrease animal use.

The following sections describe, for each of the ten KCs of carcinogens, a summary of the available data for pregabalin, computational modeling of the KC, and modeling and data gaps. Additional details of these data can be found in the references cited. Since pregabalin was not carcinogenic in rats at up to 14 (males) and 24 (females) times the maximum recommended human dose, data described in the KC focus on data from studies in mice or *in vitro*. Confidence in these data and computational approaches is assigned based on criteria described by Johnson et al. (Johnson, et al., 2022).

2 Materials and methods

2.1 Chemical information

Pregabalin (CAS Number 148553-50-8; Figure 1) is a small molecule ligand of the auxiliary α2δ subunit site of certain voltage-dependent calcium channels (VDCCs), and acts as an inhibitor of α2δ subunit-containing VDCCs. It is used to treat seizures, neuropathic pain, and generalized anxiety disorders (Mico and Prieto, 2012; Alles, et al., 2020) and is globally available. The molecular weight of pregabalin is 159 g/mol and the log P is 1.3. It is freely soluble (high solubility) in water.

2.2 Metabolism data

Pregabalin is rapidly absorbed orally, with a bioavailability of approximately 80%. There is no significant metabolism in humans or other species, with the exception of the dog. The major *in vivo* metabolite is N-methyl pregabalin, accounting for <3% of drug-related material in most species, and approximately 45% of drug-related material in the dog. The principal route of excretion is in the urine. Pregabalin is not a CYP inhibitor *in vitro* at concentrations up to 1 mM (EMA, 2005).

2.3 Carcinogenicity data

During development, pregabalin was tested for potential carcinogenicity in 2-year bioassays in mice and rats (Pegg, et al., 2012). Pregabalin did not induce tumors in rats but did in mice. The induced tumors in mice were hemangiosarcomas, primarily in the spleen, liver, and bone marrow. Additional studies were performed to elucidate the mechanism of hemangiosarcoma formation and potential human relevance (Criswell, et al., 2012a; Criswell, et al., 2012b; Criswell, et al., 2012c). These studies showed that pregabalin induces hemangiosarcomas through increased hypoxia and endothelial cell (EC) proliferation in a species-specific manner. In addition to these studies on mice, rats were included in many studies to validate that the effects observed and the mode of action were specific to the mouse.

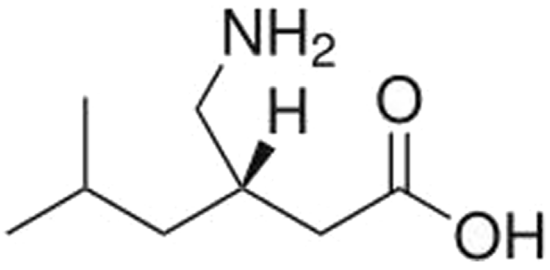

FIGURE 1
Chemical structure of pregabalin.

A search for potency (ChEMBL, 2022) values for pregabalin and structurally similar compounds was performed in ChEMBL (release 31). The Comparative Toxicogenomics Database (CTDB, 2022) was searched for data on pregabalin potentially related to mechanisms of carcinogenicity. The ClarityPV platform (CLARITY, 2023) was searched for neoplasm side effects associated with pregabalin use.

In silico predictions for pregabalin carcinogenicity and carcinogenicity potency were obtained from commercially and freely available *in silico* platforms (Leadscope Model Applier (v. 3.1.0-40), Derek Nexus 6.1.0, VEGA v. 1.3.10, Toxtree v.3.1.0, LAZAR v. 1.4.2).

2.4 Key characteristics

The available experimental data and *in silico* predictions for pregabalin were reviewed by organizing such information within the KC of carcinogens framework. Data were assessed in terms of the reliability score (RS) and relevance as discussed by Myatt et al. (Myatt, et al., 2018) and Johnson et al. (Johnson, et al., 2022), that, at the experimental level, may consider different factors such as compliance with guidelines, concordance with other studies, and/or deviations from test protocols (see Table 1). At the *in silico* prediction level, reliability refers to the extent that an *in silico* result is predictive of an experimental result. On the other hand, the expert conclusions that integrate and combine evidence from various experimental or *in silico* results (each of this can be associated with a specific RS) can be scored according to the confidence categories (high, medium, low, or no confidence), that have been specifically developed for a given toxicological assessment by Johnson et al. (Johnson, et al., 2022). Table 1 summarizes the scoring system adopted in the current work for assessing the reliability of available experimental data and *in silico* predictions (reliability scores) and for assessing the confidence of the conclusions related to the key characteristics (confidence categories).

3 Results

3.1 Key characteristics

3.1.1 KC1: Is electrophilic or can be metabolically activated

3.1.1.1 Experimental data

Pregabalin is not electrophilic based on its absence of activity in bacterial mutagenicity assays that incorporate metabolic

TABLE 1 Reliability scores (RSs) and confidence categories used to respectively assess data and conclusions on KCs in the present work. The RS is applied for assessing experimental data and *in silico* predictions (Myatt, et al., 2018; Johnson, et al., 2022); the RS framework integrates the Klimish scoring system for experimental data (Klimisch, et al., 1997). The confidence categories have been developed to grade the confidence of the assessment of a toxicological endpoint (Johnson, et al., 2022) and they can be applied here to grade the expert conclusions related to the key characteristics of carcinogens.

Reliability of experimental data and *in silico* predictions	
Reliability score	Definition
RS1	Experimental data that are well documented and accepted; data from study performed according to valid and/or accepted test guidelines, preferably following good laboratory practices (GLP). RS1 is not assigned to *in silico* predictions
RS2	Experimental data that are well documented and sufficient; data generally from study not following GLP; partially compliant with test guideline. RS2 is not assigned to *in silico* predictions
RS3	Expert review of available evidence as coming from *in silico* predictions (including read-across) and/or from low reliability experimental studies
RS4	Multiple *in silico* predictions that are in agreement
RS5	Single acceptable *in silico* result or Experimental data not reliable
Confidence of the assessment as coming from combining different pieces of evidence	
Confidence category	Definition
High	A high confidence of the assessment suggests that sufficient evidence is available to support an accurate conclusion, and further research is unlikely to increase the confidence
Medium	A medium confidence of the assessment suggests adequate evidence is available to support an accurate conclusion, but further research might increase the confidence
Low	A low confidence of the assessment suggests that available evidence is lacking to support an accurate conclusion and further research is required to derive any robust conclusion and to improve its confidence
No confidence	A no confidence of the assessment suggests that further research is required for the derivation of an assessment

activation, which are considered to be an acceptable surrogate for the electrophilicity endpoint (Ashby and Tennant, 1988). Furthermore, the lack of significant metabolism in all species tested except for the dog supports a lack of concern about a possible electrophilic metabolite, as none could be generated.

3.1.1.2 *In silico* approaches

Only one metabolite of pregabalin, N-methylpregabalin, has been experimentally isolated and it is a minor component, comprising only a few percent of the dose in all species except the dog (Pharmapendium, 2022). *In silico* predictions for bacterial mutagenicity of N-methyl pregabalin suggested the absence of electrophilicity character. Details of the *in silico* predictions are reported in the Supplementary Material.

3.1.1.3 Reliability and confidence

The experimental data are assigned a reliability score of RS1. The *in silico* prediction for N-methyl pregabalin is assigned a reliability score of RS3. Given the reliability of available evidence and its corresponding relevance for the evaluation of electrophilicity, a medium confidence can be assigned to the conclusion that pregabalin is not electrophilic (or cannot be metabolized to active intermediates).

3.1.1.4 Data gaps

Standard regulatory metabolism studies were performed with pregabalin and are complete for animal species. Additional human metabolism data would increase the confidence in extrapolation of the animal data to humans and better clarify the human relevance. Specific *in vitro* experiments for electrophilicity endpoints would be useful.

3.1.2 KC2: Is genotoxic

3.1.2.1 Experimental data

Several genotoxicity studies using standard Organisation for Economic Co-operation and Development (OECD) test guideline assays were submitted to the U.S, Food and Drug Administration (FDA) during the registration of pregabalin (Pegg, et al., 2012). In bacterial reverse mutation assays, pregabalin was reported negative in *Salmonella typhimurium* strains TA-98, TA-100, TA-1535, and TA-1537 at maximal concentrations of 5,000 μg/plate, with and without metabolic activation, and in *Escherichia coli* WP2*uvr*A at maximal concentrations of 4,980 μg/plate. Additional genotoxicity studies have been conducted on pregabalin and are shown in the Supplementary Table S1 and support a lack of genotoxic activity.

Other experimental data can be evaluated within KC2. Based on an analysis of Tox21 high throughput screening data, at concentration up to 92 μM, pregabalin was negative for induction of ELG1 or p53 and was negative for differential cytotoxicity in the chicken cell DT40 assay using several DNA repair knockout isogenic strains (ICE, 2022). At the concentrations tested in these assays pregabalin was not cytotoxic, indicating that, according to OECD criteria, these tests would not be considered adequate in terms of the maximum concentration tested.

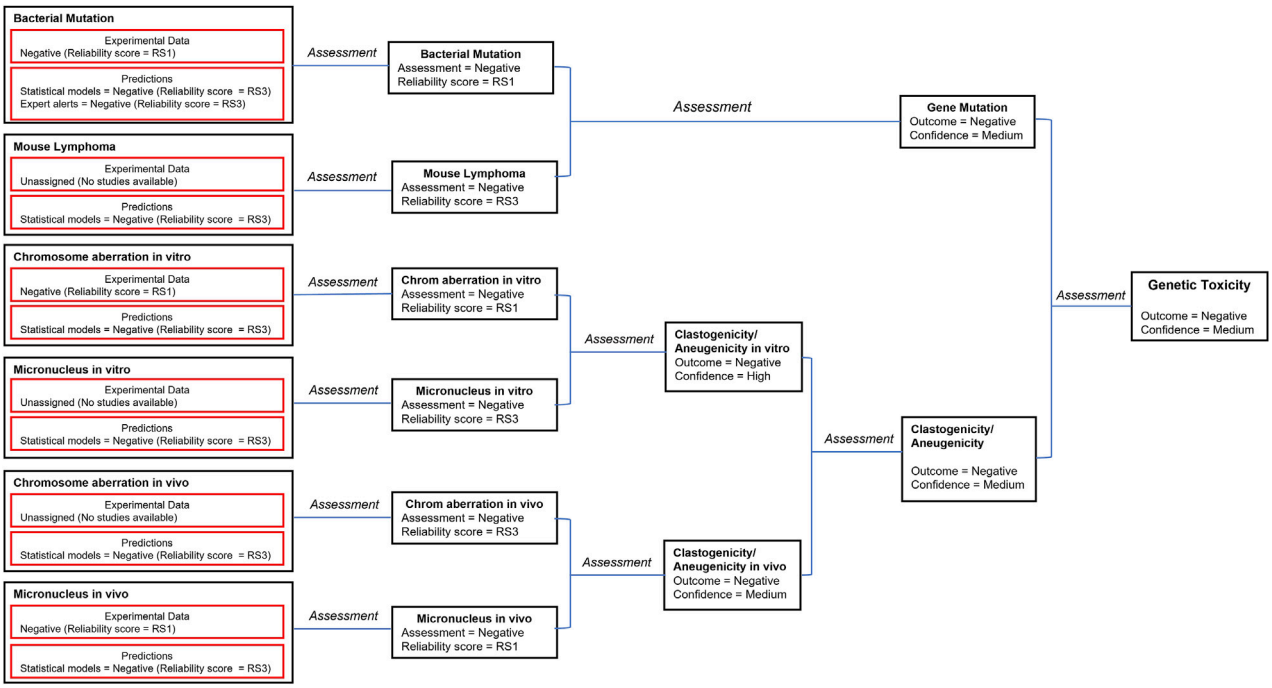

FIGURE 2
The *in silico* assessments most relevant to genotoxicity are combined with available experimental data according to the genetic toxicology *in silico* protocol (Hasselgren, et al., 2019). Selected models and corresponding reliability scores are shown in the figure. The details of available predictions are reported in the Supplementary Material. The reliability score (RS) of each prediction is documented in the Supplementary Material. This is used to derive the overall endpoint confidence (Myatt, et al., 2018; Johnson, et al., 2022) based on the published rules (Hasselgren, et al., 2019).

3.1.2.2 *In silico* approaches

A number of *in silico* models provide predictions that relate to genotoxicity including predictions for mutagenicity in bacteria and mouse lymphoma cells as well for the induction of both *in vitro* and *in vivo* chromosomal aberrations and micronuclei (Tice, et al., 2021). The *in silico* predictions leading to the genotoxicity assessment are shown in Figure 2 (the details of the corresponding predictions are reported in Supplementary Table S2). The genetic toxicology *in silico* protocol formulated by Hasselgren and co-workers (Hasselgren, et al., 2019) is used to integrate all of the available information related to genotoxicity. More specifically, the *in silico* predictions are combined with available experimental data from standardized tests to generate an overall endpoint call; in parallel, the corresponding reliability scores of the assessments are used to derive the overall endpoint confidence (Myatt, et al., 2018; Johnson, et al., 2022). Given the lack of significant metabolism for pregabalin in all species tested but the dog, and the knowledge that models and alerts for bacterial mutation based upon parent compounds using the Ames test (with and without metabolic activation) infers assessment of mutation by the metabolites, an analysis of the genotoxicity of possible metabolites was not conducted.

3.1.2.3 Reliability and confidence

The consensus outcome from the integration of the *in silico* genotoxicity models interrogated with the structure of pregabalin was that the compound was negative for genotoxicity (see Figure 2). Based on consideration of both the experimental results and the *in silico* predictions, the overall confidence is high to medium that pregabalin is not genotoxic, and pregabalin is not classified as a genotoxic compound.

3.1.2.4 Data gaps

Although some of the non-regulatory tests were conducted at lower concentrations, given the totality of the data no significant data gaps exist for genotoxicity endpoints.

3.1.3 KC3: Alters DNA repair or causes genomic instability

3.1.3.1 Experimental data

Pregabalin was inactive in the Tox21 DT40 assays that can reflect DNA repair capabilities (ICE, 2022) (Supplementary Table S3). However, this assay does not directly assess DNA repair, so no adequate data are available.

3.1.3.2 In silico approaches

There are no *in silico* methods available for evaluating this endpoint.

3.1.3.3 Reliability and confidence in the data

A confidence score cannot be assigned. Based on available evidence, a robust conclusion on whether pregabalin alters DNA repair or causes genomic instability cannot be derived.

3.1.3.4 Data gaps

Directly relevant studies in mammalian systems are needed to evaluate this KC.

3.1.4 KC4: Induces epigenetic alterations

3.1.4.1 Experimental data

No direct evidence was identified to link pregabalin to an epigenetic mode of action (LYRICA, 2018). Pregabalin functions as an anti-anxiety drug; however, the mode of action is not fully elucidated (LYRICA, 2018). Epigenetic drugs can be used to treat anxiety disorders (Peedicayil, 2020). So, it is possible that pregabalin treats anxiety by epigenetic therapy, but this relationship has yet to be proven. In addition, a number of reviews highlight the role of epigenetic mechanisms in the pathophysiology and treatment of chronic pain (Descalzi, et al., 2015; Geranton and Tochiki, 2015; Liang, et al., 2015). Therefore, although pregabalin is not currently linked to epigenetic mechanisms, there could be a yet undiscovered epigenetic mechanism in line with the therapeutic properties seen in pregabalin. This topic of epigenetic regulation of neurological activity is receiving increased research and regulatory attention (Banik, et al., 2017; EMA, 2018).

However, in contrast to this, Notartomaso et al. (Notartomaso, et al., 2017) studied different painkillers and suggested that pregabalin was not functioning via an epigenetic mechanism. In the study, mice were injected with pregabalin (30 mg/kg) over a series of different experiments. In this study (Notartomaso, et al., 2017) it was specifically chosen to use pregabalin as an active comparator; pregabalin's known interaction with the α2δ subunit of voltage-sensitive $Ca2^{+}$ channels was used to compare analgesia alongside drugs that enhance acetylation of histones or transcription factors.

There is very little experimental data directly linking pregabalin with any epigenetic interaction or modulation.

3.1.4.2 *In silico* approaches

There are no *in silico* methods for predicting the ability of pregabalin to induce epigenetic alterations.

3.1.4.3 Reliability and confidence

A confidence score cannot be assigned. Based on available evidence, a robust conclusion on whether pregabalin induces epigenetic alterations cannot be derived.

3.1.4.4 Data gaps

Expert literature review found no conclusive link documenting pregabalin with an epigenetic mechanism. As there are no models to address epigenetic modulation directly, this is a gap in our understanding, and, therefore, should be reflected in the confidence of the overall assessment.

3.1.5 KC5: Induces oxidative stress

3.1.5.1 Experimental data

A key event in the mechanism of action for pregabalin carcinogenicity is tissue hypoxia resulting from a sustained alkalosis (Criswell, et al., 2012a; Criswell, et al., 2012b). In mice, chronic tissue hypoxia leads to inflammation (discussed in detail in the next section) characterized by erythrophagocytosis, iron accumulation in macrophages and Kupffer cells, and activated macrophages that release reactive oxygen species (ROS). The inflammation then causes increases in tissue vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) which drive endothelial cell proliferation. Iron deposits in tissues can also lead to increases in ROS. These events do not occur in rats treated with pregabalin (Criswell, et al., 2012a).

Pregabalin was inactive in three p53 assays potentially related to oxidative stress listed in the National Toxicology Program's Integrated Chemical Environment database (ICE, 2022) (Supplementary Table S3).

3.1.5.2 *In silico* approaches

In silico methods are available for some mechanisms that induce oxidative stress (reviewed in (Tice, et al., 2021)). While none were used for pregabalin, models for ROS generation and ARE/Nrf-2 activation could be useful to generate additional understanding of mechanisms involved in carcinogenesis.

3.1.5.3 Reliability and confidence

The experimental data are assigned an overall reliability score of RS2. Given this reliability and consideration of their relevance for the evaluation of oxidative stress, a high confidence can be assigned to the conclusion that pregabalin causes oxidative stress *in vivo*.

3.1.5.4 Data gaps

While the data are considered reliable and relevant, the amount of data on oxidative stress is not large. Additional *in vitro* and *in silico* studies could enhance the understanding of this MOA.

3.1.6 KC6: Induces chronic inflammation

3.1.6.1 Experimental data

Female B6C3F1/CrlBR mice received 1,000 mg/kg bw of pregabalin in the diet for up to 12 months or 5,000 mg/kg bw for up to 29 days (Criswell, et al., 2012c). Dysregulation of angiogenesis and resultant cell death due to chronic hypoxia induced a chronic inflammatory state as evidenced by an increase in activated platelets and an increase in Kupffer cells in the liver and iron-laden macrophages in the bone marrow and spleen in mice, but not in rats. According to the authors (data not shown (Criswell, et al., 2012a),), pregabalin treatment resulted in a dose- and time-dependent increase in activated macrophages in the bone marrow, spleen, and liver in mice–all tissues where hemangiosarcomas were observed. No increases were observed in rats. The authors also reported an increase in the absolute number of white blood cells in treated mice, but the relative distribution of cell types was similar in control and treated animals (data not shown (Criswell, et al., 2012c). Macrophage activation has been shown in other studies to result in cytokine release and subsequent generation of ROS (Corthals, et al., 2006) and platelet activation releases platelet-derived growth factor which is a known chemotactic agent for fibroblasts, vascular smooth muscle cells, and monocytes and can stimulate eosinophils to form superoxide anions (Mannaioni, et al., 1997). In a companion study, addition of vitamin E, an antioxidant, to the mouse diet significantly decreased EC proliferation in mice treated with pregabalin, but not in untreated mice, suggesting that pregabalin treatment was activating EC growth pathways in the mouse,

most likely through ROS and inflammation pathways (Criswell, et al., 2012b).

3.1.6.2 *In silico* approaches

While some *in silico* models exist for various parts of inflammatory cascades, these mechanisms are complex and the understanding of factors that promote and sustain the effect are not fully known.

3.1.6.3 Reliability and confidence

The experimental data are overall assigned a reliability score of RS2. Given this reliability and consideration of their corresponding relevance for the evaluation of chronic inflammation, a high to medium confidence can be assigned to the conclusion that pregabalin induces chronic inflammation.

3.1.6.4 Data gaps

Although inflammation is evident with the increase in activated macrophages in liver, spleen, and bone marrow and activated platelets in the peripheral circulation, specific chemical markers of inflammation (i.e., evidence of cytokine/chemokines or myeloperoxidase in the area) were not reported. Furthermore, the presence of ROS was not experimentally verified by direct measurements, although addition of an antioxidant to the diet (vitamin E) provided indirect evidence that these reactive species are required for tumor formation.

3.1.7 KC7: Is immunosuppressive

3.1.7.1 Experimental data

Data on whether pregabalin exerts direct immunomodulatory effects in mammalian systems is mixed, but the weight of evidence suggests it likely has no direct immunosuppressive effects. Minimal to mild effects on the lymphoid system were observed only at very high doses (≥15-fold the human exposure) in rats and at high doses in monkeys. In Health Authority reviews of the data submitted for registration (US)/marketing (EU), dermatopathy on the tail skin of rats and monkeys was noted in nearly all studies; however, skin lesions in other areas were not reported and the effect was not recapitulated in clinical trials. Follow-up studies (LYRICA, 2018) (Pfizer Report 745-03326) (Pfizer Report 250-01888) evaluating the time course of dermatopathy development and its relationship to a wide variety of immune-related endpoints did not support an immune-related cause.

Pregabalin has been investigated in several animal models of disease (Jang, et al., 2012; Hundehege, et al., 2017) in both prophylactic and therapeutic treatment. No effect on the immune responses was observed. Similarly, Silva et al. (Silva, et al., 2014) showed no significant change in the levels of IL-6, IL-10, IL-27 and TGFb in lymph nodes of mice with experimental autoimmune encephalomyelitis that were treated with pregabalin. And while data from a clinical study (Mercan, et al., 2021) seemed to suggest an association between pregabalin treatment and increased immunologic markers in peripheral blood of individuals with neuropathic pain, when confounders such as comorbidities were removed the data did not show any differences.

Of interest, Gao et al. (Gao, et al., 2020) identified the adaptor protein DOK3 as a key regulator microglial cell activation in a model of neuropathic pain. Here, pregabalin was shown to reduce expression of DOK3 mRNA and the induction of inflammatory biomarkers produced by upregulated DOK3, suggesting a role (direct or indirect) on inflammatory responses. In the GLP toxicology studies in mice (Pegg, et al., 2012), there was an increase in the number of macrophages present in bone marrow, spleen, and liver (5-fold greater than controls after 1 year). In a lipopolysaccharide sepsis model in aged rats, Asci et al. (Asci, et al., 2021) showed that pregabalin can inhibit LPS-induced lesions as shown by changes in several immune system markers. The LPS-induced response, however, likely causes the damage through inflammatory processes so no conclusion can be reached from these data regarding direct immunosuppressive effects of pregabalin. Together, these data suggest a possible role for pregabalin in the inflammatory response which is discussed above, though the data are conflicting (e.g., pro-inflammatory in (Pegg, et al., 2012) and anti-inflammatory (Gao, et al., 2020; Asci, et al., 2021)).

3.1.7.2 *In silico* approaches

There are no *in silico* methods for predicting the ability of pregabalin to induce immunomodulatory changes.

3.1.7.3 Reliability and confidence

The data for pregabalin, which are derived from summaries of the original GLP toxicology studies (Pegg, et al., 2012) are assigned a reliability score of RS1. The remainder of the experimental data are assigned a reliability score of RS3. Given these reliability scores and consideration of their corresponding relevance for the evaluation of immunosuppression, a medium confidence can be assigned to the conclusion that pregabalin does not affect immune system function.

3.1.7.4 Data gaps

No other studies were found in the publicly available literature where pregabalin was specifically evaluated for immunosuppressive activity in normal animals (e.g., T-cell-dependent antibody response, assessment of cell-mediated or innate immunity, or evaluation in host resistance models).

3.1.8 KC8: Modulates receptor-mediated effects

3.1.8.1 Experimental data

Criswell et al. (Criswell, et al., 2012b) described the effects of pregabalin treatment in mice on VEGF, PDGF, bFGF, and thrombopoietin (TPO), as well as VEGFR2. There was no increase in serum VEGF or TPO in the study, while serum PDGF increased by 4-fold after 12 months of treatment, and by 47% after 24 months. No increase was observed after 18 months of treatment. Bone marrow and splenic macrophages and erythroid precursor cells were positive for bFGF staining after 6 months of treatment and were strongly positive after 12 months. VEGF levels were increased in spleen at the highest dose level tested after 6 months of treatment, and VEGF was increased in spleen and sternal bone marrow at 1,000 mg/kg after 12 months. No increased VEGF staining was observed in liver. VEGFR2 levels were increased in EC in the liver of female mice at 1,000 mg/kg after 12 months, but not at lower dose levels.

In vitro, the only activity in a panel of 182 assays in the ICE database was for estrogen receptor agonist activity, with an AC50 of 11.7 μM (ICE, 2022) (Supplementary Table S3). Pregabalin was considered inactive in the other receptor-mediated assays conducted.

3.1.8.2 *In silico* approaches

The authors of the original studies performed during the development of pregabalin (Criswell, et al., 2012a; Criswell, et al., 2012b; Criswell, et al., 2012c; Pegg, et al., 2012) did not conduct any computational study on their endpoints of interest. Though not necessarily relevant to hemangiosarcoma formation, QSAR models were applied to evaluate androgenic activity, estrogenic (ER) activity, and thyroid peroxidase activity of pregabalin with the Leadscope model applier (Instem, Inc.) and ADMET Predictor (SimulationsPlus) platforms. Such predictions can provide insights on the potential interactions with receptors relevant to other mechanisms of carcinogenicity. Predictions were negative for different endpoints including androgen receptor (AR) binding, aromatase inhibition, thyroperoxidase (TPO) inhibition, thyroid hormone receptor binding and transactivation. Details of the predictions are reported in the Supplementary Table S4.

3.1.8.3 Reliability and confidence

The experimental data are assigned a reliability score of RS2 and standard relevance. The *in silico* results are assigned a RS5. Overall, there is medium confidence that pregabalin does not affect receptor-mediated pathways known to be associated with carcinogenicity.

3.1.8.4 Data gaps

Several computational models have been published for VEGF interactions with VEGFR2 and subsequent proliferation of cells (Mac Gabhann and Popel, 2005; Kleinstreuer, et al., 2013; Clegg and Mac Gabhann, 2015) and use of these models may have strengthened the associations postulated for the mechanism proposed in (Criswell, et al., 2012a; Criswell, et al., 2012c). Similarly, models exist for PDGF activity, and recently a model was reported for PDGF-VEGF interactions with VEGFR2 (Mamer, et al., 2017). However, all of these models are computational biology models and do not predict growth factor activity based on the chemical structure of the binding ligand.

3.1.9 KC9: Causes immortalization

3.1.9.1 Experimental data

No data are available on immortalization of cells exposed to pregabalin, other than the presence of hemangiosarcomas in mice treated with the compound, which implies immortalization of cells.

3.1.9.2 *In silico* approaches

While some *in silico* methods for predicting immortalization in the SHE cell assay are available (Tice, et al., 2021), none were used for pregabalin.

3.1.9.3 Reliability and confidence

Based on a lack of evidence, a robust conclusion on whether pregabalin causes immortalization cannot be derived.

3.1.9.4 Data gaps

Immortalization of cells is typically considered to be an *in vitro* property, relating to the lack of senescence after long-term passaging of cells in culture. Cells taken from malignant tumors usually are immortal when cultured, and non-malignant cells can become immortal in culture when manipulated with certain viruses, proteins, etc., or arise from spontaneous mutations. The utility of *in silico* modeling for immortalization is unknown, as not all immortal cells will progress to malignant tumors.

3.1.10 KC10: Alters cell proliferation, cell death, or nutrient supply

3.1.10.1 Experimental data

Criswell et al. (Criswell, et al., 2012a) described several experiments where EC proliferation was measured in mouse liver, bone marrow, or spleen, and rat liver. Pregabalin increased hepatic endothelial and Kupffer cell proliferation in mice after 12 months of treatment at 200 and 1,000 mg/kg bw, while there was no effect at 50 mg/kg. In another mouse experiment, 5,000 mg/kg bw pregabalin increased the number of proliferating ECs in the liver after 2 and 4 weeks of treatment, and in the bone marrow after 12 weeks. Only minor increases in absolute numbers of proliferating ECs were found in the spleen. Vitamin E supplementation in the diet abolished the EC proliferation in the liver observed after 2 weeks. No increased proliferation of EC was seen in rat liver after up to 18 months of treatment at the maximum tolerated dose. Increases in release of tissue growth factors (VEGF, bFGF, PDGF) could also play a role in proliferation of EC.

No data were found for cell death or nutrient supply.

3.1.10.2 *In silico* approaches

No computational studies were performed on the EC data. As described in Tice and Bassan et al. (Tice, et al., 2021), global *in silico* methods for cell death, cell proliferation, and alteration of nutrient supply are not available.

3.1.10.3 Reliability and confidence

The experimental data are assigned a reliability score of RS2 with standard relevance. Confidence is high to medium that pregabalin affects cell proliferation.

3.1.10.4 Data gaps

The understanding of the mechanistic drivers for proliferation of EC are incomplete, and *in silico* models for the processes involved in KC10 are not available.

3.2 Other data related to carcinogenicity

A ChEMBL search retrieved no evidence of activity at targets related to known mechanisms of carcinogenicity. For pregabalin itself, no pChEMBL values were found other than for its recognized target: voltage-dependent calcium channels, α2δ subunit. A search for compounds with ≥50% structural similarity to pregabalin retrieved only two compounds (50% and 53% similar to pregabalin), both with only very low potencies (>30 μM) at four

targets: CYP1A2, putative fructose-1,6-bisphosphate aldolase, carbonic anhydrase II, and solute carrier family 22 member 20.

Data for pregabalin in the Comparative Toxicogenomics Database (CTDB, 2022) shows gene interactions with angiotensin-related receptors and a few other genes that have no direct relationship to carcinogenesis.

A safety signal analysis of spontaneous reporting systems was performed with the ClarityPV platform (https://claritypv.com/) (CLARITY, 2023). The analysis was based on a total number of 397,205 unique spontaneous reports deposited between 1 January 2005 and 31 August 2023 (average reporting rate of 1,773 reports/month or 21,278 reports/year) in FAERS (250,457), VigiBase (130,039), JADER (13,973) and VAERS (2,736). A list of 22 side effects disproportionally reported (PRR05 > 2.0) for pregabalin was identified (Supplementary Table S5), of which pituitary tumour benign shows also levels of suspiciousness and instantiation that warn some cautionary vigilance. Pregabalin was first approved for marketing in the US in 2004 and in the EU in 2005. Of note, some patients treated with pregabalin may also have been treated with dopamine-active agents, which are associated with pituitary tumors.

3.3 Carcinogenicity models and their predictions

The *in silico* models available for predicting *in vivo* carcinogenicity are based on resources that collect carcinogenicity results from the corresponding animal studies (Benigni, et al., 2008; Golbamaki and Benfenati, 2016; Bossa, et al., 2018; Bower, et al., 2020). The specific carcinogenicity predictions for pregabalin are reported in Table 2, where detailed results are included for both statistical- and expert-based systems (i.e., structural alerts). No alerts for both the genotoxic and the non-genotoxic carcinogenicity mechanisms are reported and, similarly, the statistical-based predictions falling in the applicability domain of the corresponding models (as in the case of the *in vivo* rodent carcinogenicity models for female rat, male rat, female mouse and male mouse (Instem, 2022)) are negative. There are also models providing a quantitative prediction (TD50); however, these models generally have a limited predicting capacity. Two of these software programs, ADMET Predictor and LAZAR, predict carcinogenicity potency expressed as TD50 (the oral daily dose administered over the course of lifetime required to produce tumors in 50 percent of animals); these values were compared with those reported by Pegg, et al. (2012). Both ADMET predictor and Lazar are statistically-based models that use Carcinogenicity Potency Database (CPDB, 2022) data to model endpoints of interest. In the case of Lazar prediction for rat carcinogenicity, the confidence in the prediction was considered low by the program. Comparison of the predictions by ADMET predictor (predicted TD50 rat = 92.2 mg/kg/day and predicted TD50 mouse = 376.8 mg/kg/day) to the bioassay results shows the *in silico* predictions to be far away from the actual TD50 of >5,000 mg/kg in the mouse. The reason for ADMET prediction of pregabalin being more potent for rat carcinogenicity than mouse is not clear. Overall, the *in silico* outcome for carcinogenicity for pregabalin *in vivo* does not highlight any element of concern. However, reliability scores of the predictions (Myatt, et al., 2018; Johnson, et al., 2022) as evaluated by means of analysis and expert review of the results are not high (mostly RS5) and this lowers the confidence of the negative overall assessment for *in vivo* carcinogenicity. The models did not predict the experimental outcome in the mouse, indicating that while they were correct in predicting a low likelihood of carcinogenicity with pregabalin across species, consideration of mechanisms of carcinogenicity is not a strength of these models.

3.4 Species differences/human relevance

Pregabalin treatment for up to 2 years caused hemangiosarcoma formation in mice, but not in rats (Pegg, et al., 2012). The mode of action of pregabalin-induced hemangiosarcomas is formulated in (Criswell, et al., 2012a; Criswell, et al., 2012b; Pegg, et al., 2012). The data and conclusions in these publications are consistent with the mode of action for hemangiosarcoma formation described in (Cohen, 2017). Criswell et al. (Criswell, et al., 2012c) further describe the relevance of mouse hemangiosarcomas to humans, including some data from studies with human blood or cells, and *in vivo* from human subjects. Regarding human relevance of results from rodent studies, it is recognized that tumors observed in animal studies that result from genotoxic mechanisms are generally considered to be relevant to humans even when occurring in tissues with no direct human equivalent (ECHA, 2017). On the other hand, non-genotoxic compounds causing tumors in animals may act through modes of action that are not human relevant (ECHA, 2017; Goodman, 2018). The available data point to a lack of relevance of the pregabalin-induced mouse hemangiosarcomas to humans.

4 Discussion

Lifetime rodent carcinogenicity studies are extremely resource intensive, requiring the use of over 500 rodents, costing over $1 million and taking approximately 3 years of time to complete. As it is not possible or desirable to test all chemicals and drugs under these conditions, the development and use of *in vitro* and *in silico* tools to predict carcinogenicity is imperative. Tice and Bassan et al. (Tice, et al., 2021) described the state-of-the-art for the use of *in silico* tools to predict the outcome of *in vitro* and *in vivo* assays (other than the traditional rodent carcinogenicity assay) relevant to carcinogenicity hazard assessment. Using pregabalin as a case study, we reviewed the *in vivo*, *in vitro*, and *in silico* data organized within the framework of the 10 KCs of carcinogens. We show where the experimental and *in silico* models give results that are useful in predicting carcinogenicity, and where there are gaps in the data and models that need to be addressed to more reliably predict nongenotoxic compound carcinogenicity.

Pregabalin is a single species, nongenotoxic rodent carcinogen. The MOA for pregabalin carcinogenicity has been proposed by (Criswell, et al., 2012c) and has been accepted by regulators globally. This MOA is consistent with the MOA of other agents that cause hemangiosarcomas in rodents (Cohen, 2017). Figure 1 in (Tice, et al., 2021) showed the relationship between the KCs of carcinogens

TABLE 2 Carcinogenicity predictions for pregabalin.

Tool	Endpoint	Model	Data/Prediction[a]	Applicability domain	Call	Comments
Leadscope Model Applier (v. 3.1.0-40)	Carcinogenicity *in vivo*	Carc female mouse v3	Negative (PPP = 0.13)	In domain	Negative	1) Low positive prediction probability provided by the statistical model (PPP = 0.13)
						2) The identified model features are mainly represented in experimentally negative compounds and the identified negative features provide a higher contribution to the result, resulting in an overall negative prediction call (PPP = 0.13). However, the structure of the target molecule is not fully covered by the features used to derive the prediction, i.e., the methylamine moiety is not covered
						3) No relevant training set analogs, meaning that the target molecule is only limited represented in the training set.
						4) Concordance of the analogs: the mostly similar training analog, i.e., Gabapentin, has positive experimental data in disagreement with the prediction
						5) Prediction accuracy of the analogs: the mostly similar training analog, i.e., Gabapentin, is not correctly predicted by the model introducing an uncertainty in the prediction derived for the target molecule
						6) Based on the poor coverage of the structure of the target molecule and the not optimal concordance and accuracy of the mostly similar training analog, the reliability score cannot be upgraded from the default RS5
		Carc male mouse v3	Negative (PPP = 0.223)	In domain	Negative	1) Low positive prediction probability provided by the statistical model (PPP = 0.223)
						2) The identified model features provide a good coverage of the structure; they are mainly represented in experimentally negative compounds and the identified negative features provide a higher contribution to the result, resulting in an overall negative prediction call (PPP = 0.223)
						3) No relevant training set analogs, meaning that the target molecule is only limited represented in the training set.

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TABLE 2 (*Continued*) Carcinogenicity predictions for pregabalin.

Tool	Endpoint	Model	Data/Prediction[a]	Applicability domain	Call	Comments
						4) Concordance of the analogs: the mostly similar training analog, i.e., Gabapentin, has positive experimental data in disagreement with the prediction
						5) Prediction accuracy of the analogs: the mostly similar training analog, i.e., Gabapentin, is not correctly predicted by the model introducing an uncertainty in the prediction derived for the target molecule
						6) Based on the not optimal concordance and accuracy of the mostly similar training analog, the reliability score cannot be upgraded from the default RS5
		Carc male rat v3	NEGATIVE (PPP = 0.0987)	In domain	Negative	1) Low positive prediction probability provided by the statistical model (PPP = 0.0987)
						2) The identified model features provide a good coverage of the structure; they are mainly represented in experimentally negative compounds and the identified negative features provide a higher contribution to the result, resulting in an overall negative prediction call (PPP = 0.0987)
						3) No relevant training set analogs, meaning that the target molecule is only limited represented in the training set.
						4) Concordance of the analogs: the mostly similar training analog, i.e., Gabapentin, has positive experimental data in disagreement with the prediction
						5) Prediction accuracy of the analogs: the mostly similar training analog, i.e., Gabapentin, is not correctly predicted by the model introducing an uncertainty in the prediction derived for the target molecule
						6) Based on the not optimal concordance and accuracy of the mostly similar training analog, the reliability score cannot be upgraded from the default RS5
		Carc female rat v3	Negative (PPP = 0.161)	In domain	Negative	1) Low positive prediction probability is provided by the statistical model (PPP = 0.161), meaning that the target molecule is predicted as negative
						2) The identified model features provide a good coverage of the structure; they are mainly represented in experimentally negative compounds and the identified negative features provide a higher contribution to the result, resulting in an overall clear negative prediction call (PPP = 0.161)

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TABLE 2 (*Continued*) Carcinogenicity predictions for pregabalin.

Tool	Endpoint	Model	Data/Prediction[a]	Applicability domain	Call	Comments
						3) Training set analogs were inspected and no concern arose by this analysis. Analogs are characterized by a limited structural similarity with respect to the target molecule, meaning that the target molecule is only limited represented in the training set.
						4) The mostly similar analog is Gabapentin, which is experimentally negative and correctly predicted by the model
						The reliability score is then upgraded to RS3
Derek Nexus: 6.1.0, Nexus: 2.3.0	Carcinogenicity	Expert alerts	No alerts fired	Not applicable	Not assigned	No alerts associated with carcinogenicity are fired by the expert system. Because of the nature of the model, this is not a negative prediction. It, however, supports any negative result from other model(s)
VEGA (v. 1.3.10)	Carcinogenicity *in vivo*	Carcinogenicity model (CAESAR) 2.1.10	Positive	Outside the applicability domain (AD = 0)	Rejected	This prediction is rejected given its low reliability
		Carcinogenicity model (ISS) 1.0.3	Negative	Outside the applicability domain (AD = 0)	Rejected	This prediction is rejected given its low reliability
		Carcinogenicity model (IRFMN-ISSCAN-CGX) 1.0.1	Possible NON-Carcinogen	Outside the applicability domain (AD = 0.52)	Rejected	This prediction is rejected given its low reliability
		Carcinogenicity model (IRFMN-Antares) 1.0.1	Possible NON-Carcinogen	Outside the applicability domain (AD = 0.555)	Rejected	This prediction is rejected given its low reliability
		Carcinogenicity oral classification model (IRFMN) 1.0.1	Carcinogen	The predicted compound could be out of the Applicability Domain of the model (AD = 0.754)	Rejected	This prediction is rejected given its low reliability
		Carcinogenicity in male rat (CORAL) 1.0.0	Predicted TD50 [mg/kg bw/day]: 2.48	Outside the applicability domain	Rejected	This prediction is rejected given its low reliability
		Carcinogenicity in female Rat (CORAL) 1.0.0	Predicted TD50 [mg/kg bw/day]: 8588.44	Outside the applicability domain	Rejected	This prediction is rejected given its low reliability
Toxtree v 3.1.0	Carcinogenicity	Genotoxic and/or non-genotoxic carcinogenicity alerts by ISS	No alerts fired (Negative for genotoxic carcinogenicity and Negative for nongenotoxic carcinogenicity)	Not available	Not assigned	This model is also available in the OECD QSAR Toolbox
LAZAR (v. 1.4.2)	Carcinogenicity *in vivo*	Carcinogenicity (Mouse (TD50))	-	Out of domain	-	-
		Carcinogenicity (Rat (TD50))	2560.0 (mg/kg_bw/day)	Low confidence (Insufficient number of neighbors for regression model, using weighted average of similar substances)	-	-

[a]The predictions may be associated with statistical value such as the PPP, that is the positive prediction probability (the positive prediction probability is given as the likelihood value between 0 (non-toxic) and 1 (toxic)).

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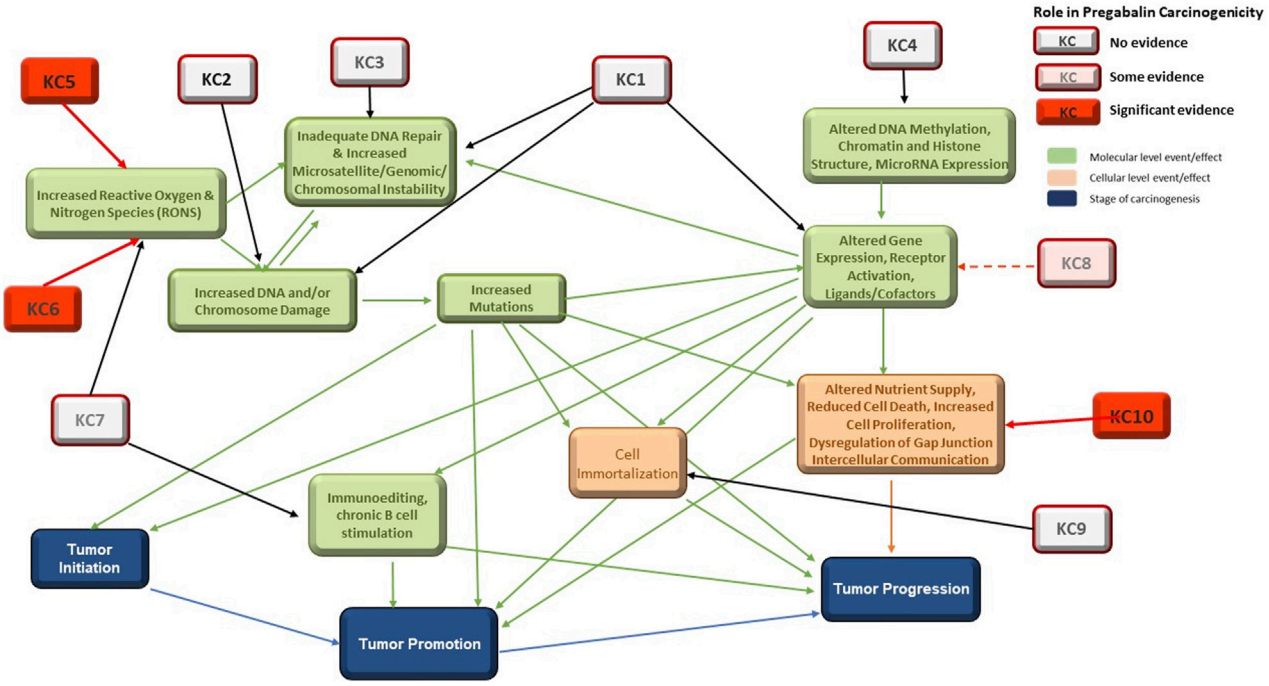

FIGURE 3
Interactions among the 10 KCs and with the Three Stages of Carcinogenesis. KC1 = is electrophilic, KC2 = is genotoxic, KC3 = alters DNA repair or causes genomic instability, KC4 = induces epigenetic alterations, KC5 = induces oxidative stress, KC6 = induces chronic inflammation, KC7 = is immunosuppressive, KC8 = modulates receptor-mediated effects, KC9 = causes immortalization, KC10 = alters cell proliferation, cell death, or nutrient supply. Specific evidence for involvement in pregabalin carcinogenicity is highlighted in red.

TABLE 3 Summary of data reliability and confidence. Reliability scores and confidence levels are assigned according to (Myatt, et al., 2018; Johnson, et al., 2022). Confidence considers the reliability, relevance, and coverage of information available. KCs listed in red are involved in the mode of action of pregabalin carcinogenicity in mice.

Key characteristic	Reliability		Confidence
	Experimental	In Silico	
KC1: Is Electrophilic	RS1	RS3	Medium
KC2: Is Genotoxic	RS1	RS1-RS3	Medium to High
KC3: Alters DNA Repair or Causes Genomic Instability	[a]	[a]	No Confidence
KC4: Induces Epigenetic Changes	[a]	[a]	No Confidence
KC5: Induces Oxidative Stress	RS2	[a]	High
KC6: Induces Chronic Inflammation	RS2	[a]	Medium to High
KC7: Is Immunosuppressive	RS1-RS3	[a]	Medium
KC8: Modulates Receptor-Mediated Effects	RS2	RS5	Medium
KC9: Causes Immortalization	[a]	[a]	No Confidence
KC10: Induces Cell Proliferation, Cell Death, Nutrient Supply	RS2	[a]	Medium to High

[a]Insufficient data to make assignment.

and the stages of carcinogenesis. Here, the figure has been revised to highlight the roles of the KCs involved in the carcinogenic process for pregabalin (Figure 3).

Outcomes for KC1 (is electrophilic or can be metabolically activates), KC2 (is genotoxic), and KC8 (modulates receptor-mediated effects) can, at least in part, be reliably predicted with *in vitro* systems and *in silico* models (Table 3). These are discussed in detail in (Tice, et al., 2021). While models to predict electrophilicity may be overly sensitive, bacterial mutagenicity models that incorporate metabolic activation are an acceptable and more accurate surrogate for the electrophilicity endpoint. Prediction of KC2 (genotoxicity) is the most well-developed area of *in vitro* and *in*

silico tools for carcinogenicity assessment and *in vivo* tests are rarely needed. Prediction of KC8 outcomes (modulates receptor-mediated effects) was applied in this work for some nuclear receptor activities, with the most effort centered on ER and AR which have been linked to certain mechanisms of carcinogenicity. Interactions with some additional receptors, CYPs and AhR can be modeled (Vedani, et al., 2012). While these activities are not part of the pregabalin carcinogenicity MOA, they could play a role in the carcinogenicity of endocrine-disrupting chemicals (Heusinkveld, et al., 2020). Some receptor-mediated mechanisms of carcinogenicity, such as PPARα and AhR driven tumors, could lack relevance to humans, which highlights the need for species-specific understanding of mechanisms and models.

A variety of *in vitro* systems are available for prediction of KC5 (induce oxidative stress), but *in silico* model development has lagged. As described in (Tice, et al., 2021) *in silico* models are available for several hard chemistry endpoints related to oxidative stress, such as peroxide and quinone formation. Of note, a quantum model for Nrf-2/ARE activation has been reported in the literature to identify the structures predicted to activate the Nrf2-antioxidant response element pathways (Williamson, et al., 2012). As oxidative stress plays a role in the MOA for pregabalin carcinogenicity, a readily available model for the prediction of these types of effects would be desirable.

In contrast, no significant *in silico* models exist for prediction of KC3 (alerts DNA repair or causes genomic instability), 4 (induces epigenetic alterations), 6 (induces chronic inflammation), 7 (is immunosuppressive), 9 (causes immortalization), and 10 (alters cell proliferation, cell death, or nutrient supply). *In silico* models exist for only a small number of the endocrine and hormonal endpoints that can be associated with carcinogenicity. *In vivo* and *in vitro* assays are available for some aspects of these KCs, but prediction from the chemical structure of the molecule (i.e., drug, xenobiotics) is not possible at this time. This is a major obstacle for prediction of nongenotoxic carcinogenicity, as the MOA is often dependent on KC6 (inflammation) and/or KC10 (cell proliferation). DNA repair (KC3) and epigenetic factors (KC4 (Shin, 2020)) could play significant roles in carcinogenicity of agents that are not positive in traditional genotoxicity assays (KC2). While many immunosuppressive drugs carry a warning for increased cancer risk (Cangemi, et al., 2019), the extent of immunosuppression associated with this risk in humans is unknown. Additional *in vitro* assays and *in silico* models to predict these effects would be extremely useful.

Data from other studies such as Tox21-type screening, toxicogenomics, target activity profiles such as ChEMBL, and human data, when available, can add value to carcinogenicity assessments, but *in silico* models are not available for any of these. Cook et al. (Cook, et al., 2018) investigated three additional, structurally diverse compounds that caused hemangiosarcomas in mice (fenretinide, troglitazone, and elmiron), further testing the MOA proposed in (Cohen, et al., 2009; Criswell, et al., 2012c). These studies included some of the same analyses used in the pregabalin studies (bone marrow, hematology, and hypoxia parameters) as well as transcriptomics. The results indicated that the three additional compounds initiated the same MOA as pregabalin, with the potency of biological effects following the potency of hemangiosarcoma formation by these compounds. Additionally, the studies showed that transcriptomics were consistent with the MOA and potency of the compounds but was not more sensitive than hydroxyprobe for detection of hypoxia. Given the structural diversity of compounds that cause hemangiosarcomas in mice, the availability of *in silico* models to predict some of the key elements of this MOA could save significant amounts of effort, time, and animals. The use of *in silico* models to predict hazard for the different KC could be very useful in determining what targeted biological assays to perform to confirm an effect.

While known human carcinogens are almost exclusively genotoxic compounds, nongenotoxic carcinogenicity has been shown in experimental systems and in most cases the relevance to humans is not known with any degree of confidence (Silva Lima and van der Laan, 2000; Cohen, 2017). This is the case for environmental chemicals, food source chemicals, and drugs, highlighting the need for reliable methods for predicting the key events in an MOA, and KCs of carcinogens can be a useful method as an initial step to organize and process the data. As an example of where reliable *in silico* methods for prediction of nongenotoxic carcinogens would be of use, the ICH has revised the S1B guideline to allow for drug developers to develop a weight of evidence (WoE) argument to assess whether a rat carcinogenicity study would add value over existing data for determination of human carcinogenicity. The proposed WoE assessment (ICH, 2022) combines certain factors such as target biology, genotoxicity, secondary pharmacology, immunomodulation, hormonal perturbation, and repeated-dose histopathology into an integrated human risk assessment. The KCs framework may be one approach to support the identification and interpretation of relevant evidence and assays for each factor supporting how such evidence might be combined, and relevant *in silico* predictions would provide additional insights into the ICH S1B weight of evidence approach. This can be particularly useful when a specific MOA is not postulated.

5 Conclusion

The overall goal of this exercise was to evaluate the ability of *in silico* models to predict nongenotoxic carcinogenicity with pregabalin as a case study while being guided by the KC framework in the organization and combination of the collected information. Pregabalin is a single-species carcinogen producing only one type of tumor, hemangiosarcomas. The established MOA is triggered by tissue hypoxia, leading to oxidative stress (KC5), chronic inflammation (KC6), and increased cell proliferation (KC10) of EC (Criswell, et al., 2012a). Of these KCs, *in silico* models are available only for selected endpoints in KC5, limiting the usefulness of computational tools in prediction of pregabalin carcinogenicity. KC1, KC2, and KC8, for which predictive *in silico* models exist, do not play a role in this MOA. Additionally, as the pregabalin MOA is considered not relevant to humans, experimental assays and *in silico* models used to predict endpoints for the KC involved must either account for species differences or produce results that can be interpreted in the context of species-specific biology.

We investigated the availability of *in silico* models to predict the ten KCs of carcinogens for a nongenotoxic compound, pregabalin. *In silico* approaches are available for some of the mechanisms associated with the KCs but are particularly lacking for the KCs involved in the MOA specific for pregabalin carcinogenicity. Development of reliable *in silico* models for prediction of oxidative stress, chronic inflammation, immunosuppression, and cell proliferation will be critical for the ability to predict nongenotoxic compound carcinogenicity.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

DK: Conceptualization, Investigation, Writing–original draft, review and editing. AB: Conceptualization, Investigation, Writing–review and editing. AA: Investigation, Writing–review and editing. LB: Investigation, Writing–review and editing. JC: Investigation, Writing–review and editing. KC: Conceptualization, Investigation, Writing–review and editing. FH: Investigation, Writing–review and editing. GJ: Investigation, Writing–review and editing. AL: Investigation, Writing–review and editing. SM: Investigation, Writing–review and editing. JM: Investigation, Writing–review and editing. AM-B: Investigation, Writing–review and editing. DW: Investigation, Writing–review and editing. RT: Conceptualization, Supervision, Investigation, Writing–review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DK, LB, GJ and RT are independent contractors. AB is employed by Innovatune. AA is employed by Sanofi. JC, KC and FH are employed by Instem. AL is employed by Medline Industries. SM is employed by Nestle Research. JM is employed by Chemotargets SL. Amy Mihalchik-Burhans is employed by ToxStrategies, LLC. David Wooley is employed by ForthTox.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Developing a pragmatic consensus procedure supporting the ICH S1B(R1) weight of evidence carcinogenicity assessment

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The ICH S1B carcinogenicity global testing guideline has been recently revised with a novel addendum that describes a comprehensive integrated Weight of Evidence (WoE) approach to determine the need for a 2-year rat carcinogenicity study. In the present work, experts from different organizations have joined efforts to standardize as much as possible a procedural framework for the integration of evidence associated with the different ICH S1B(R1) WoE criteria. The framework uses a pragmatic consensus procedure for carcinogenicity hazard assessment to facilitate transparent, consistent, and documented decision-making and it discusses best-practices both for the organization of studies and presentation of data in a format suitable for regulatory review. First, it is acknowledged that the six WoE factors described in the addendum form an integrated network of evidence within a holistic assessment framework that is used synergistically to analyze and explain safety signals. Second, the proposed standardized procedure builds upon different considerations related to the primary sources of evidence, mechanistic analysis, alternative methodologies and novel investigative approaches, metabolites, and reliability of the data and other acquired information. Each of the six WoE factors is described highlighting how they can contribute evidence for the overall WoE assessment. A suggested reporting format to summarize the cross-integration of evidence from the different WoE factors is also presented. This work also notes that even if a

2-year rat study is ultimately required, creating a WoE assessment is valuable in understanding the specific factors and levels of human carcinogenic risk better than have been identified previously with the 2-year rat bioassay alone.

KEYWORDS

carcinogenicity assessment, WoE, ICHS1B, 2-year rat bioassay, integrated assessment, pharmaceuticals, drug development

1 Introduction

The International Council on Harmonization (ICH) S1B(R1) guideline provides a framework for evaluating the carcinogenic potential of pharmaceuticals to enhance the assessment of human carcinogenic risk, increasing efficiency and consistency in testing approaches across regulatory agencies. The original guideline was revised in 2022 and adopted across multiple regulatory jurisdictions (ICH S1B(R1), 2022). The addendum of this guideline introduces a detailed weight of evidence (WoE) approach supporting a robust scientific strategy for assessing human carcinogenic risk of pharmaceuticals. The addendum identifies six WoE factors to assess whether conducting a 2-year rat carcinogenicity study (bioassay) would add value to the existing data supporting a human carcinogenicity risk assessment. In certain cases (Figure 1), the fully integrated WoE approach is proposed as a potential alternative to the 2-year rat bioassay thus reducing animal testing without compromising human safety. This pivotal change introduced in the ICH S1B(R1) guideline is expected to increasingly rely on new and alternative strategies for determining carcinogenic risk. This is in line with the 3Rs [Replacement, Reduction, and Refinement (Russell and Burch, 1959)] approach of animal use in science (Van Der Laan et al., 2023), that is embraced by several programs. For example, the FDA Modernization Act 2.0 gives the drug development industry the option to use alternatives to animal testing to determine safety and efficacy of drugs, empowering the use of innovative non-animal methods in the most rigorous and scientific way (US Congress, 2022; Wadman, 2023). Furthermore, there are calls from members of the European Parliament to accelerate the transition to an animal-free research and testing (EU, 2021), which is also being mapped by the European Food Safety Authority (EFSA) (Escher et al., 2022; Cattaneo et al., 2023), the European Chemicals Agency (ECHA) (ECHA, 2023) and the European Medicines Agency (EMA) (EMA, 2020).

Rodent carcinogenicity studies of pharmaceuticals are usually initiated in the late drug development phase, following the completion of shorter repeat-dose toxicity studies (which are used as dose ranging studies for the 2-year rat bioassay) and Phase I and Phase II clinical trials. The rat carcinogenicity study is usually the last nonclinical study completed prior to submission of the Marketing Application. Without intent to extend the drug development timeline, the novel strategy described in the ICH S1B(R1) guideline encourages early planning of carcinogenicity assessment based on the integration and combination of relevant evidence from standard *in vitro* and *in vivo* studies. It also highlights the use of additional investigative approaches to address concerns and data gaps identified by the WoE evaluation. The outcome of the WoE assessment is a determination whether a 2-year rat study adds value after all the data (including chronic toxicology data) are available, and then agreement with regulators is pursued; therefore it is essential that the approach to the WoE assessment is planned timely so that decision regarding the need for a 2-year rat study can be achieved early enough; consequently, if needed, the bioassay can be started without major impact to the project timeline.

The integrated WoE approach that applies to molecules requiring carcinogenicity assessment according to ICH S1A (1995) is supported by experience with a similar WoE framework described for biotechnology-derived therapeutics in ICH S6(R1) (2011). The assessment for biotechnological products includes analysis of data from multiple sources, including published data (e.g., information from transgenic, knock-out or animal disease models, and human genetic diseases), information on class

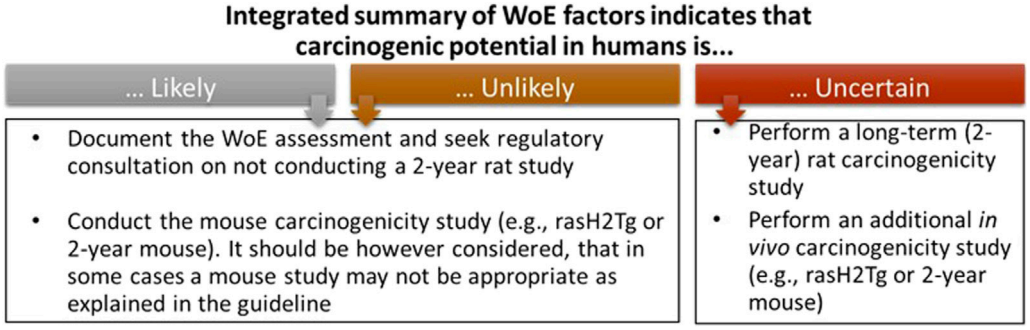


FIGURE 1
The three outcomes of the WoE integrated assessment as defined by the ICH S1B(R1) guideline. These outcomes and actions provide a basis for Sponsors to define project goals for logistics around how to best fit the WoE approach into the project timeline so that the decision that a 2-year rat study is needed does not result in a major impact to the project timeline.

TABLE 1 Description of the WoE factors and their interpretation in the WoE assessment as included in the ICH S1B(R1) guideline (ICH S1B(R1), 2022). As discussed in the guideline, decision making is driven by the evidence collected to assess carcinogenic risk from each of the six WoE criteria. The guideline addendum also notes that in addition to cases where all the WoE factors indicate no risk, the 2-year rat bioassay is likely not to add value in the case of unequivocal genotoxicity risk or observed effects of broad immunosuppression.

WoE factor short name	Description[a]	2-year rat study and/or investigative approaches more likely if . . . [a]	2-year rat study and/or investigative approaches less likely if . . . [a]
Target biology	"Data that inform carcinogenic potential based on drug target biology and the primary pharmacologic mechanism of the parent compound and major human metabolites; this includes drug target distribution in rats and humans along with the pharmacologic activity and potency of the parent compound and major metabolites in these species; available information from genetically engineered models; human genetic association studies; cancer gene databases; and carcinogenicity information on class effects, if available."	"Poorly characterized biologic pathways, unknown class effects"	"Well characterized biologic pathways, known class effects"
Secondary pharmacology	"Results from secondary pharmacology screens for the parent compound and major metabolites that inform selectivity and off-target potential, especially those that inform carcinogenic risk (e.g., binding to nuclear receptors)."	"Low target selectivity, off-target activity"	"High target selectivity, no off-target activity"
Histopathology chronic studies	"Histopathology data from repeated-dose toxicity studies completed with the compound, with particular emphasis on the 6-month rat study, including plasma exposure margin assessments of parent drug and major metabolites."	"Hyperplastic or other lesions of concern"	"No findings of concern or human-irrelevant findings"
	"Histopathology findings from 6-month rat toxicity studies of particular interest for identifying carcinogenic potential in a 2-year rat study include cellular hypertrophy, cellular hyperplasia, persistent tissue injury and/or chronic inflammation, foci of cellular alteration, preneoplastic changes, and tumors. It is important to provide an understanding of the likely pathogenesis, and/or address the human relevance of such findings. While the 6-month rat toxicity study is the primary study to be used for assessing the likely outcome and value of conducting a 2-year rat study, shorter-term rat studies can sometimes also provide histopathologic conclusions of value. Data from long-term toxicity studies in non-rodents and mice may also be useful for providing additional context on the human relevance of rat study findings (e.g., species-specific mechanistic differences) and whether there is value in conducting a 2-year rat study."		
Hormonal effects	"Evidence for hormonal perturbation, including knowledge of drug target and compensatory endocrine response mechanisms; weight, gross and microscopic changes in endocrine and reproductive organs from repeated-dose toxicity studies; and relevant results from reproductive toxicology studies, if available."	"Endocrine/reproductive organ perturbation"	"No findings of concern or human-irrelevant findings"
	"Findings from rat toxicity studies suggesting hormonal perturbation may include microscopic changes in endocrine or reproductive tissues of atrophy, hypertrophy, and hyperplasia and/or biologically significant endocrine and reproductive organ weight changes which are not explained as findings secondary to processes such as stress or altered body weight. Changes of this nature may be considered evidence of functional hormonal perturbation even when changes in hormone levels are not documented. Such findings may be suggestive of potential carcinogenic risk unless investigated for human relevance and demonstrated otherwise."		
Genotoxicity	"Genetic toxicology study data using criteria from ICH S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (ICH S2(R1), 2012); equivocal genotoxicity data that cannot be resolved in accordance with ICH S2(R1) recommendations increases uncertainty with respect to the carcinogenic potential."	"Positive genotoxicity data of uncertain human relevance"	"No genotoxicity risk or unequivocal genotoxicity"
Immune modulation	"Evidence of immune modulation in accordance with ICH S8 Immunotoxicity Studies for Human Pharmaceuticals (ICH S8, 2006). Evidence of broad immunosuppression may provide sufficient concern for human risk that would not be further informed by standard rat and mouse carcinogenicity studies."	"Immune effects of uncertain human relevance"	"No effects on immune cell/tissues or broad immunosuppression in humans"

[a]Description and summary interpretation as originally taken from the ICH S1B(R1) guideline.

effects, detailed information on target biology and mechanism of action, *in vitro* data, chronic toxicity studies, reproductive toxicology studies and clinical data. If this WoE assessment is not sufficient to clearly assess carcinogenicity, under ICH S6(R1), alternative studies can be proposed to reduce remaining uncertainties or to address data gaps and inform more clearly the potential risk.

The ICH S1B(R1) WoE factors should be considered in a holistic and integrative manner to determine the need, timing, and design of carcinogenicity studies in drug development. Accordingly, the factors bring together pharmacological, biological, and toxicological data that can be integrated for human carcinogenicity risk assessment leading to a decision on whether carcinogenic potential of the therapeutic agent in humans is: A) likely and a 2-year rat carcinogenicity study would not add value; B) unlikely and a 2-year rat carcinogenicity study would not add value; or C) uncertain and a 2-year rat carcinogenicity study would add

value to the overall safety assessment for humans (Figure 1). The WoE criteria include evidence from public sources and relevant drug development studies, and they cover six different factors described in Table 1: 1) target biology; 2) secondary pharmacology; 3) histopathology from chronic studies; 4) hormonal effects; 5) genotoxicity; and 6) immune modulation. In general, a robust assessment of the absence of concern for all the WoE criteria supports a conclusion that a 2-year rat bioassay would not add value to the overall human carcinogenicity risk assessment. The 2-year rat bioassay is less likely to be of value also in the case of evidence of unequivocal genotoxicity or broad immunosuppression indicating a carcinogenic risk to humans (ICH S1B(R1), 2022). In these cases, the risk can be clearly stated in the product label.

Notably, the ICH S1B(R1) strategy supports the incorporation of results from different investigative approaches such as molecular biomarkers and emerging technologies and the use of published data on related molecules. Targeted nonstandard clinical data can also be collected in clinical trials to help to address hypothesized concerns of carcinogenic drug actions and determine relevance of animal findings to humans. These additional results can be used to inform the WoE factors and support the decision making on the need and value of conducting the 2-year rat bioassay. The guideline notes that a rasH2-Tg mouse study is not expected to be completed to support a WoE assessment. However, if rasH2-Tg mouse study results are available, they should be included as evidence, and, for example, they can inform the strength of association of target modulation with rodent tumor development when sufficient pharmacologic activity is documented.

The present work leverages the rationale of the *in silico* toxicology protocols initiative (Myatt et al., 2018; Myatt et al., 2022), where an international network of experts has been working to identify principles for generating, recording, communicating, archiving and then evaluating toxicity assessments (employing *in silico* methods when appropriate) in a uniform, consistent and reproducible manner.

The present work proposes a pragmatic standardized procedure framing the ICH S1B(R1) human carcinogenicity assessment in the spirit of the ideas underlying the *in silico* toxicology protocols, thus aiming to make decisions (i.e., on whether a 2-year rat carcinogenicity study adds value) that are transparent, consistent, documented, repeatable and defendable. In general terms, WoE analyses integrate numerous pieces of evidence to make a scientifically defensible conclusion, that may be inherently based on subjective judgment and thus affected by potential bias, as, for example, discussed by the Organisation for Economic Co-operation and Development (OECD) in relation to weight of evidence for chemical assessment (OECD, 2019). Therefore, an established procedure that drives the process of collating, weighing and evaluating such evidence ensures that the analysis and the conclusions are clearly understood, documented and thus transparent to all stakeholders. The pragmatic consensus procedure described here is meant to support the creation of the Carcinogenicity Assessment Document (CAD), which reports the expected utility of the 2-year rat study as derived from the WoE assessment.

Determination in certain infrequent instances of whether a mouse study may not be needed for the carcinogenicity assessment is discussed in ICH S1B(R1) and is not further addressed in this work. Moreover, strategies for exact timing of study activities and regulatory interactions are also considered out of scope of this publication.

2 Background

An international network of experts from different organizations has been working to develop *in silico* toxicology protocols for combining evidence coming from different sources (e.g., *in vitro* and *in vivo* experimental data and *in silico* results) and to establish an overall assessment and confidence score for a given toxicological endpoint (Myatt et al., 2018; 2022). In general, a protocol is a standardized procedure that frames the hazard assessment process to facilitate transparent, consistent and documented decision-making. This protocol concept has been applied for genetic toxicology (Hasselgren et al., 2019), skin sensitization (Johnson et al., 2020) and acute oral toxicity (Zwickl et al., 2022), and has been discussed in a number of other publications covering carcinogenicity (Tice et al., 2021), organ toxicity (Bassan et al., 2021a; Bassan et al., 2021b), neurotoxicity (Crofton et al., 2022), and confidence of a general integrated assessment (Johnson et al., 2022). In the present work the *in silico* toxicology protocol concept (Myatt et al., 2018; Myatt et al., 2022) is applied to guide the ICH S1B(R1) assessment.

3 Overview of the proposed pragmatic consensus procedure

The *in silico* toxicology protocol approach (Myatt et al., 2018; 2022) is applied here in a more specific fashion to the ICH S1B(R1) WoE assessment, where the endpoint of interest is understanding the added value of a 2-year rat study to the assessment of human carcinogenic risk. There is no "one size fits all" approach for such a novel carcinogenicity assessment strategy and its application must be tailored to the specific pharmaceutical being evaluated and the logistics surrounding the project development timeline. This work attempts to standardize as much as possible the procedure that guides the integration of data associated with the different ICH S1B(R1) WoE criteria (Table 1). The result of this effort is meant to be a pragmatic consensus procedure providing indications and suggestions that guide holistic, science-based and intelligent conclusions as well as facilitating the creation and successful submission of the CAD that would be deemed to be sufficiently comprehensive, objective and balanced, and both reasonable and convincingly conclusive.

The pragmatic consensus procedure is intended to discuss best-practices for both the organization of the studies and presentation of the data in a suitable format as well as to clarify expectations in terms of the types of integrated evidence to be presented in the CAD. Indeed, definition of a reporting format for collected evidence, results and conclusions helps clarify what is expected in terms of the types of evidence to be included and critical questions to be answered.

The procedure contains proposals on: 1) the strategy of the integrative WoE carcinogenicity assessment; 2) approaches for the

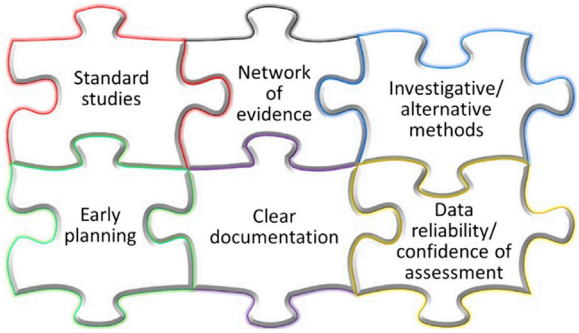


FIGURE 2
Attributes of the proposed pragmatic consensus procedure for the ICH S1B(R1) integrated assessment.

collection and organization of data and information; 3) analysis of available evidence; 4) reporting of the results. In order to establish a pragmatic consensus procedure for the integrated WoE assessment, several general aspects are considered and examined as summarized in Figure 2 and described below.

3.1 Network of evidence

The six ICH S1B(R1) WoE factors are related to each other, since the evidence belonging to a specific WoE area (e.g., histopathology from chronic studies) can be used to inform other WoE criteria (e.g., hormonal effects) as illustrated in Figure 3. Different observations are collected from the analysis of target biology, secondary pharmacology and histopathology from chronic studies. Such observations are integrated with the evaluation of the other endpoints associated with the remaining WoE factors (hormonal effects, genotoxicity, and immune modulation). In general, the assessment of some WoE factors can be supported by evidence and signals collected from other WoE factors. The six WoE factors can thus be viewed as a network of evidence within a holistic assessment framework that is used synergistically to analyze and explain signals (and/or absence of signals), in order to demonstrate that the ICH S1B(R1) integrated assessment has been conducted thoroughly, and that all appropriate aspects of the WoE approach have been considered. For example, a histopathological finding from the 6-month rat study may be connected to data coming from the secondary pharmacology screening to aid interpretation and give a better understanding of the evidence presented based on assessing coherence of observed responses.

3.2 Mechanistic analysis

Human relevance of the findings from the different WoE areas needs to be established. Mechanistic analysis of effects of potential concern is critical to determine whether the mode of action is relevant to humans, and to support interpretation of signals and findings (an example will be given further below when discussing chronic inflammation in relation to the histopathology from chronic studies factor in Section 4.3). The Adverse Outcome Pathway (AOP) framework (OECD, 2023) can help to organize the mechanistic understanding that is being built while performing the ICH S1B(R1) integrated assessment. The AOP framework describes a sequence of events that is triggered by an initial interaction between a stressor and a biomolecule (i.e., Molecular Initiating Event, MIE) and can progress through a dependent series of intermediate key events (KEs) involving structural and functional changes. This sequence of events, potentially part of a larger network, ultimately culminates in the adverse outcome (AO) relevant to an organism (OECD, 2017). Existing consensus about a given AOP should be carefully evaluated before using the AOP. Translational mechanistic or safety biomarkers that can reflect animal study findings linked to carcinogenesis and serve as bridges for monitoring for such potential drug actions at therapeutic exposures in clinical trials, are also useful for addressing human relevance.

3.3 Alternative methodologies and novel investigative approaches

Evidence sources from *in vivo* studies are primarily from standard toxicology studies on the drug candidate (e.g., histology from subchronic and chronic rodent studies, reproductive toxicology studies and the standard genetic toxicology battery) to the fullest extent possible to minimize the need for additional, unwarranted animal studies. Potential elements of concern identified during the evaluation of the six WoE factors could be further inspected by applying alternative methodologies such as network biology approaches (e.g., Wang, 2022), quantitative systems toxicology (e.g., Bloomingdale et al., 2017), or other novel investigative approaches such as organotypic cultures (e.g., Hayden and Harbell, 2021), organs-on-a-chip (e.g., Ingber, 2022; Leung et al., 2022), humanized mice (e.g., Ye and Chen, 2022), disease models (e.g., Loewa et al., 2023). These approaches are selected as appropriate to improve the mechanistic understanding and to interpret and explain the relevance of findings to humans.

3.4 Early planning

Early, pragmatic and flexible planning of the integrative WoE carcinogenicity assessment is advisable for anticipation of the ICH

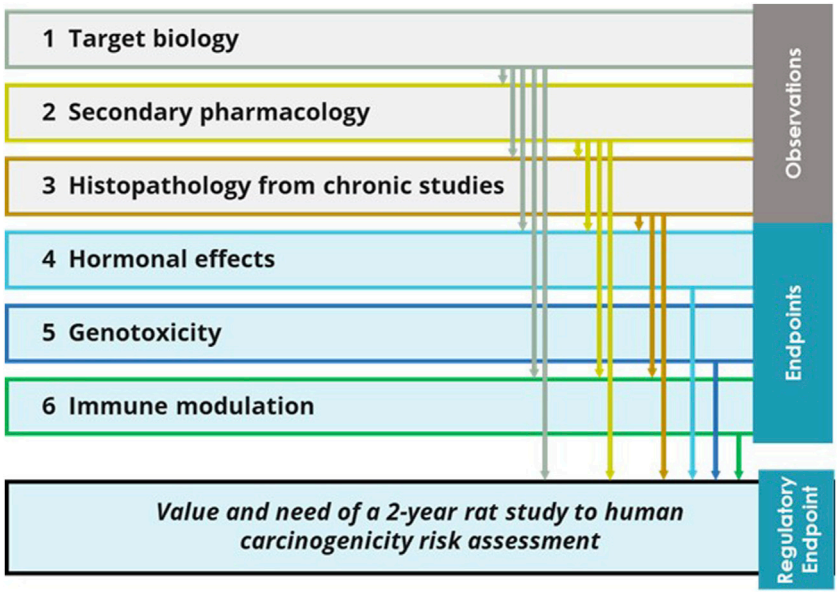


FIGURE 3
Potential relationship among the six ICH S1B(R1) WoE factors. Observations from the target biology analysis, secondary pharmacology and histopathology from chronic studies provide evidence that can inform the human carcinogenic risk and the added value of the 2-year rat study; such evidence would also inform the other endpoints forming the other WoE factors: hormonal perturbation and immune modulation. In general, the six WoE factors form a network of evidence where the analysis of each WoE factor can be integrated with input from the other WoE factors.

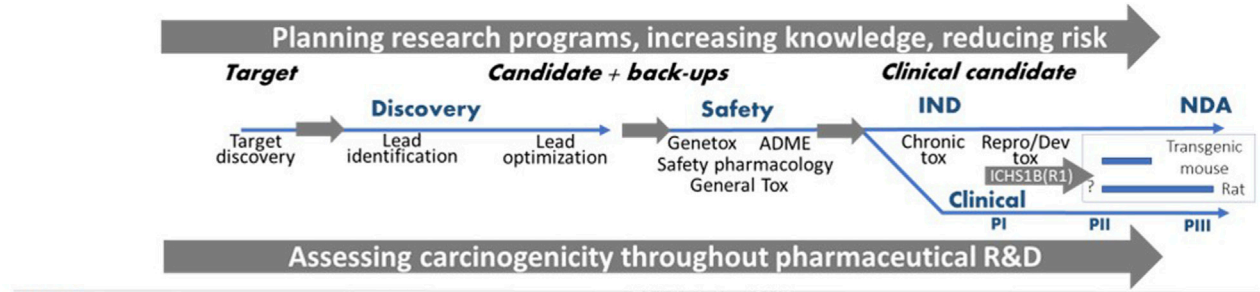
S1B(R1) assessment as it allows one to capture signals for carcinogenicity concern at an early stage of the drug discovery and development process (i.e., carcinogenic potential is likely) and also to make early decisions as to whether a WoE approach is reasonable. The Benefit/Risk balance can be considered as each new set of data is collected. Methodologies such as (Quantitative) Structure Activity Relationship, (Q)SAR (including read-across) (e.g., Myatt et al., 2022; 2018), may be useful to collect evidence for early internal decision of the Sponsor. The potential integrative assessment of the evidence in ICH S1B(R1) throughout the drug discovery and development process is illustrated in Figure 4. As discussed earlier, the goal of the WoE assessment is to determine whether a 2-year rat study provides additional value as early as possible during the project so that, if necessary, a late start of the study does not impact the project timeline. To this end, an early start of the chronic rat study might be appropriate for promising projects, to allow for an earlier completion of the WoE assessment. However, in order to minimize animal use on projects that might terminate early, this approach should generally be applied to high priority projects (e.g., expected to enter Phase III clinical trials or have shown early Proof of Concept). Of course, decisions to progress may differ between companies for strategic and scientific reasons. Still, the WoE approach becomes a progressive assessment that collates and absorbs relevant evidence as the project develops; it provides an early decision on whether a rat study is needed or not, and will minimize risk to the project timeline.

3.5 Reliability and confidence

Evaluation of the reliability of the data or in general of the acquired information (e.g., available experimental evidence, information from literature), is an essential component of the integrative assessment. Various factors have been suggested for evaluating data reliability (Myatt et al., 2018; Johnson et al., 2022), and these can be taken into account if relevant, including: a) compliance with internationally accepted best practice guidelines; b) agreement with test guidelines; c) data availability for independent inspection; d) concordance with other relevant assessments; e) transparency with respect to deviation from guidelines and protocols as well as discussion of outliers or extreme values (Johnson et al., 2022). In addition to data reliability, it is also critical to evaluate the overall confidence of an assessment (i.e., the strength of the assessment and its uncertainty). Reliability and confidence are different concepts as confidence in the assessment depends on reliability and relevance; relevance of experimental data refers to adequacy for the endpoint and the fit-for-purpose of the test and the corresponding evidence as further discussed by Johnson et al. (2022). The development of a scoring confidence system that can properly grade the different WoE factors is a challenging and complex task. Any assessment, intermediate or final, with a confidence less than high may prompt additional investigations and analysis to strengthen the conclusions. According to Johnson et al. (2022), a high confidence of the assessment suggests that sufficient evidence is available to support an accurate conclusion, and further research is unlikely to increase the confidence.

3.6 Metabolites

Consideration should also be given to major human metabolites. Metabolites identified only in human plasma or human metabolites present at greater than 10% of total drug-related exposure that are not present at comparable levels and cannot be qualified by high doses in



WoE #1 Target biology	• Target Carcinogenicity Assessment (TCA)	• TCA	• TCA (includes ADME, pharmacology)	• TCA (includes ADME, pharmacology)
WoE #2 Secondary pharmacology		• *In silico* screens • Targeted *in vitro* screens	• Secondary pharmacology screens	• (Additional) secondary pharmacology screens
WoE #3 Histopathology		• Read-across • 5-day *in vivo* rat	• 2-4-week repeated dose studies • Other relevant studies	• 3-month rat, repeated dose study • 6-month rat, (potentially started early to shorten timeline of WoE assessment) • Other relevant studies
WoE #4 Hormonal effects		• *In silico* screens • *In vitro* screens	• Teratology and fertility studies • Other *in vivo* and *in vitro*	• DART, endocrine, or related studies • Clinical biomarkers or other investigative approaches
WoE #5 Genotoxicity		• *In silico* screens • Non-GLP *in vitro* screens	• GLP genetic toxicology (ICH S2(R1))	• GLP genetic toxicology (ICH S2(R1)) • Investigative genotoxicity studies
WoE #6 Immune modulation			• Relevant *in vivo*, *in vitro*, and *in silico* results	• Immune modulation (ICH S8) • Clinical biomarkers • Other investigative approaches

FIGURE 4
Assessing carcinogenicity throughout pharmaceutical research and development (R&D) incorporating evidence from the ICH S1B(R1) factors. Abbreviations: IND (Investigational New Drug); NDA (New Drug Application); PI (Phase I); PII (Phase II); PIII (Phase III); ADME: absorption, distribution, metabolism and excretion; GLP: Good Laboratory Practice; DART: Developmental & Reproductive Toxicity.

animal test species, generally require additional safety assessment (FDA, 2020). Therefore, a section describing the metabolic profile and potential carcinogenic risk of major human metabolites is warranted. Discussion of the metabolites for each WoE factor can be incorporated along with the discussions of the parent compound. Various studies (e.g., *in vitro*, short-term dosing major human-specific metabolites) may need to be performed to fill in gaps in the WoE factors for these metabolites.

3.7 Reporting

The integrated assessment is to be clearly documented in the final report. A recommended structure of the report is outlined in Section 6. For example, the report would provide both information on timelines and search terms used for a particular search in the case of target biology analysis as well as summary search results. Information derived from toxicity studies will need to be summarized in the WoE assessment with reference links back to the original study reports. In general, the WoE report includes a summary section of each factor complemented with additional details supporting the conclusions in Appendices. A more extended discussion on information gathered for each WoE factor and other supportive information is presented in Sections 4 and 5.

4 The six WoE factors

The following sections discuss the elements to be considered when gathering and evaluating evidence from the different WoE areas. The six different WoE factors, as outlined in ICH S1B(R1), are examined below in varying levels of detail depending on how thoroughly the underlying procedures and corresponding best practices are already developed and established. Accordingly, the target biology analysis is presented here in detail highlighting recommended approaches to perform such analysis and gather relevant evidence. The secondary pharmacology WoE factor is discussed in terms of what additional aspects of the standard approaches may be considered to support the ICH S1B(R1) WoE assessment. A similar level of discussion is presented for the histopathology WoE factor from chronic toxicity studies, but it is noted that the guideline already specifies the type of relevant alerting signals that need to be evaluated. The discussion on the genotoxicity WoE factor is brief as the ICH S2(R1) guideline cited in the ICH S1B(R1) addendum fully covers such an assessment. On the other hand, the discussion on hormonal perturbation and immune modulation is hampered by the complexity of the topics. While there are specific examples of hormonal perturbation that are linked to certain carcinogenic outcomes (e.g., estrogen, thyroid hormones), for the majority of cancers these relationships are poorly understood. Similarly, the mechanisms by which effects on the immune system influence human cancer development are still being discerned.

4.1 Target biology WoE factor

4.1.1 Background on the target biology factor

The purpose of this individual WoE factor investigation is to determine whether any biological pathways related to the primary pharmacology of the drug candidate (either at the intended tissue site, or as well at other tissue sites where the target may be expressed

but therapeutic benefit is not expected) are involved in the development of human cancer. As part of such an assessment, different lines of evidence can be explored, including:

1. Empirical carcinogenicity data on target selective drugs within the same primary pharmacological class. Comparisons to other drugs within a class could (where possible) include an analysis of the similarity of the biological pathways involved, the mechanism of any carcinogenic effects for any previously tested molecules with a positive response in a 2-year rat bioassay (i.e., was the positive result related to target biology or some other factors?), relative potency for any carcinogenic activities related to the primary target for targets with multiple activities, and potentially other aspects such as clinical relevance of the effects, ADME characteristics or considerations based on the principles of read-across (Schultz et al., 2015; 2019).
2. The extent to which the responding biological pathways are well-characterized (e.g., knowledge of the receptor and down-stream or up-stream receptors/genes, interactions with other receptor pathways), and their potential involvement in cancer development (e.g., biological effects of the target exclude a role in immunosuppression, chronic inflammation, oxidative stress, functional interaction with nuclear receptors, and epigenetic effects such as modifications of histones and other structural cellular components). This will also include known human genotypes associated with cancer. Examples of resources to collect such evidence are included in Supplementary Table S1.
3. Relevant carcinogenicity risks related to the pharmacology of any major human metabolites whether related to the intended target of the parent or if there is interaction at closely related isoforms of the target or unintended targets.
4. Any additional links of the target to any of the ICH S1B(R1)-defined WoE factors (e.g., immunosuppression, hormonal effects).

Based on the description of the target biology WoE factor provided in the ICH S1B(R1) addendum, Table 2 outlines several topics to consider in documenting the findings and conclusions pertaining to this area. The outcome of the analysis is any interpretation from the literature/database searches supporting key findings, with the raw results from the literature and database searches included as archived supplementary information.

Broadly speaking, a 2-year rat bioassay will be considered to add value to the human carcinogenic risk assessment in uncertain situations, when the target biological pathway is either poorly characterized or there are up- or downstream events that are likely to lead to cancer, or the class effects of drugs with activity within this pathway are unknown (or include a risk of cancer). In addition, a first-in-class therapeutic has a higher chance to be considered for carcinogenicity testing unless additional supportive evidence is provided to fill in knowledge gaps to reduce cause for carcinogenic concern for the class. Conversely, if the target is involved in a well-characterized pathway and/or the compound of interest is from a class with well documented effects with positive or negative cancer risk, then it is unlikely that a 2-year rat bioassay will add value.

4.1.2 Target biology WoE evaluation

The target biology evaluation should use a repeatable, transparent, unbiased, and extensive analysis to provide a convincing conclusion regarding the risk of carcinogenicity. This evaluation includes analysis of the literature and relevant biological databases, utilizing similar approaches that have been used for wider assessments of target safety (Brennan, 2017). Integration of data from a variety of genomic and cancer-based resources (examples of which are included in Supplementary Table S1) will inform an assessment of carcinogenic potential (Carss et al., 2023). Emerging approaches such as network biology models may also be considered (e.g., Krämer et al., 2014). Individual literature searches and database queries should be documented, and it is advisable to preserve the unfiltered results. The results should be reviewed for relevance by the domain expert(s) and all key findings discussed to determine whether there is an overall and demonstrable risk of carcinogenicity. Importantly, evaluation of reliability and potential uncertainties should also be conducted for the data used in the analysis of target biology and primary pharmacology.

The report on the target biology analysis should include a balanced integrated evaluation of "negative" findings (i.e., where cancer risks have been investigated and no association with target biology was identified) as well as assessment of the relevance of any potential positive, equivocal, or incomplete information. It is likely that this assessment will broadly cover all aspects of target biology and is performed early in the project timeline [e.g., Target Safety Assessment (TSA)]. A data subset analysis of the main target biology evaluation report(s) used in the WoE assessment would need to focus on carcinogenicity risk endpoints identified in the early target safety assessment. These elements will be extracted into the overall carcinogenicity risk assessment. Notably, the main conclusions from the target biology analysis related to carcinogenicity would be summarized in the WoE report, whereas the corresponding broader, more detailed report can be included in the Appendices of the WoE report, as discussed further below in Section 6.

It should be noted that, although the target biology and primary pharmacology evaluations are needed to support regulatory conversations aligned with ICH S1B(R1), they can also be considered as part of a more proactive strategy started early in the drug discovery and development process (see Figure 4) with initial data (e.g., target biology, genetic toxicity studies) and further data being added to the assessment as it is generated (e.g., histopathology from the chronic toxicology studies is likely the last piece of evidence). Such upfront evaluation, coupled with increasingly informative experimental results from chronic studies, can provide input into product stewardship, and potentially avoid costly and unforeseen impact to the project timeline if a 2-year rat bioassay is determined to be necessary during late-stage clinical trials. Many pharmaceutical companies currently perform a version of this general assessment of target risk (e.g., TSA) either internally or by outsourcing. The TSA could be modified to increase the focus on carcinogenicity endpoints. This early-stage assessment can be used for determining any gaps in carcinogenicity risk assessment which may be filled by incorporation of endpoints into upcoming planned studies or investigational studies [e.g., need for

TABLE 2 Outline of the content related to the evaluation of the target biology WoE factor. Notably, evaluation of reliability and potential uncertainties should also be conducted for the data used in the analysis of target biology and primary pharmacology. The detailed report of the target biology analysis is used to draw conclusions on the corresponding WoE factor.

Sections	Description
1. Executive summary	Summary addressing the following points, where appropriate: (1) an evaluation of whether the target biological pathways are well characterized and are demonstrably associated or involved in human cancer development; (2) an assessment of any relevant carcinogenicity data available for other chemicals within the same pharmacological class (or absence in the case of first-in-class drugs); (3) a carcinogenicity evaluation of major human metabolite(s) and their associated target(s); (4) assessment of data reliability and confidence of the analysis with reference for need for further analyses and/or uncertainty clarification; (5) a conclusion regarding whether a 2-year rat study would add value to the human carcinogenicity risk assessment.
2. Materials and methods	Description and record of databases examined, literature searches performed and any other data science procedures (e.g., data analysis, artificial intelligence, machine learning, data processing, and modelling).
3. Summary of target pathway(s) and pharmacological class	Background biology information related to normal physiological role of the target pathway and pharmacological class. This could include: • summary of the signaling pathways in which the target is involved; • cell, tissue, and organ/organ system function; • comparison of tissue distribution between species; • links to any of the identified WoE factors (e.g., hormonal effects or immune modulation). The potential association of target pathways with tumor development would be summarized and assessed for human and target relevance, including examples such as: • classification of the target as an oncogene/tumor suppressor or its potential to lead to or exacerbate tumorigenesis; • associations made at the pathway level, rather than separately, assessing upstream/downstream pathway components; this analysis would likely involve the interrogation of multiple structured and unstructured (e.g., literature) data sources; • use of human genomics databases [e.g., Carss et al. (2023)] to inform wider assessments of target safety, including carcinogenicity risk evaluations; • use of gene ontology terms as derived from the database interrogations and mapped onto cancer hallmarks (Chen et al., 2021); hallmarks of cancer represent a conceptual framework that recapitulates the functional capabilities of cells collectively leading to malignant growth (Hanahan and Weinberg, 2000; 2011; Hanahan, 2022); • any evidence from the scientific literature and phenotypic databases that directly implicates modulation of target function (such as modulated, hyperactive and hypoactive states) with cancer. All of the evidence will be qualified (where appropriate) by species, anatomical location and intervention type.
4. Summary of drug mechanism of action	Information on the pharmacological activity of the drug, and any known human metabolites. This is discussed alongside relevant information regarding the drug class including a description of known/proposed mechanism(s) of action, and a listing of commonly used drug and target synonyms. Also, an assessment can be made of how active the drug is likely to be against rat orthologues, and how this may translate to effective doses in rat and human. Closely related "off target" subtypes (subtypes or isoforms of the primary target) should also be considered when rat carcinogenicity study exposures would be likely to reach pharmacologically active drug concentrations. Relative human/rodent affinities at target exposures at these off-target subtypes in rats and humans can be assessed accordingly to help address human relevance.
5. Carcinogenicity assessment of primary pharmacological class	Discussion on the human relevance of carcinogenicity data for pharmacological class. These data could be obtained from: • labels and package inserts obligated by regulatory authorities (noting both the presence or absence of relevant data), and related relevant documentation; • published clinical studies including clinical trials and post market surveillance/pharmacovigilance and other human data; • published rodent carcinogenicity data including knock-out or other genetically engineered animal models; for example, studies completed by sponsors early in the rasH2Tg model (Sistare et al., 2011; Morton et al., 2014; Hisada et al., 2022) can be helpful for anticipating an association of target modulation with tumor outcome in rodents. Additional information, such as the results from (Q)SAR or read-across models (considering substances with the same pharmacology), may be included where they contribute to the mechanistic understanding or support an evaluation of the structural basis of carcinogenicity (or lack of) across chemicals in the drug class.

(Continued on following page)

TABLE 2 (*Continued*) Outline of the content related to the evaluation of the target biology WoE factor. Notably, evaluation of reliability and potential uncertainties should also be conducted for the data used in the analysis of target biology and primary pharmacology. The detailed report of the target biology analysis is used to draw conclusions on the corresponding WoE factor.

Sections	Description
6. Analysis of cancer risk of major human metabolite(s)	When information on major human-relevant metabolites becomes available, their pharmacological target(s) should be addressed with particular reference to target biology. Carcinogenic potential of such metabolites could be investigated, for example, using (Q)SAR methods. However, an evaluation of secondary pharmacology (e.g., in instances where the principal pharmacological target for a metabolite differs from that of the parent compound) is the subject of WoE factor 2. Comparison (e.g., exposure ratios and differences highlighted) of rat and human metabolites could be performed. Results from non-rodent species may be supportive of the assessment of such metabolites.
7. Conclusions	General conclusions drawn based on the topics discussed above reiterating the conclusion from the Executive Summary regarding whether a 2-year rat study would add value to the human carcinogenicity risk assessment.
8. Appendices	Additional information may be gathered including information on: • the molecular profile (DNA, RNA and protein structure, binding domains, isoforms, variants, interactions, orthologues, paralogues, degradation, cellular location); • anatomical distribution (i.e., a comprehensive review of RNA, protein and operational/functional expression across different cell types, tissues, organs and systems across a range of species); Links to archived raw output as as supplementary data file(s) may be provided. Where applicable, a metabolic pathway could be included.
9. Supplementary Information	Raw output from the different literature and database searches can be made available.

additional nonclinical or clinical data approaches as listed in Figure 2 of ICH S1B(R1)] to minimize the performance of additional studies late in the project.

4.2 Secondary pharmacology WoE factor

4.2.1 Background on the secondary pharmacology WoE factor

Documentation of safety risks in humans includes studies of the mode of action and/or effects of a compound not related to the desired therapeutic target. Characterization of the off-target interactions has been termed secondary pharmacology profiling in contrast to primary pharmacology and safety pharmacology studies (ICH S7A, 2000). The safety pharmacodynamic effects of a drug candidate may result from functional interaction with the primary molecular target, secondary targets or non-specific interactions (Valentin and Hammond, 2008).

To investigate the off-target interactions leading to potential safety concerns (secondary pharmacology), industry uses *in vitro* assay panels against multiple unintended targets (i.e., receptors, ion channels, enzymes including kinases, and transporters) with the aim of exploring off-target interactions to focus on selecting more specific molecules to move forward and thus of reducing liabilities potentially leading to toxicity (Valentin et al., 2018; 2023; Jenkinson et al., 2020). The number of targets and target classes tested vary across the industry (Bowes et al., 2012; Bendels et al., 2019; Lynch et al., 2017); however, a trend is emerging with significant overlap in the screening strategies across organizations (Valentin et al., 2018; Jenkinson et al., 2020). The physiological and/or histopathological role of the targets and potential clinical implications usually determine the battery of targets that are selected for the screening. After this, off-target effects are evaluated extensively in *in vivo* regulatory toxicology and safety pharmacology studies.

The guidance for industry on safety pharmacology studies for human pharmaceuticals generally indicates that the design of safety pharmacology studies should consider ligand binding or enzyme assay data suggesting a potential for adverse effects, but it does not recommend the selection of specific targets that should be screened in a secondary pharmacology profiling (ICH S7A, 2000); the only example is the screening against Kv11.1 (i.e., hERG) encapsulated under the ICH S7B (ICH S7B, 2005). As Valentin and Leishman (2023) have recently observed, secondary pharmacology studies are not described in any dedicated guideline but they are sparsely referenced in ICH S7A despite these studies being critical to support hazard identification and human risk assessment, management and mitigation, and they are included in the regulatory submission process together with primary and safety pharmacology studies.

This leads to a potential gap on what targets relative to carcinogenicity assessment are necessary to include in a secondary pharmacology panel to support the discussion on the secondary pharmacology WoE factor. Thus, current panels should be reviewed to ensure that it is clear which targets are relevant to carcinogenicity assessment as it will be discussed further below.

Frequently *in vitro* secondary pharmacology testing is initially conducted at a single concentration, and in such cases the test concentration of 10 μM is used by over 50% of sponsors (Valentin et al., 2018). The 10 μM concentration was historically selected because it offered a >100-fold exposure multiple over the therapeutic free plasma exposure of most small molecule drugs. That said, alternative approaches do exist based on the modalities, the therapeutic, or pharmacological classes and individual organizational strategies. This initial testing narrows the number of targets to be submitted for further evaluation of full concentration-response curves in follow-up functional assay tests. This is required to characterize the drug's potency, mode of action (e.g., agonist, partial agonist, antagonist) and it also allows to rank

compounds of interest with respect to their levels of concern to help guide lead selection. Using this data in conjunction with the drug's potency on its primary target, an exposure margin at the expected clinical plasma exposure can be estimated for all secondary targets that are suspected to play a role in carcinogenesis. The margin of safety (MOS) is the ratio between the drug's *in vitro* potency and the unbound clinical plasma concentration; as a rule of thumb, all off-target specific safety margins should typically exceed 30-fold (Redfern et al., 2003; Muller and Milton, 2012; Papoian et al., 2015). In relation to off-target activities, the Cmax (free or unbound) drug concentration is typically used to calculate the MOS. However, the recently released ICH E14/S7B IWG (2022) refers to using both the free and total (i.e., bound) drug concentration especially when species differences in human plasma protein binding (PPB) exist, and for highly PPB drugs. Additionally, the AUC should be considered for MOS determination when appropriate.

4.2.2 Secondary pharmacology WoE evaluation

The secondary pharmacology WoE factor integrates results from off-target profiling for both the specific pharmaceutical being evaluated (see Table 1) and any major human specific metabolites (FDA, 2020). In the context of the ICH S1B(R1) integrated assessment, secondary pharmacology screening is assessed based on promiscuity of the pharmaceutical towards secondary targets (which are not necessarily mechanism-related to cancer). As shown in Table 1, "low target selectivity, off-target activity" is an indication that the 2-year rat carcinogenicity study would add value as compared to "high target selectivity, no off-target activity" (ICH S1B(R1), 2022). As such, a pharmaceutical with a high selectivity and no off-target activity at a large human exposure multiple would provide confidence for a low carcinogenic risk and therefore for a low added value of conducting a 2-year bioassay study. In addition, the ICH S1B(R1) WoE should take into consideration the inclusion of cancer-relevant targets in the secondary pharmacology screen. The screening should evaluate off-target interactions for specific targets "that inform carcinogenic risk (e.g., binding to nuclear receptors)" (ICH S1B(R1), 2022). The ICH S1B(R1) addendum discusses several case studies providing examples of the assessment of secondary pharmacology results (e.g., "No evidence of off-target interactions at drug concentrations up to 10 μM, including no interaction with estrogen, androgen, glucocorticoid receptors"; "Antagonist binding interaction identified for one off-target receptor with Ki 8-fold higher than Cmax at maximum clinical dose"; "Known pharmacology of off-target receptor not associated with tumorigenesis").

As indicated above, major human specific metabolites should also be evaluated for off-target interactions. Major metabolites currently considered for safety assessment are those identified only in human plasma and present at greater than 10% of total drug-related exposure at steady state (FDA, 2020).

Since most secondary pharmacology targets traditionally tested are human targets, the results are by default of human relevance. However, the sponsor might also consider conducting secondary pharmacology screens on other species-specific or disproportionate metabolites that are evaluated in animal models using a panel of species-specific targets or by means of computational modelling techniques. This might help to shed light into any functional and/or histopathological findings of concern for carcinogenicity that may be species specific, and possibly lacking human relevance.

In the absence of a single "carcinogenicity risk-specific" secondary pharmacology screen, the data from the multiple screens performed during drug development can be summarized for the integrated ICH S1B(R1) summary by pointing out results that inform on cancer risk. For example, no interaction in standard off-target and kinase panels, including binding to pro-inflammatory targets, hormone receptors and/or nuclear receptors, would be relevant outcome generally supporting no value of the 2-year rat bioassay. Insights from secondary pharmacology may be used to explain histopathological findings of concern from the animal models and support the identification and assessment of human-relevant effects (Ribeiro et al., 2020).

Any interaction with secondary targets would prompt an analysis of other supporting evidence assisting an active relationship between such molecular targets and carcinogenesis pathways (including associations with hormonal perturbation and immune modulation that may manifest as histologic findings after 6 months of exposure). An approach like the analysis of the target biology and primary pharmacology mechanism may be envisaged, if necessary, where the human-relevance of any off-target interactions and its possible association with carcinogenicity can be explored. It is also important to remember that secondary pharmacology screens represent human sequence targets, and not those of the rodent, so significant potency differences may exist.

In summary, understanding the characteristics of both on-target and off-target hits through the integrated analysis described above, along with the relative potency and activity compared to the intended target activity at anticipated human exposures, enables development of an integrated risk assessment to further characterize and interpret the functional and/or histopathological findings in animal models and their potential human relevance. Relevant elements useful to summarize the experimental findings from secondary pharmacology results within the ICH S1B(R1) assessment are displayed in Table 3.

4.2.3 Cancer-related off-targets panels

The addendum emphasizes the importance of targets that inform carcinogenic risk such as binding to nuclear receptors (Table 1). In general, several targets, such as aryl hydrocarbon receptor (AhR) (Murray et al., 2014), p38 kinase (Kudaravalli et al., 2022) or epigenetic targets (Herceg et al., 2013), have a demonstrated role in development of some types of tumors, but a full comprehensive list of targets critically associated with a carcinogenic risk has not been identified. Screening panels specifically including cancer-related targets are being proposed, where to our knowledge, the scientific rationale on the association between the targets and carcinogenic potential has not been fully elucidated (Eurofins, 2023). Comprehensive literature searches based on cancer-gene databases might support the identification of cancer-relevant targets and currently activities are under way to isolate, review, and describe targets (Rider, 2023) that might then be used as biomarkers in assessing the carcinogenic potential of chemicals. When associations are identified, further investigation of available evidence is needed to demonstrate the causal relationship between a given target and cancer, as well as its human-relevance.

As summarized by Tice et al. (2021), numerous targets can be involved in carcinogenesis, e.g., activation of PI3/AKT signaling through G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (Martini et al., 2014). Carcinogens may act through modulation of receptor-mediated effects (e.g., estrogen receptor (ER), peroxisome proliferator-activated receptor (PPAR), and AhR) or modulation of endogenous ligands (including hormones) (Smith et al., 2016; 2020). Attention has been devoted to nuclear receptors (Dhiman et al., 2018; Zhao et al., 2019) and their co-regulators (Lonard and O'Malley, 2012) that play crucial roles in normal physiological processes, and alterations of such receptors impact the development of cancer. Examples of nuclear receptors' involvement in cancer are hormone-dependent cancers (e.g., estrogen-dependent breast cancer) (Emons, 2022). There is a considerable overlap between the processes involved in receptor-mediated effect modulation and hormonal effects given the involvement of receptor-based signaling in both cancer and endocrine disruption. Receptors involved in receptor-mediated rodent carcinogenesis include constitutive androstane receptor (CAR), PPAR alpha, and AhR (Klaassen, 2019).

Notably, some targets that are usually employed in secondary pharmacology screening (Bowes et al., 2012; Lynch et al., 2017) are associated with cancer-related AOPs as derived from the AOP wiki (AOP Knowledgebase, 2023); these targets are, for example, AR Human Androgen nuclear hormone receptor (NHR), D2S Human Dopamine GPCR, Beta-2 Human Adrenoceptor GPCR, and Human PPAR gamma NHR. The off-target panels described by Bowes et al. (2012) and Lynch et al. (2017) also include several targets associated with immune effects (e.g., Cannabinoid receptor CB_2, Lymphocyte-specific protein tyrosine kinase, Adenosine A_{2B} Receptor) and endocrine effects (e.g., Dopamine receptor D_2 and Serotonin 1A receptor 5-HT_{1A}). The effects associated with a given target are specifically reported by Bowes et al. (2012) and Lynch et al. (2017) as derived from the analysis of adverse drug reactions (ADRs) described in the literature.

The development of a cancer-related off-target panel would need to pay special attention to the human relevance of the pathways underlying a specific off-target activity. For example, Beta-2 Human Adrenoceptor GPCR is associated with the AOP involving Beta-2 adrenergic agonist activity leading to mesovarian leiomyomas in the rat and mouse, but this pathway is considered human irrelevant by the scientific community (Kelly et al., 1993; ECETOC, 2006). On the other hand, the human relevance of anti-dopaminergic activity (D2S Human Dopamine GPCR) leading to mammary adenomas and carcinomas in the Sprague-Dawley rat is still controversial (Harvey, 2005). Additionally, the relationship between targets and AOPs should be ultimately evaluated in terms of relevance to clinical use according to the elements in Table 3.

A cross sector effort involving safety scientists from academia, industry, service and technology providers and health authorities should be established to support the development of a cancer-related panel of targets to support the ICH S1B(R1) secondary pharmacology factor. Similar initiatives have led to the successful identification of targets associated with key safety risks as in the case of seizure liability (Easter et al., 2009; Roberts et al., 2021).

4.3 Histopathology from chronic studies WoE factor

Histopathology evaluation of toxicology studies, especially chronic toxicology studies, may identify proliferative or pre-neoplastic lesions as specified in the ICHS1B(R1) histopathology WoE category. These lesions may also provide information that contributes to the assessment of other WoE categories, including hormonal effects and immune modulation. Lesions that may be expected from the targeted pharmacology, or the secondary pharmacology that are described in the earlier sections of the WoE, may also be observed in the chronic toxicology study histopathology.

The presence or lack of proliferative or pre-neoplastic changes in the chronic toxicology studies is certainly an important factor in the WoE evaluation. When proliferative or pre-neoplastic changes are identified, the pathologist or toxicologist is left with interpreting the relevance or non-relevance of the findings to humans. Rodent specific findings considered not relevant to humans have been described and are documented in the public literature. Findings of unknown clinical significance will shift the WoE assessment to identifying that additional investigative studies may be needed and/or that a 2-year rat study may add value to the carcinogenicity risk assessment. The ICH S1B(R1) addendum provides a detailed description of relevant histology findings from chronic studies that would be considered alerts for carcinogenic potential. The 6-month rat study is expected to be the main source of information but other types of studies (shorter-term rat studies, longer-term non-rodent studies, longer-term mouse studies, and early clinical data) can be integrated to build the WoE assessment or provide earlier alerts to potential carcinogenic risk.

The original description of the preneoplastic constellation of observations (e.g., cellular hypertrophy, cellular hyperplasia, persistent tissue injury and/or chronic inflammation, foci of cellular alteration, preneoplastic changes, and tumors) gathered from repeated-dose toxicity studies (with emphasis on the 6-month rat study) is reported in Table 1. The full pathology report and individual animal findings should be examined for proliferative findings that may not be highlighted in the main summary. It should be noted that standard terminology for cancer-relevant histopathological findings should be utilized in study reports and histopathology interpretations. An example of this terminology is the INHAND criteria (www.goreni.org). Participation of an expert pathologist in this part of the WoE evaluation is necessary.

The evaluation of this WoE factor should include presentation and discussion of the plasma exposure margin of the parent and any major metabolites relative to clinical exposure. The dose corresponding to the plasma exposure at which pre-neoplastic effects are observed from animal studies (and if it is dose-dependent) can be extrapolated to a human equivalent dose (HED) in the early phases of the WoE evaluation or, if human exposures are known, animal exposures can be directly compared to the human AUC or C_{max}, as appropriate. The occurrence of proliferative findings at a high exposure multiple that will not be reached in the clinic could mitigate the need for a 2-year rat study when the WoE data are integrated. This potential human exposure

TABLE 3 Elements to summarize the secondary pharmacology results for each molecular target within the ICH S1B(R1) assessment.

Title	Details
Molecular target	Name of the molecular target (including details such as gene and IUPHAR names and/or Uniprot ID)
Tested chemical	Chemical being tested with indication on whether it is the parent drug or metabolite(s)
Methodology	Short description of methodology including information providing confidence in the assay (e.g., positive and negative controls, number of replicates)
Efficacy	Percentage of maximal response
Potency	*In vitro* binding affinity (IC_{50}, K_i) or cellular functional activity (EC50)
Mode of action	Details on mode of action, e.g., agonist, partial agonist, biased agonist, and antagonist
Human plasma exposure	Cmax and AUC, both total and free
Exposure multiple	Test concentration of drugs/metabolites in relation to the measured or anticipated clinical exposure (e.g., 10-, 30-, 100-, 300-, and/or 1000-fold multiples)
Margin of safety	Assessment of *in vitro* off-target potency in relation to human exposure (e.g., the ratio between the *in vitro* activity and the unbound clinical plasma concentration)
Likelihood of carcinogenic risk to humans with evaluation of the confidence	Conclusion on carcinogenic risk to humans

Abbreviations: AUC: the area under the plot of plasma concentration of drug against time after drug administration; Cmax: the maximum or "peak" concentration of a drug observed after its administration; EC_{50}: half-maximal effective concentration; IC_{50}: half-maximal inhibitory concentration; IUPHAR: International Union of Basic and Clinical Pharmacology (IUPHAR/BPS, 2023); K_i: inhibition constant; UniProt: universal Protein Resource (UniProt, 2023).

risk relative to exposures in animal studies is used as part of the overall WoE assessment.

It is important to discuss the relevance of rodent lesions (proliferative and non-neoplastic) that occur with an incidence level above study matched controls or appropriate historical controls. Spontaneous genetic alterations occur in commonly used rodent strains, and genetic drift should be considered if unexpected findings occur when changing animal suppliers or test facilities. Also, especially as new mouse models of disease are investigated, unexpected histologic pre-neoplastic findings may be observed and must be interpreted in conjunction with mouse genetics and strain background (e.g., Alison et al., 1994; Szymanska et al., 2014). An example of a rodent-specific finding is the induction of alpha 2u-globulin nephropathy in male rats, which has data to support that it is not relevant in human risk assessment (Swenberg, 1993). The goal of investigative studies would be to increase the understanding of the relevance of changes present in toxicology studies to humans, potentially due to differences in anatomy/physiology, metabolism or because of differences in sensitivity, with human exposure being below the threshold at which homeostasis is perturbed. Overall, understanding of the pathogenesis of the lesions and the underlying mechanism would support the evaluation of human relevance as well as the WoE integrated assessment.

As regards to mechanistic interpretation, chronic inflammation, for example, creates a local microenvironment that can induce genomic instability in cells (Smith et al., 2016; 2020; Tice et al., 2021). Inflammation generates various mediators including cytokines, reactive oxygen and nitrogen species (ROS and RNS respectively), serine and cysteine proteases, membrane perforating agents, matrix metalloproteinase (MMP), tumor necrosis factor alpha (TNFα), interleukins (IL-11, IL-6, and IL-8), interferons (IFNs) and enzymes, as cyclooxygenase-2 (COX-2), lipooxygenase-5 (LOX-5) and phospholipase A2 (PLA2), which activate or are activated by transcription factors such as nuclear factor-κB (NF-κB) and signal transducers and activators of transcription-3 (STAT3) (Vendramini-Costa and Carvalho, 2012). These events induce oxidative stress and facilitate mutations, epigenetic changes, or genomic instability (Multhoff et al., 2012; Vendramini-Costa and Carvalho, 2012; Wu et al., 2014; Ding et al., 2019) while prolonged release of the inflammatory mediators facilitates growth, progression, and tumor invasion. Potential investigative studies that examine the key elements of chronic inflammation could serve as additional data for the overall WoE.

4.4 Genotoxicity WoE factor

Genetic toxicology testing assesses whether a compound can cause DNA damage that leads to heritable defects and thus potentially cancer. There is abundant evidence that genetic alterations constitute a cancer risk and may be a prerequisite to tumor development. Thus, genetic toxicology assessment has been a standard for evaluation of cancer risk for many decades. In the drug discovery and development process, the genotoxicity potential of a drug candidate is assessed by means of a series of genetic toxicity tests according to a core battery well defined by the regulatory guideline ICH S2(R1) (2012). ICH S2(R1) should be used in conjunction with ICH S1B(R1) for understanding the interpretation of the results of the genotoxicity battery for the WoE determination. Unequivocally negative (or resolved positive or equivocal findings resulting in a WoE conclusion that genetic toxicity is of low risk) or positive genetic toxicity results as defined by ICH S1B(R1) provide evidence that a 2-year rat bioassay is less likely to add value to the carcinogenicity risk assessment. Alternatively, genetic toxicity results that are of uncertain relevance to humans (which cannot be resolved by investigative approaches described in

relevant guidelines) will indicate that a 2-year rat bioassay will add value to the human carcinogenicity risk assessment.

The ICH S2(R1) core battery includes two options. In option 1, *in vitro* tests (a bacterial reverse mutation assay and a cytogenetic test for chromosomal damage or a mouse lymphoma *Tk* gene mutation assay) are conducted to evaluate gene mutations and chromosomal damage followed by an *in vivo* evaluation of chromosome level effects. Additional *in vivo* tests may be needed as a follow-up strategy for positive or equivocal results in option 1. The option 2 battery includes the *in vitro* bacterial reverse mutation assay and *in vivo* testing of two genotoxic endpoints in two tissues. Other tests that are conducted in addition to the ICH S2(R1) core battery to investigate the genotoxicity mechanisms and the relevance of the response to humans (as appropriate) are, for example, (Nicolette, 2017): a) *in vitro* comet or alkaline elution (different cell types) conducted as early screening and for mechanistic evaluations; b) *in vivo* comet conducted to further investigate positive bacterial or mammalian *in vitro* tests from the core battery; c) transgenic rodent gene mutation to further investigate *in vitro* gene mutation results; d) mammalian Erythrocyte Pig-a Gene Mutation Assay particularly following Ames positive results (Robison et al., 2021). Further reading on the combination of genotoxicity results for genotoxicity assessment is in the publication by Hasselgren et al. (2019).

4.5 Hormonal perturbation WoE factor

The evaluation of hormonal effects potentially leading to carcinogenic risk is a critical component of the weight of the evidence evaluation originating from different sources as outlined in the ICH S1B(R1) addendum (see Table 1). This assessment is illustrated in Figure 5. The evaluation of hormonal perturbation is primarily based on findings from repeated-dose toxicity studies and relevant signals from reproductive toxicology studies that suggest hormonal perturbation. These include microscopic changes in endocrine or reproductive tissues of atrophy, hypertrophy, and hyperplasia and/or biologically significant endocrine and reproductive organ weight changes which are not explained as findings secondary to processes such as stress or altered body weight (ICH S1B(R1), 2022). If there is concern for potential endocrine effects early in the development program, hormonal measurements can be made during the 4-week or 6-month toxicology studies and results compared to clinical data to assess the relevance to patients. Alternatively, targeted hormonal studies can be conducted once a specific concern is identified. In designing these studies, care must be taken to ensure that samples are taken at appropriate time points to minimize impact of diurnal or reproductive cycles on the results.

As outlined by the guideline, knowledge of drug target and compensatory endocrine response mechanisms is also an element to consider, and this knowledge can be acquired within the analysis of the target biology WoE factor. Notably, secondary pharmacology screening may inform on potential interactions with targets that have been associated with the endocrine system (Bowes et al., 2012; Lynch et al., 2017). Additionally, investigative approaches (e.g., *in vitro* studies with cells from endocrine-controlled tissues) may help to clarify potential concerns. Moreover, confirmation of hormonal changes identified in animal studies with samples taken in clinical trials may confirm the relevance of the animal findings to humans.

As mentioned earlier, it is essential to understand pathogenesis and human relevance of hormonal perturbations. This would also include discussion of the plasma exposure margins.

4.6 Immune modulation WoE

4.6.1 Immune modulation WoE assessment

The WoE integrated assessment requires the evaluation of the immune modulation factor according to the ICH S8 guideline, which applies to new human pharmaceuticals (ICH S8, 2006). The ICH S8 guideline restricts immunotoxicity to "unintended immunosuppression and immunoenhancement, excluding allergenicity or drug specific autoimmunity". Evaluation of immune modulation is based on a weight of evidence that requires additional immunotoxicity testing based on the following constellation of observations (a single positive signal prompts additional in-depth studies on the potential concern for immunotoxicity):

- Preliminary toxicology findings indicating immune modulation from standard toxicity studies (rodent and non-rodent studies from early short term to more chronic repeated-dose studies); the ICH S8 guideline lists the relevant signals indicating potential immunosuppression or enhanced activation of the immune system.
- Pharmacological properties of the compound that indicate potential modulation of the immune function.
- The intended indication and patient population to evaluate whether the intended patient population is already in an immunocompromised state.
- Structural similarities to known immunomodulators.
- Disposition properties of the drug to evaluate whether the drug is retained at high concentrations in cells of the immune system.
- Clinical observations in case of on-going clinical trials.

The new FDA guidance on Nonclinical Evaluation of the Immunotoxic Potential of Pharmaceuticals (FDA, 2023b) provides additional information on assessment of immune function relating to carcinogenicity specifically noting the need to consider the potential for a drug candidate to increase tumor promotion, growth, and metastasis. Additional points of consideration include "effects of the pharmaceutical on key immune components thought to be involved in tumor surveillance (e.g., NK cells, T cells, antigen-presenting cells), such as downregulation or functional impairment of key immune-cell populations" (FDA, 2023b). Figure 6 summarizes examples of elements that can inform cancer risk assessment for immunomodulators (Lebrec et al., 2016) framed into the ICH S1B(R1) assessment.

4.6.2 Immunosuppression

Several carcinogens can act largely via immunosuppression and this is particularly true of drugs intended to prevent

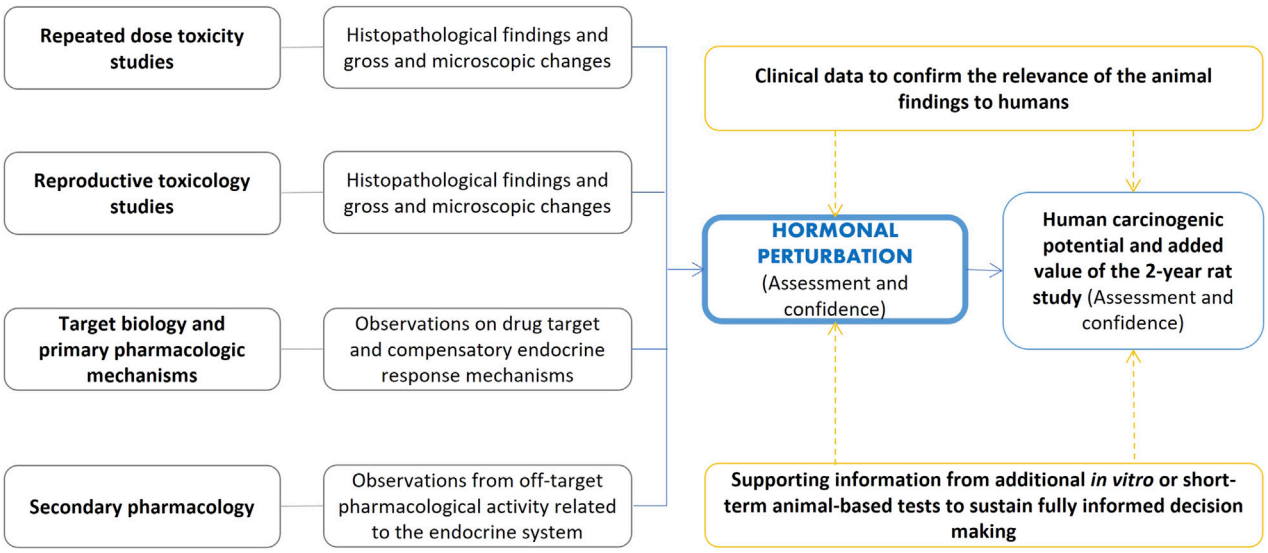


FIGURE 5
Assessment of hormonal perturbation contributing to the evaluation of human carcinogenic potential and the added value of the 2-year rat study. The ICH S1B(R1) addendum refers to relevant signals from repeated-dose toxicity studies and reproductive toxicology studies (including, for example, changes in organ weights, see Table 1). Secondary pharmacology screens may also inform on the interactions with targets associated with the endocrine system. Supporting evidence from other investigative approaches may aid intelligent decision making.

transplant rejection [e.g., cyclosporin (Rafferty et al., 2012)] and some classes of agents intended to treat inflammatory diseases. Immunosuppression may not directly transform normal cells into potential tumor cells. Instead, immunosuppression can both inhibit and potentiate neoplasia with pre-neoplastic cells that manage to evade mechanisms of elimination thereby having their survival and/or replication facilitated (Bugelski et al., 2010; Lebrec et al., 2016; Smith et al., 2016).

The relationship between the immune system and development of cancer (Lebrec et al., 2016; Ponce, 2018) has been related to different mechanisms including tumor immunoediting (Dunn et al., 2002), oncogenic viruses (Engels et al., 2008), chronic inflammation (Mantovani et al., 2008), and chronic B cell stimulation (Küppers, 2005). Though not true for all cases, many of the cancers known to be associated with chronic immunosuppression (e.g., transplant recipients, HIV/AIDS) appear to be related to chronic infection (e.g., viruses, bacteria, parasites). Each of these mechanisms can occur simultaneously (Ponce, 2018).

An FDA and HESI funded workshop (Lebrec et al., 2016) concluded there is a limited understanding of the quantitative relationship between immunosuppression and cancer risk, stating that an increased focus on new approaches for monitoring immune function and early detection of cancer risk in humans is needed. Information from nonclinical experiments, clinical epidemiology and immunomodulatory therapeutics show that the complex link between immunosuppression and cancer risk is multifactorial and does not correlate well with the 2-year rodent bioassay (Bugelski et al., 2010; Lebrec et al., 2016). This view is supported by ICH S1B(R1) and the recent FDA guidance (FDA, 2023b) which notes that "animal models, including rodent carcinogenicity studies, have been shown to be of limited help in identifying an increased cancer risk that may arise in patients as a consequence of immunosuppression", an observation that is "particularly true when the increased tumor risk is caused by recrudescence of latent viral oncogenes, infectious agents, or chronic inflammatory states, for which significant species differences exist that make clinical translatability challenging". Furthermore, cancer risk associated with immunosuppression cannot be assumed to be similar for all immunomodulatory molecules. Any evaluation therefore needs to be a mechanism-based weight-of-evidence approach, including data from immune function tests and their relationship to tumor initiation, immunosurveillance, and tumor promotion, in addition to the consideration of underlying human disease (ICH S8, 2006; Lebrec et al., 2016; FDA, 2023b). Of interest, the lack of human predictivity in rodents may be related to differences in structure, development and function of the immune system between rodents and humans (Haley, 2003; Holsapple et al., 2003; Kotturi et al., 2009; Bugelski et al., 2010). The use of human cells *in vitro*/*ex vivo* to screen for potential immunomodulatory effects has demonstrated encouraging potential (Phadnis-Moghe and Kaminski, 2017) and may serve to augment current immunologic investigations.

As noted in the ICH S1B(R1) guideline, a 2-year rat study is less likely to add value when there are either no effects on the immune system (e.g., in a 6-month rat or 9-month non-rodent study) or when broad immunosuppression is expected based on target biology evaluation or results of standard toxicology studies and immunotoxicity follow-up testing (as recommended by ICH S8). In the latter case, while a human carcinogenicity risk is expected, this can be addressed by appropriate discussion in the WoE document and product labeling. Findings of tumors in clinical trials of immunosuppressive agents will guide stricter labeling (e.g., boxed warning). Assessment of the impact of immunosuppressive or immunomodulatory activity on

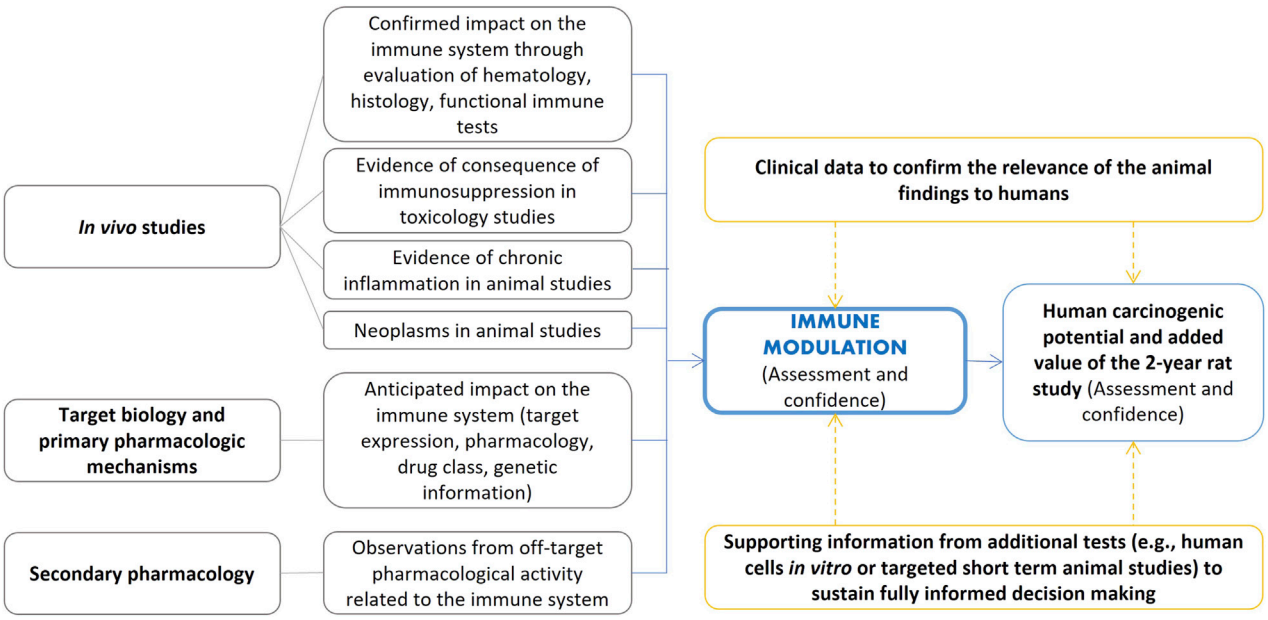


FIGURE 6
Examples of elements that can inform cancer risk assessment for immunomodulators as adapted from Lebrec et al. (2016) and framed in the ICH S1B(R1) assessment.

carcinogenic risk is expected to gain no further insights from the conduct of a 2-year rat study.

5 Other information

5.1 Additional studies

It is expected that additional studies including novel technologies that target identified knowledge gaps in the WoE assessment and support the understanding of human relevance of signals, could complement the evidence from the six WoE factors. These would help to clarify potential concerns and aid intelligent decisions. In general, any novel investigative approach that is based on rigorous scientific methods may provide useful evidence. An example of this may be the quantitation of clones with cancer driver mutations (Marchetti et al., 2023). Importantly, attention should be paid to the quality of conduct of these studies and how widely accepted the proposed studies are (scientifically and by regulatory agencies).

The ICH S1B(R1) mentions (but not limited to):

- Nonclinical approaches: special histochemical stains, molecular biomarkers, serum hormone levels, immune cell function, *in vitro* or *in vivo* test systems, data from emerging technologies.
- Clinical approaches: generated to inform human mechanistic relevance at therapeutic doses and exposures (e.g., drug concentrations in urine and evidence of crystal formation; targeted measurements of clinical plasma hormonal alterations; human imaging data).

5.2 *In silico* approaches

For the assessment of complex endpoints, there are known issues and limitations to employing *in silico* approaches including (Q)SARs in isolation; however, their use within an integrated assessment framework to help explain specific experimental signals, is justified.

For example, while not routinely performed, application of appropriate *in silico* methods can support secondary pharmacology screening to fill in data gaps in experimental profiling (Jenkinson et al., 2020) and they may become more commonplace in the future. Experimental screening can be combined with predictions from computational models (e.g., statistical- or expert-based systems) if they are developed using an adequate experimental dataset for cancer-related targets and covering an appropriate chemical space. However, such models must be used with caution to avoid the problem of unpredictable events, and furthermore are not a prerequisite but only one tool to aid the expert judgement.

Moreover, *in silico* approaches can make use of resources that collect carcinogenicity study findings with details on the histopathological findings from the corresponding animal studies. Various publications have reviewed the carcinogenicity databases together with available (Q)SAR models that are based on such databases (Benigni et al., 2008; Golbamaki and Benfenati, 2016; Bossa et al., 2018; Bower et al., 2020). Additionally, various platforms are available to search these databases and/or run the (Q)SAR models [e.g., (Myatt et al., 2017; Roncaglioni et al., 2022; LCDB, 2023)]. Notably, *in silico* models are also being discussed to predict the human carcinogenic potential based on relevant PubChem bioassays (Chung et al., 2023). The Cancer Potency Database (CPDB) is a key repository of chronic, long-term animal cancer bioassays (Gold et al., 1984; Gold et al., 2005) that classifies chemicals based on multiple-organ

toxicity data. It provides access (NIH, 2023; Instem, 2023; LCDB, 2023) to several other data sources including histopathological findings on neoplastic and non-neoplastic lesions, such as those described in the NTP reports of short-term toxicity and long-term carcinogenicity (CEBS, 2020). CPDB has been serving as the basis for the development of several *in silico* models, including organ-specific (Q)SARs (Lagunin et al., 2018). To facilitate the construction of such organ-specific carcinogenicity models, the CPDB has been used by FDA to develop a liver cancer specific database (Young et al., 2004).

A repository of data from 2-year rodent bioassays is also maintained by FDA's Center for Drug Evaluation and Research (CDER) (Matthews and Contrera, 1998; Bourcier et al., 2015) and it has been used to develop *in silico* models (e.g., Matthews et al., 2008; Kruhlak et al., 2015; Guo et al., 2017).

The EPA Toxicity Reference Database (ToxRefDB) is an example of repository where chemicals are classified as positive or negative for preneoplastic or neoplastic lesions in rat and mouse for multiple tissues (Watford et al., 2019).

The Registry of Toxic Effects of Chemical Substances (RTECS) [initially maintained by US National Institute for Occupational Safety and Health (NIOSH)] is a database which collects tumorigenic dose data from positive or equivocal tumorigenic reports and affected organ, tissue or functional systems; RTECS classifies the test-compounds as carcinogenic, neoplastic (evidence for tumors lacking invasiveness but that could not definitely be classified as either benign or malignant), or equivocal (NIOSH, 1997).

The application of read-across supported by the use of *in silico* techniques, can be useful within the WoE assessment framework. This approach aids the examination of similarities and differences between a data-poor substance (the target chemical) and a chemically similar data-rich substance. Overall, the use of computational models such as artificial intelligence (AI), expert systems, statistical machine learning methods like QSARs and emerging methodologies could be considered in the context of fit-for-purpose evaluations to be added to the integrated WoE assessments. AI may become an increasingly valuable asset in the future (Hartung, 2023). Today, *in silico* or computational methods can provide screening, targeted read-across, review of similar analogues and identification of areas of concern or toxicophores in a target compound. However, to avoid generation of unnecessary data and potential false (positive or negative) results, such models should be used in a judicious and targeted manner and not in isolation. Several considerations must be evaluated when selecting models including, for example, training set breadth, endpoints, model performance, validation and applicability domain. Expert judgement can guide such selection and interpretation. However, as the technology exists today, the use and application of computational methods should be carefully considered and the results evaluated and integrated alongside the other considerations outlined herein.

5.3 First-in-class

First-in-class drugs, those as defined by the FDA that "have mechanisms of action different from those of existing therapies" (FDA, 2023a), may require particular attention and review under the ICH S1B(R1) framework. For novel drug targets, the integrative WoE assessment is still considered eligible, though higher evidentiary standard to compensate for the lack of precedent experience with the drug target would be required to demonstrate no cause for concern.

In such cases, the target biology analysis may still be used to demonstrate with strong evidence that target biology is not associated with cancer development showing that the pharmacology and pathways are sufficiently well-characterized and no plausible links to cancer development related to the primary pharmacology biological pathways are identified (the best example would be a non-mammalian target). A lack of proliferative changes or tumor signal in any organs/tissues should be demonstrated at a high multiple of exposure in the 6-month rat study (or pharmacologically relevant species, such as the 9-month non-rodent). In such situations where this may be questionable, it may be prudent to generate additional supporting evidence (e.g., special histochemical stains, molecular biomarkers, serum hormone levels, data from emerging technologies, or immune cell function integrated into the 6-month rat study) and/or compare the No Observed Adverse Effect Levels (NOAELs) from the 1-, 3- and 6-month rat studies taking into account that exposure margins may change with an increase in the duration of exposure. Collaborative initiatives (e.g. (Corton et al., 2022)) have been launched to investigate the value of emerging technologies that may provide such a higher evidentiary standard. Sponsors can apply customized and creative investigative approaches that could address the uncertainty or inform human relevance of the identified risk. Clinical data generated to inform human mechanistic relevance at therapeutic doses and exposures may provide potential evidence. In addition, data from longer-term toxicity studies in non-rodents and mice may also be instrumental in providing additional information on the human relevance of rat study findings (i.e., demonstrating that the rat study findings are species specific).

When the results from the rasH2-Tg mouse study are available, they should be included in the WoE document and a negative result can contribute with other available evidence to further derisk first-in-class drugs when pharmacologic target engagement can be demonstrated in the rasH2-Tg model.

6 Suggested WoE report structure

The WoE integrated carcinogenicity risk assessment addresses the six WoE factors (as noted in the above sections) and could include considerations of metabolites, evidence from additional special studies and clinical data coupled with the integrated assessment according to the following suggested table of contents:

- Executive summary that summarizes the integrated assessment
- Target biology
- Metabolite profile and ADME
- Secondary pharmacology
- Genetic toxicity
- Histopathological findings in chronic toxicity studies
- Hormonal perturbation
- Immune modulation

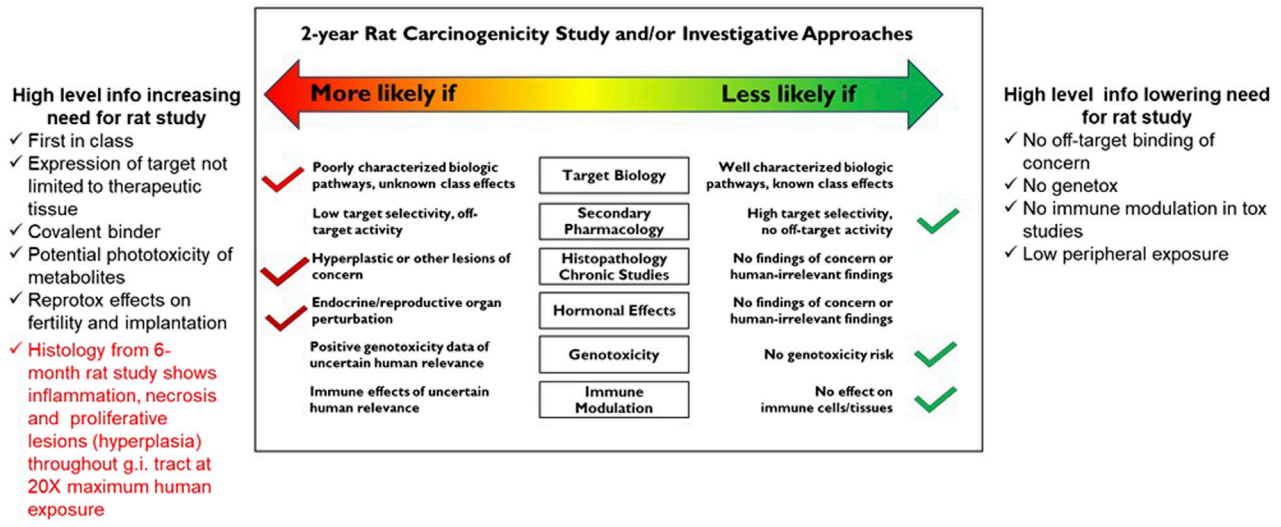


FIGURE 7
The format that can be used to summarize relevant evidence and corresponding conclusions. The core image is originally taken from the ICH S1B(R1) guideline, and it can be updated with relevant evidence as soon as it becomes available. Notably, the 2-year rat bioassay is less likely to be of value also in the case of evidence of unequivocal genotoxicity or broad immunosuppression indicating a carcinogenic risk to humans (ICH S1B(R1), 2022). This figure is a "living" sliding scale to be updated at each stage gate. In this example, the 6-month rat study histology confirms lesions consistent with carcinogenic risk and may be used as the critical information to spur a decision on the need for a 2-year rat carcinogenicity study. The results from the 1-month or 3-month studies can also be useful to get an early indication of a problem, but if negative they will not be definitive.

- Additional special studies
- Clinical data
- Guidance/Advice from other regulatory authorities (if any)
- Data integration and human relevance including overall conclusions
- Appendices

The different sections summarize the findings and relevant conclusions for the integrated assessment whereas additional details of the assessments can be included in the Appendices. Summary tables may be included for each WoE factor reporting information such as the types of studies (e.g., human, animal, and *in vitro*), strengths/limitations of evidence from each study (if applicable), confidence in the outcomes for each study and any other data considered (e.g., ADME and clinical data). The evidence assessment of each study should address the relevance of the *in vitro* or *in vivo* findings to a biologically plausible mechanism in humans.

A final table in the "Data integration and human relevance including overall conclusions" can then condense the conclusions and confidence from the WoE factor tables. Overall strength of evidence from each WoE factor and human relevance conclusions provides the overall rationale in support of the integrated assessment conclusion of whether or not a 2-year rat bioassay will add value to the human cancer risk assessment. This summary table can work in concert with the visualization provided in Figure 7 where each factor can be commented in relation to the overall balance of data towards the WoE assessment.

Figure 7 can also serve as a "living" sliding scale to be updated during the project timeline. Applying the data to the summary table and to this figure and adding new data from subsequent studies as they become available, can help identify gaps in information that might need special assessment in upcoming studies (e.g., clinical data or other assessments of human relevance or histology endpoints in a repeat dose toxicity study) and track whether knowledge gaps have been filled. Figure 7 exemplifies the cumulative data gathering approach to the WoE integrated assessment. The use of this type of approach can aid in making an early decision as to which of the three WoE outcomes is expected (carcinogenic potential in humans is likely, unlikely or uncertain) and to evaluate whether a 2-year rat study would add value to the human carcinogenicity risk assessment. This will allow for a timely decision to begin the activities on running a 2-year rat bioassay to be made with minimal impact to the project timeline.

7 Discussion

The current work presents a procedural framework that helps develop and apply the WoE integrated approach to support a derivation of a scientifically-sound opinion on whether the 2-year rat study provides relevant additional information on carcinogenic risk to humans. Experts from multiple organizations have collaborated to propose a transparent and pragmatic consensus procedure supporting the ICH S1B(R1) WoE carcinogenicity assessment. First, this paper presents each of six WoE factors and describes how these factors contribute to add evidence for the overall WoE assessment. These factors are discussed with varying degrees of thoroughness, reflecting the current development and best practices associated with the evaluation of each factor. Second, the proposed procedure recommends an organized timely approach to data collection that highlights the importance of transparency in presenting the data and how the data itself is collected, and it advocates the evaluation of data

reliability and the estimation of confidence in the assessments leading to the final outcome. The six Weight of Evidence (WoE) factors, as outlined within the ICH S1B(R1) guidelines, can be conceptualized as interconnected components within a comprehensive assessment framework, collaboratively employed to scrutinize and elucidate observed signals (or the lack thereof). Cross-integration of evidence from the different factors leads to a network of evidence for critical discussion and presentation of a structured WoE document. The systematic approach presented here also includes a framework for preparing the carcinogenicity risk assessment document both for presentation to the regulatory authorities or for internal use.

The progressive nature of the integrative WoE carcinogenicity assessment adopted by sponsors over the course of their own development programs, encourages addition of new evidence as it becomes available. In general, this progressive approach, is a critical process to reach an early conclusion on the added value and need of the 2-year rat carcinogenicity study thereby enabling timely product stewardship.

The application of the procedural framework proposed herein is expected to consistently support application of the scientifically-based integrated approach and to increasingly promote the successful implementation of the WoE approach to carcinogenicity assessment and further the elimination of unnecessary animal studies by reduction of the need to conduct the 2-year rat carcinogenicity study. Even if a 2-year rat study is ultimately required, creation of a WoE assessment is valuable in understanding the specific factors and levels of human carcinogenic risk better than have been identified previously.

Author contributions

AB: Writing–review and editing, Writing–original draft, Methodology, Investigation, Conceptualization. RS: Writing–review and editing, Writing–original draft, Methodology, Investigation, Conceptualization. DK: Writing–review and editing, Writing–original draft, Methodology, Investigation, Conceptualization. LB: Writing–review and editing, Investigation. PB: Writing–review and editing, Investigation. FB: Writing–review and editing, Investigation. WB: Writing–review and editing, Investigation. LB-N: Writing–review and editing, Investigation. JC: Writing–review and editing, Investigation. KC: Writing–review and editing, Investigation, Supervision. MD: Writing–review and editing, Investigation. RE: Writing–review and editing, Investigation. DF: Writing–review and editing, Investigation. FH: Writing–review and editing, Investigation. JH: Writing–review and editing, Investigation. GJ: Writing–review and editing, Investigation. FK: Writing–review and editing, Investigation. EMcD: Writing–review and editing, Investigation. FS: Writing–review and editing, Investigation. J-PV: Writing–review and editing, Investigation. DW: Writing–review and editing, Investigation. DZ: Writing–review and editing, Investigation. GM: Writing–review and editing, Investigation, Conceptualization, Supervision.

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Conflict of interest

AB is employed by Innovatune; RS is employed by Takeda; LB is employed by Toxicology Solutions; PB, JC, FH, and KC are employed by Instem; GM was employed by Instem; FB is employed by Merck Healthcare; WB is employed by Brock Scientific Consulting; LB-N is employed by Magnolia Toxicology Consulting; DF is employed by BioXcel Therapeutics; MD is employed by MBX Biosciences; DZ and JH are employed by Gilead; FK was employed by ADAMA; EM is employed by Neurocrine Biosciences; FS is employed by Sanofi; J-PV is employed by UCB Biopharma; DW is employed by ForthTox.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2024.1370045/full#supplementary-material

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ICH S1 prospective evaluation study: weight of evidence approach to predict outcome and value of 2-year rat carcinogenicity studies. A report from the regulatory authorities subgroup

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Introduction: The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) initiated a process in 2012 to revise the S1B Guideline "Testing for Carcinogenicity of Pharmaceuticals". Previous retrospective analysis indicated the importance of histopathological risk factors in chronic toxicity studies, evidence of endocrine perturbation, and positive genetic toxicology results as potentially predictive indicators of carcinogenic risk. In addition, a relationship between pharmacodynamic activity and carcinogenicity outcome in long-term rodent studies has been reported. It was postulated that these factors could be evaluated in a Weight-of-Evidence (WoE) approach to predict the outcome of a 2-year rat study.

Methods: The ICH S1B(R1) Expert Working Group (EWG) conducted a Prospective Evaluation Study (PES) to determine the regulatory feasibility of this WoE approach. Drug Regulatory Authorities (DRAs) evaluated 49 Carcinogenicity Assessment Documents (CADs), which describe the WoE for submitted pharmaceutical compounds. Each compound was categorized into a carcinogenic risk category including a statement of the value of the 2-year rat study. The outcome of the completed 2-year rat studies was evaluated in relation to the prospective CAD to determine the accuracy of predictions.

Results: Based on the results of the PES, the EWG concluded that the evaluation process for assessing human carcinogenic risk of pharmaceuticals described in ICH S1B could be expanded to include a WoE approach. Approximately 27% of 2-year rat studies could be avoided in cases where DRAs and sponsors unanimously agreed that such a study would not add value.

Discussion: Key factors supporting a WoE assessment were identified: data that inform carcinogenic potential based on drug target biology and the primary pharmacologic mechanism of the parent compound and major human

metabolites; results from secondary pharmacology screens for this compound and major human metabolites that inform carcinogenic risk; histopathology data from repeated-dose toxicity studies; evidence for hormonal perturbation; genotoxicity data; and evidence of immune modulation. The outcome of the PES indicates that a WoE approach can be used in place of conducting a 2-year rat study for some pharmaceuticals. These data were used by the ICH S1B(R1) EWG to write the R1 Addendum to the S1B Guideline published in August 2022.

KEYWORDS

carcinogenicity, bioassay, weight of evidence, ICH S1B(R1), risk assessment, pharmaceuticals, 3R principles

Introduction

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) is a key international organization involving regulators and industry that develops internationally harmonized scientific and technical guidelines to support global licensing of human medicines. The ICH S1B guideline "Testing for Carcinogenicity of Pharmaceuticals" provides recommendations on approaches for evaluating the carcinogenic potential of pharmaceuticals which can include the conduct of a 2-year rat carcinogenicity study. An Addendum to the ICH S1B guideline was recently introduced to include a Weight of Evidence (WoE) approach (ICH S1B(R1), 2022) which involves an assessment of WoE factors to inform whether a 2-year rat carcinogenicity study adds value to the assessment of human carcinogenic risk. The recommendations outlined in the Addendum are in part based on the outcome of a Prospective Evaluation Study (PES) conducted under ICH S1(R1) Proposed Change to Rodent Carcinogenicity Testing of Pharmaceuticals–Regulatory Notice Document (ICH, 2013) between 2013–2020.

The primary impetus for updating the guidance was the retrospective analysis of a dataset of 182 blinded compounds from 13 PhRMA companies and a further dataset of 76 IARC Class 1 and 2A compounds (Sistare et al., 2011) which indicated that the absence of (1) histopathologic risk factors for rat neoplasia in chronic toxicology studies, (2) evidence of hormonal perturbation or intended endocrine pharmacology, and (3) positive genetic toxicology results predicted a negative tumor outcome in 82% of 2-year rat carcinogenicity studies evaluated. The rat tumor findings in the remaining 18% of compounds were judged to be of questionable human relevance. It was proposed that compounds meeting these criteria would have a low likelihood of being rat carcinogens and therefore an adequate assessment of human carcinogenic risk could be based on these criteria and completed without results from a 2-year rat study.

Furthermore, a retrospective analysis of a dataset of 255 unblinded compounds from industry and regulatory agencies showed a relationship between pharmacodynamic activity and histopathology findings in rats after 6 months of treatment and subsequently with carcinogenicity outcome in the 2-year rat study (Van der Laan et al., 2016a). Both a positive and a negative relationship was observed and indicated that a more complete knowledge of drug target pharmacology may contribute to the improved prediction of carcinogenicity outcome in the 2-year rat study (Van der Laan et al., 2016a). In another dataset of 289 human pharmaceuticals, the ability to predict rat non-carcinogens based on pharmacology and histopathology had a success rate of 92% whereas the ability to predict rat carcinogens was 98% (Van der Laan et al., 2016b).

These retrospective analyses supported the hypothesis put forward by the ICH S1B(R1) expert working group (EWG) in a Regulatory Notice Document (ICH, 2013). That is, knowledge of pharmacologic target(s) and signaling pathway(s), together with toxicological data, is sufficient to characterize the carcinogenic potential of a pharmaceutical and therefore sufficient to determine whether the conduct of a 2-year rat study would add value to the assessment of human carcinogenic risk. Prospective studies had not been conducted to discern the predictivity of a WoE approach that includes information on drug target pharmacology together with compound-specific toxicology to assess the outcome of a 2-year rat study and its relation to assessing human carcinogenic risk. Moreover, there was no information that addressed if Drug Regulatory Authorities (DRAs) and industry could align on reasonably consistent safety and regulatory decisions based on the conclusion of a WoE assessment, and in regard to the need for a 2-year rat bioassay in assessing human carcinogenic risk.

A PES was therefore conducted to determine the regulatory feasibility of this WoE approach and conclusions from these retrospective analyses in a real-world setting, where prior knowledge of the 2-year rat carcinogenicity study outcome is not available. The specific objectives of the PES were as follows:

- To determine if the WoE approach is sufficiently robust to predict the outcome and value of a 2-year rat carcinogenicity study,
- To define the specific factors that contribute to a WoE assessment leading to a conclusion that a 2-year rat study does, or does not, contribute to the assessment of human carcinogenic risk,
- To assess concordance of predictions and statements of value among DRAs and between DRAs and pharmaceutical sponsors.

Methods

The PES called for sponsors to conduct a prospective assessment addressing human carcinogenic risk of a pharmaceutical under active development and the anticipated

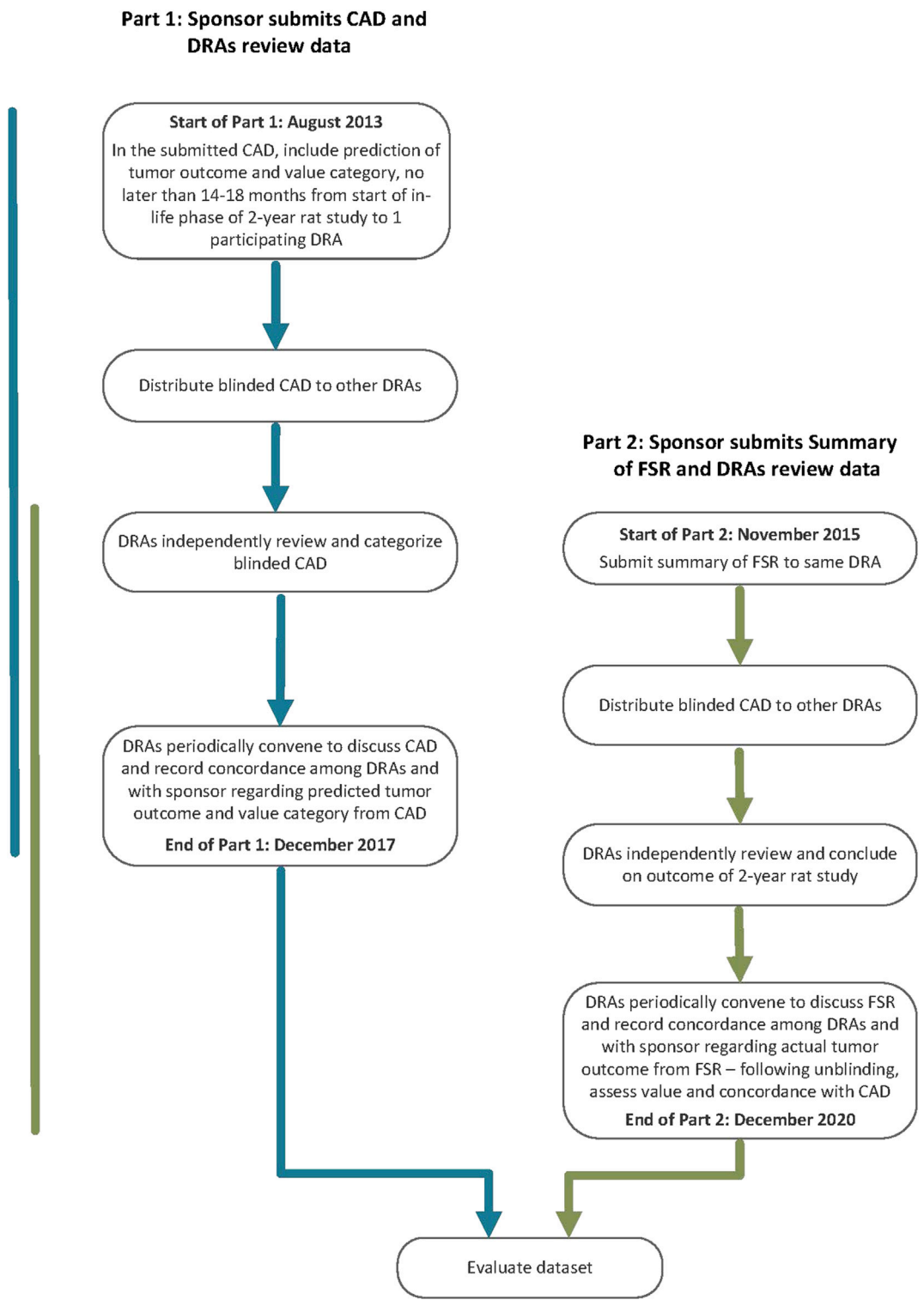

FIGURE 1
Flow chart outlining the design of the Prospective Evaluation Study.

outcome and value of a 2-year rat study to that assessment using specific WoE criteria (ICH, 2013). The assessment, referred to as a Carcinogenicity Assessment Document or CAD, was submitted to one of the five participating DRAs (Figure 1: Part 1). The outcome of the prospective assessment was then compared with the outcome of the 2-year rat study (Figure 1: Part 2). Therefore, following completion of the 2-year rat study, a summary of the final study report (FSR) was submitted to the same DRA receiving the CAD

TABLE 1 Carcinogenicity risk categories.

Category 1	Highly likely to be tumorigenic in humans such that a 2-year rat, 2-year mouse, or transgenic mouse carcinogenicity studies would not add value.
Category 2	Tumorigenic potential for humans is uncertain and rodent carcinogenicity studies are likely to add value to human risk assessment.
Category 3a	Highly likely to be tumorigenic in rats but not in humans through prior established and well recognized mechanisms known to be human irrelevant, such that a 2-year rat study would not add value.
Category 3b	Highly likely not to be tumorigenic in both rats and humans, such that a 2-year rat study would not add value.

TABLE 2 Drug Regulatory Authority (DRA) participation in Prospective Evaluation Study.

European medicines agency (EMA)	Contributed to categorization of all 49 submitted CADs
Pharmaceuticals and medical devices agency (PMDA)	
U.S. Food and drug administration (FDA)	
Health Canada (HC)	Contributed to categorization of 41 submitted CADs after entry into PES
Swiss Agency for Therapeutic Products (SMC)	Contributed to categorization of 23 submitted CADs after entry into PES

All DRAs participated in evaluative comparison of CADs to associated 2-year rat carcinogenicity study outcomes.

submission. After completion of Part 1 and Part 2 of the PES, WoE criteria addressed in the CADs were re-evaluated for the dataset regarding the value for predicting tumor outcome and assessing overall human carcinogenic risk.

Carcinogenicity Assessment Documents

Participating sponsors submitting a CAD were requested to address specific WoE factors considered pertinent to the assessment of carcinogenic potential (ICH, 2013). Based on the level of certainty regarding carcinogenicity risk and its potential human relevance, sponsors were requested to include a prediction of tumor outcome from the planned or ongoing 2-year rat study and assign the pharmaceutical to one of 4 carcinogenicity risk categories described in Table 1. The sponsor was also requested to state the projected value of the rat carcinogenicity study outcome.

Each CAD had to be completed prior to or within 14–18 months of an ongoing 2-year rat study and could not be informed by any interim 2-year study data. Sponsors submitted their CADs to one of the participating DRAs (Table 2) using a dedicated email address. The submitted CADs were shared with the other DRAs. Each participating DRA independently reviewed the submitted CADs, and the rationale for concurrence or non-concurrence with the sponsor's assessment and carcinogenicity risk category was documented. DRA review staff were blinded to the sponsor, compound identification, and the regulatory status of the pharmaceutical. In some cases, DRAs sought limited clarification regarding completeness of information from the sponsor via an unblinded assistant.

Category 3a and 3b cases were considered to have the greatest potential impact on the overall outcome of the PES in terms of defining the criteria to support a WoE assessment *in lieu* of conducting a 2-year rat study as these cases would result in a conclusion that a 2-year rat study would not add value to the assessment of human carcinogenic risk. Therefore, receipt of at least 20 CADs that were categorized as either 3a or 3b from a DRA perspective (i.e., at least one DRA agreed with the sponsor's category 3a/3b designation) was considered necessary to gain sufficient experience to support a potential revision to the ICH S1B guideline.

Initially, the DRA group included the three founding regulatory members of ICH (EMA, FDA, PMDA, September 2014) as confidentiality agreements were available between these Agencies. Periodically, DRAs met by teleconference to discuss each CAD and to assess concordance in categorizations reached by each region's independent review of the CADs. Industry members of the EWG were not included in these discussions because of the proprietary nature of the data. However, at various timepoints the full EWG, which included Industry members, as well as DRAs that did not have mutual confidentiality agreements, convened to discuss the results (following anonymization of the data by DRAs), determine study success criteria, and to develop the framework for the ICH S1B(R1) Addendum. At the start of the PES, it was agreed to have a single agreed-upon category for each CAD (i.e., case #s 101 to 107, and 140), even in cases where unanimity for a category was not reached across the DRAs. Health Canada (HC) joined in 2015 after a confidentiality agreement was established, increasing the number of DRAs to 4. From then on, a single final category could not be based on a majority decision, since there could not always be a majority decision with 4 parties involved. Rather, the DRAs communicated any differing viewpoints with a supporting rationale to the ICH S1B(R1) EWG. This approach remained in place when Swissmedic (SMC) joined as the 5th DRA in 2016. The DRAs have reported periodically the progress of the PES in a series of Status Reports (ICH, 2016; ICH, 2017; ICH, 2019; ICH, 2021).

Determination of rat carcinogenicity study outcome

A summary of the FSR of the completed 2-year rat carcinogenicity studies was submitted to DRAs that contained an executive summary with sufficient information to enable independent assessment of tumor outcome (e.g., tumor incidence

tables and statistical analysis). When available, complete FSRs were also submitted. Outcomes of the 2-year rat studies were reviewed by DRAs without prior knowledge of the associated CAD. Each DRA evaluated the carcinogenicity study in a manner consistent with the practice in each regulatory region and concluded whether the carcinogenicity study outcome was either:

- Positive: substantive evidence of treatment-related tumors,
- Negative: no evidence of treatment-related tumors, or
- Equivocal: numerical imbalance in tumor incidence relative to concurrent control without clear relationship to treatment. For example, relation to dose-response or historical controls was unclear, statistical significance was not achieved, or a different statistical approach to tumor incidence data was applied (e.g., trend analysis vs. pair-wise testing thresholds).

Following each DRA's independent assessment, a teleconference was held to discuss the submitted FSR summary and to seek alignment on study outcome(s). It was agreed to designate a single outcome for each FSR summary, even in cases where unanimity on study outcome was not reached across the DRAs.

Evaluation of CAD and carcinogenicity outcome

Following assessment of tumor outcome for each 2-year rat carcinogenicity study, the associated CAD was unblinded, and the carcinogenicity study outcome was compared with the CAD's predicted human risk category and predicted rat tumor outcome. The data for each CAD/FSR summary pair were discussed to determine if the CAD was consistent with the 2-year rat study outcome and if the 2-year rat study added value to the assessment of human carcinogenic risk. DRAs also discussed specific WoE attributes, particularly those that suggested the conduct of a 2-year rat study would add value, to determine if identified areas of uncertainty could be addressed with additional investigative studies. In March and April 2020, DRAs held a series of teleconferences to discuss each CAD/FSR summary pair in further detail and to begin to map out the WoE framework by identifying WoE attributes that would likely necessitate a 2-year rat study and those that would support a WoE assessment *in lieu* of a 2-year rat study.

Results

Prospective evaluation study (PES) data set

Acceptance of CADs was initiated following publication of the RND in August 2013 (ICH, 2013). A total of 49 CADs were submitted by 25 sponsors by the closing date of December 2017. In one case, interim data of an ongoing rat study was found in the CAD, and the case was subsequently excluded from the dataset. The sponsors of three CADs (two Category 2, one non-unanimous Category 3a) indicated that the associated rat carcinogenicity study report could not be submitted, leaving a total of 45 CADs with FSRs from 22 sponsors for evaluation. The 45 cases that provided complete information (CAD and associated 2-year rat FSR) comprised the final data set for evaluation in this manuscript. The data set includes 24 Category 3a or 3b CADs with associated 2-year rat FSRs, meeting one study objective of receiving at least 20 Category 3a/b cases as designated by at least one of the participating DRAs, which lead to the closing date of 31 December 2020. The investigational compounds represented approximately 18 different pharmacological targets in active development in approximately 11 different therapeutic areas or clinical indications.

CAD categories and concordance

Table 3 summarizes the categories designated by the sponsors and the corresponding category designated by the DRAs of the 45 completed CAD/FSR summary cases. Among the 31 cases designated by the sponsor as Category 3a or 3b, at least one DRA concurred with this designation in 24 (77%) of these cases. No DRA concurred with the sponsor's designation of Category 3a/b in 7 cases, concluding instead that the prospective WoE assessment supported the need for a 2-year rat study to adequately assess human carcinogenic risk (i.e., Category 2).

As not all category designations by DRAs were unanimous, Table 4 indicates the extent of concordance among the participating DRAs in categorizing the CADs. DRAs reached a unanimous conclusion in 1 of 3 Category 1 cases and in 15 of 18 Category 2 cases. Among the 24 cases designated as Category 3a or 3b, the DRAs reached a unanimous decision in 12 cases and a non-unanimous decision, typically between Categories 2 and 3, in an additional 12 cases.

Outcome of 2-year rat carcinogenicity studies

Tumor outcomes of the 45 two-year rat carcinogenicity studies were reported as positive, negative, or equivocal. As interpreted by the DRAs, 24 studies yielded a negative outcome while 13 yielded a positive outcome in tumor incidence. An equivocal outcome was observed in 8 cases. With one exception (case #140), the sponsors of the equivocal cases reported the study outcomes as negative; thus, the sponsors designated 31 studies as negative and 14 studies as positive for tumor outcome[1].

Table 5 represents the number of negative, equivocal, and positive tumor outcomes of the 2-year rat studies grouped by CAD category, as designated by the DRAs. The highest percentage of negative tumor outcomes was associated with Category 3b designations, consistent with this category being defined as compounds unlikely to be carcinogenic in rats or humans based on the CAD WoE evaluation. Category 3a designations were associated with a higher percentage of positive tumor outcomes, relative to Category 3b, consistent with the WoE evaluation supporting the higher likelihood of a positive tumor

1 Case #140 was designated as Category 3b by the sponsor and Category 2 by the DRAs. The sponsor called the 2-year rat study positive based on a statistically significant trend in urinary bladder papilloma, whereas DRAs considered the outcome equivocal due to the lack of a dose-response and the marginal increase above historical controls.

TABLE 3 Concordance between DRA and sponsor category designations for 45 completed CAD/FSR cases.

CAD category	Number of CADs	
	Sponsors	DRAs
1	3	3
2	11	18
3a	14	12
3b	17	12

Category 1, highly likely to be tumorigenic in humans; Category 2, tumorigenic potential for humans is uncertain; Category 3a, highly likely to be tumorigenic in rats but not in humans; Category 3b, highly likely not to be tumorigenic in both rats and humans.

TABLE 4 Concordance among DRAs on category designations for 45 completed CAD/FSR cases.

CAD category	Number of CADs		
	DRAs		
	Total	Unanimous	Non-unanimous
1	3	1	2
2	18	15	3
3a	12	7	5
3b	12	5	7

outcome in rats for these compounds. For Category 2 designations, where the carcinogenic potential was indeterminate based on the CAD WoE, a similar number of 2-year studies yielded a negative or positive tumor outcome.

Outcomes in relation to CAD category designation

The basis for CAD categorizations and the outcome of the associated 2-year rat carcinogenicity studies are summarized in Tables 6-12 for Categories 3b (Table 6), 3a (Tables 7, 8), 2 (Tables 9–11), and 1 (Table 12). These tables also describe the rationale underlying concordance or lack of concordance between the DRAs and sponsors, and among DRAs regarding CAD categorization and 2-year rat tumor outcomes.

TABLE 5 Tumor outcome of 2-year rat studies for cases designated as Categories 1, 2, 3a, and 3b by DRAs.

CAD category	DRA-determined 2-year rat study outcome		
	Positive	Negative	Equivocal
1	2	1[a]	0
2	6	9	3
3a	4	5	3
3b	1[b]	9	2

Category 1: highly likely to be tumorigenic in humans; Category 2: tumorigenic potential for humans is uncertain; Category 3a: highly likely to be tumorigenic in rats but not in humans; Category 3b: highly likely not to be tumorigenic in both rats and humans.
[a]Case 123, discussed in the text below.
[b]Case 122, discussed in the text below.

Category 3b

Category 3b was designated when the prospective WoE assessment supported a conclusion that the predicted carcinogenic risk is low or absent for both rats and humans, such that the outcome of a 2-year rat study would not add value to the assessment. The sponsors designated 17 cases as Category 3b of which the DRAs agreed fully or partially with 12 of those cases (Tables 3, 4). Among these 12 Category 3b cases, 11 were reported by the sponsors as having a negative tumor outcome, and 1 case was reported as positive (Table 6). The DRAs assessed two sponsor-designated negative cases as equivocal. In one equivocal case (#137), there was a dose-dependent numerical imbalance in the incidence of pancreatic islet neoplasms which exceeded the historical range in the high-dose group but was not statistically significant by trend or pairwise testing. In addition, a dose-independent numerical imbalance for uterine endometrial neoplasms showed an incidence higher than in the concurrent control group but remained within the historical rate for the test site. In the second equivocal case (#149), there were numerical imbalances in 3 dermal neoplasms (squamous cell papilloma, carcinoma, and keratoacanthoma) in males that reached statistical significance only when combined, driven primarily by a higher incidence of keratoacanthoma at the high dose. The latter exceeded the historical control for rats from the study site.

The tumor outcome of one Category 3b case was determined to be positive and treatment-related by both the sponsor and the DRAs (case #122) for uterine carcinoma. Retrospective examination of the 6-month toxicology study revealed a marked increase in uterine weight with abnormal contents, with microscopic evidence of dilatation at doses that were associated with uterine neoplasms in the 2-year study. The occurrence of uterine carcinoma at low multiples of clinical exposure in the 2-year rat study was not consistent with the original WoE assessment of low carcinogenic risk (Category 3b). At the time of CAD assessment, these uterine findings in the 6-month study were not recognized as a risk factor for development of uterine neoplasia by the sponsor or DRAs. It is now noted that an increase in reproductive organ weights with or without histological correlates observed in a 6-month study may be interpreted as a predisposing risk factor for neoplasia upon long-term administration. Further investigative studies to understand the underlying mechanism and human relevance would be appropriate in such cases as part of a WoE evaluation in determining whether a 2-year rat study is warranted.

Category 3a

Category 3a was designated when the prospective WoE supported a conclusion that the predicted cancer risk is low in humans, but that a positive tumor outcome is likely in the 2-year rat study by a species-specific and human irrelevant pathway. The sponsors designated 14 cases as Category 3a of which the DRAs agreed either fully or partially with 12 of those cases (Tables 3, 4). Among the 12 cases designated as Category 3a by the DRAs,

TABLE 6 Unanimous and non-unanimous Category 3b: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	Basis for alternative category	2-year rat tumor outcome	Discussion on CAD or outcome
103	3	3b	Therapeutic Indication: Migraine Target: G-protein-coupled receptor (novel drug target) ● Literature reports no effect or potential anti-tumor effects related to drug target inhibition; negative 2-year rat data available for comparable compound ● No off-target activity in secondary pharmacology screen ● No histological findings of concern at clinically relevant exposures in 6- month rat study ● No genotoxicity, hormonal or immunosuppressive effects	N/A	DRA: negative Sponsor: negative	The absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans, such that a 2-year study would not add value
122	3	3b	Therapeutic indication: Cardiomyopathy and arrhythmias Target: Ion channel ● Literature reports potential role of channel activation in promoting tumor invasiveness. Compound 122 inhibits ion channel activity ● No off-target activity in secondary pharmacology screen ● Increased uterine weight, abnormal contents, microscopic dilatation in 6- month rat study ● No genotoxicity or immunosuppressive effects	N/A	DRA: positive Sponsor: positive Uterine carcinoma (not predicted in CAD)	Doses resulting in increased uterine weight in the 6- month toxicity study resulted in uterine carcinoma and polyps in the 2-year rat study at exposures ~2 times the anticipated clinical exposure The outcome of the 2-year rat study indicated that an increase in reproductive organ weights with or without histological correlates observed in a 6- month study may be evidence of functional hormonal perturbation and suggest a potential carcinogenic risk Further investigative studies required to assess causality and human relevance for inclusion in a WoE assessment
128	3	3b	Therapeutic indication: Viral infection Target: Viral protein ● Non-mammalian target with no mammalian equivalent ● No off-target activity in secondary pharmacology screen ● No histological findings of concern in 6-month rat study ● No genotoxicity, hormonal or immunosuppressive effects	N/A	DRA: negative Sponsor: negative	The absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans, such that a 2-year study would not add value
129	3	3b	Therapeutic Indication: Viral infection Target: Viral protein ● Non-mammalian target with no mammalian equivalent ● No off-target activity in secondary pharmacology screen ● No histological findings of concern in 6-month rat study ● No genotoxicity, hormonal or immunosuppressive effects	N/A	DRA: negative Sponsor: negative	The absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans, such that a 2-year study would not add value
130	3	3b	Therapeutic indication: Severe asthma Target: G-protein-coupled receptor inhibitor (novel drug target) ● Knock-out mice lacking the drug target do not exhibit findings indicative of a potential carcinogenicity risk after 1 year of observation ● No interactions with receptors/transporters screen (<10 μM) ● No histological findings of concern at a 54-fold human plasma exposure margin in 6-month rat study ● No genotoxicity, hormonal or immunosuppressive effects	N/A	DRA: negative Sponsor: negative	The absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans, such that a 2-year study would not add value

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TABLE 6 (*Continued*) Unanimous and non-unanimous Category 3b: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	Basis for alternative category	2-year rat tumor outcome	Discussion on CAD or outcome
118	3	3b, 2	Therapeutic indication: Viral infection Target: Viral protein (novel drug target) Category 3b • Non-mammalian drug target with no mammalian equivalent • No off-target activity in secondary pharmacology screen • No histological findings of concern at clinically relevant exposures in 6- month rat study • No genotoxicity, hormonal, or immunosuppressive effects	Category 2 • Novel drug target • Incomplete information on metabolite characterization • Inadequate assessment of off-target activity • Demonstrating a negative 2- year study outcome considered of value to risk assessment	DRA: negative Sponsor: negative	Adequacy of compound characterization for a novel target varied across DRAs For DRAs selecting Category 3b, the absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans, such that a 2-year study would not add value For DRAs selecting Category 2, the absence of drug-related tumorigenicity in the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk
137	3	3b, 2	Therapeutic indication: Alzheimer's disease Target: Protease (novel drug target) Category 3b • No evidence of carcinogenic concern in knock out mice lacking the drug target • No off-target activity in secondary pharmacology screen • No histological findings of concern at clinically relevant exposures in 6-month rat study • No genotoxicity, hormonal, or immunosuppressive effects	Category 2 • The compound modulates a novel drug target exhibiting complex biology that has not been well characterized which precludes confident prediction of tumorigenic outcome in rats and humans • Data from knock out mice insufficient to conclude no carcinogenic concern related to drug target biology	DRA: equivocal Pancreatic islet adenoma, uterine adenoma/carcinoma Sponsor: negative	For DRAs selecting Category 2, the negative/equivocal outcome in the 2-year rat study characterized tumor outcome following pharmacological inhibition of the novel drug target and provided value to the assessment of human carcinogenic risk
144	3	3b, 2	Therapeutic indication: Hypertension Target: Steroidal receptor Category 3b • Target biology and selectivity profiles do not raise a concern • No off-target activity in secondary pharmacology screen • Histological findings of adrenal hypertrophy and/or renal juxtaglomerular cells in 6-month rat study; however, similar histological findings for compounds that target the same receptor do not show adrenal tumors in 2-year rat studies and renal tumors in 2-year rat studies were not considered human relevant • No genotoxicity, hormonal or immunosuppressive effects	Category 2 • Structural dissimilarity between compound 144 and other compounds in the class limits extrapolation of 2-year rat findings to compound 144 • Findings of gastrointestinal erosion/inflammation in 6-month rat study not adequately addressed	DRA: negative Sponsor: negative	Adequacy of addressing complexity of target biology sufficient for confident prediction of outcome varied across DRAs For DRAs selecting Category 3b, the absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans, such that a 2-year study would not add value For DRAs selecting Category 2, the negative outcome in the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk
146	3	3b, 2	Therapeutic indication: Inflammatory disease Target: Phosphodiesterase Category 3b • Target biology and selectivity profiles do not raise a concern • No off-target activity in secondary pharmacology screen • Human metabolites adequately assessed • No histological findings of concern in 6-month dermal rat study, the intended clinical route of administration • Uterine tumors observed with similar compound in the class from oral dosing in 2-year rat study not applicable to intended dermal application (2-year dermal mouse study was negative) • No genotoxicity, hormonal, or immunosuppressive effects	Category 2 • Insufficient information provided on carcinogenic potential of drug target, potential hormonal effects, and potential immunotoxic effects • Uterine granular cell tumors observed with similar compound in the class following oral dosing suggest tumorigenic potential • Potential oral exposure from dermal application in pediatric population supports conduct of rat oral study	DRA: negative Sponsor: negative	Adequacy of information provided for target biology, hormonal, and immunotoxic endpoints varied across DRAs Relevance of tumors observed following oral dosing to the route of clinical administration, and potential systemic clinical exposure, also varied across DRAs For DRAs selecting Category 2, the negative outcome in the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk

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TABLE 6 (*Continued*) Unanimous and non-unanimous Category 3b: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	Basis for alternative category	2-year rat tumor outcome	Discussion on CAD or outcome
136	3	3b, 2, 1	Therapeutic indication: Inflammatory disease Target: Tyrosine kinase (novel drug target) Category 3b ● Target biology involved in immunity, but no direct role in tumorigenesis ● No pharmacologically relevant off- target activity in secondary pharmacology screen for either compound 136 or major metabolites ● No histological findings of concern in 6-month rat study or in other species tested (mice, monkeys) ● No genotoxic effects for parent compound or major metabolites ● No hormonal effects	Category 2 ● Different target selectivity limits extrapolation from related congeners in class, which exhibit an inconsistent rodent tumor profile. Compound-specific assessment considered of potential value Category 1 ● Immunosuppressive profile (decreased peripheral blood lymphocyte counts, decreased lymphoid cellularity, suppression of T-cell-dependent antibody response in 6-month rat study; suppression of T-cell-dependent antibody response in 9-month non- rodent study) ● Potential cross-reactivity with related kinase presents a human carcinogenicity risk that would not be further informed by a 2-year rat study	DRA: negative Sponsor: negative	Relevance of data for related congeners with differing target selectivity, some of which present a human carcinogenic risk, varied across DRAs
124	3	3b, 3a, 2	Therapeutic indication: Adjuvant cancer treatment Target: Tyrosine kinase Category 3b ● Target biology related to growth inhibition and not considered a concern ● No tumor findings from 2-year rat studies with congeners in class ● No off-target activity in secondary pharmacology screen ● Bile duct hyperplasia, a finding of concern observed in a 14-day study, was not confirmed in the 6-month study ● No genotoxicity, hormonal, or immunosuppressive effects	Category 3a ● Potential for hemangiosarcoma based on a numerical but non- significant increase observed in TgRasH2 mice by a human-irrelevant pathway Category 2 ● Inadequate characterization of toxicities in 6-month rat study (e.g., chronic GI inflammation, villous atrophy in the ileum, mammary gland atrophy, bile duct/liver injury) ● Incomplete characterization of metabolites ● Different target selectivity profile limited extrapolation of carcinogenicity data from congeners in class to compound 124 ● Liver, vascular tumors in 2- year rat study observed with one congener in the class was not addressed by sponsor	DRA: negative Sponsor: negative	Adequacy of information provided for relevant toxicities observed in the 6-month rat study, extent of metabolite characterization, and data for related congeners varied across DRAs For DRAs selecting Category 3b or 3a, the absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in rats and humans (3b), or humans (3a), such that a 2-year study would not add value For DRAs selecting Category 2, the absence of drug-related tumorigenicity in the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk
149	3	3b, 3a, 2	Therapeutic indication: General absorption enhancer Target: Fatty acids Category 3b ● Compound related to dietary ingredient and not considered a tumorigenic risk ● Gastric mucosal hypertrophy/hyperplasia in 6-month rat study interpreted as rat-specific, not being observed in dog toxicity studies ● Pancreatic adenoma in 6-month rat study observed in one low dose male but not at higher doses or in a repeat 6-month study ● No genotoxicity (only Ames test performed) or hormonal effects	Category 3a ● Possible tumorigenicity in forestomach and pancreas through a rat-specific and human irrelevant pathway Category 2 ● Insufficient information regarding relevance and extent of dietary intake compared to exposure to compound ● Gastric mucosal findings in the main 6-month rat study and occurrence of pancreatic adenoma in a supportive 6-month rat study require further characterization ● Pancreatic adenoma reported in rats with related congener ● Insufficient testing for genotoxicity ● Inadequate plasma exposure margin assessment (rat to human)	DRA: equivocal Skin squamous adenoma/ carcinoma (not predicted in CAD) Sponsor: negative	Adequacy of information regarding dietary intake and relation to compound exposure was a key point of disagreement across DRAs Relevance of gastric mucosal findings and characterization of pancreatic toxicity also varied across DRAs

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4 yielded a positive tumor outcome in the 2-year rat study as assessed by the DRAs and sponsors. Another 5 cases yielded a negative tumor outcome as assessed by the sponsors and DRAs, and 3 cases yielded an equivocal tumor outcome as assessed by the DRAs. The sponsors of the 3 DRA-designated equivocal cases interpreted the 2-year rat studies as being negative (case #s 109, 135 of Table 7; case 125 of Table 8). In some cases, tumor types that were observed in the 2-year rat study were not anticipated based on the WoE assessment (case #s 109, 125, 135, 139, 145), and not all tumor types anticipated from the WoE assessment were observed in the 2-year rat study (case #s 106, 109, 116, 117, 125, 131, 133, 135, 139, 142, 145 of Tables 7 and 8). However, none of the tumor types observed in the positive studies were interpreted as presenting a human carcinogenicity risk due to either human irrelevance based on anticipated tumorigenic mechanism and/or the high exposure multiple at which tumors emerged.

Category 2

Category 2 was designated when the prospective WoE assessment indicated that human carcinogenic risk is uncertain, and results from a 2-year rat study would add value to the assessment. Sponsors submitted 11 CADs with a Category 2 designation and the DRAs unanimously agreed with the sponsor's designation in 8 of those cases. Table 9 lists key observations recognized by both sponsors and the DRAs as presenting substantial uncertainty regarding human carcinogenic risk, and describes the anticipated value of the 2-year rat study to the overall risk assessment. In each case, uncertainty was identified from more than one WoE factor and often derived from several observations. In general, substantial uncertainty was identified from the compound's pharmacological mechanism or compound-specific toxicology findings and the absence of information from rat carcinogenicity studies with other compounds of the drug class. In one case (#108), a diverse rodent tumor profile associated with the drug class contributed to the concerns identified from compound-specific findings of potential genotoxicity and a low incidence of vascular tumors in the chronic rat toxicology study. In another case (#114), a 3-month rat study was submitted as the longest repeat-dose toxicity study in the WoE assessment, and no data were submitted following 6 months of repeat-dosing in rats. Given these uncertainties, a positive or negative tumor outcome in the 2-year rat study would be interpreted as adding value to the overall assessment of human carcinogenic risk.

For the 8 cases unanimously designated as Category 2 by the DRAs and sponsors, a positive tumor outcome, as interpreted by both the DRAs and the sponsor, was observed in 3 of the 8 cases. These tumor outcomes consisted of duodenal adenocarcinoma (case #101), hepatocellular and hepatocholangiocellular adenoma (case #138), and pituitary adenoma (case #132) (Table 9). The sponsor reported a negative tumor outcome for the remaining 5 cases and the DRAs agreed with this interpretation in 4 cases, citing an equivocal outcome for 1 case (#120) based on a numerical imbalance of pancreatic islet adenoma and carcinoma.

For 7 cases submitted by sponsors proposing a Category 3a or 3b designation, the DRAs placed these cases unanimously in Category 2 because of identified concerns not sufficiently addressed in the CAD. A 2-year rat study would be warranted to establish an adequate assessment of carcinogenic risk in these cases (Table 10). In many of these cases, DRAs cited insufficient information regarding the relevance of histological findings identified in the 6-month rat study to potential human carcinogenic risk (e.g., hypertrophy, hyperplasia, injury/regeneration of various tissues). Findings indicative of hormonal perturbation in rats without sufficient explanation was additionally cited in three cases (#s 102, 105, 148). Additional reasons included insufficient knowledge of drug target pathways given the novelty of the target or the multiplicity of drug targets, and insufficient information provided on metabolite profiles, genetic toxicology testing, and uncertain relevance of experience with the associated drug class. For one case (#102), the CAD did not include sufficient information about the compound's immunomodulatory activity or an adequate characterization of a signal in female reproductive tissues for the DRAs to concur with the sponsor's conclusion of low human risk and category 3b designation.

Among these 7 cases, a negative tumor outcome in the 2-year rat study was observed for #s 102, 104, 105, and 148, and a positive or equivocal tumor outcome was observed for #s 140, 107, and 141. For case #140, a potential signal of urinary bladder papilloma was reported in the 2-year study which was not anticipated in the CAD despite the occurrence of bladder hypertrophy in the 6-month toxicology study. Hepatocellular adenoma was observed in the 2-year rat study for case #107 and was consistent with the sponsor's expectation of liver tumors based on increased liver weight/hypertrophy in the 6-month toxicology study. Details of case #141 are undisclosable; however, the positive tumor outcome was only partially consistent with the sponsor's expectation in the CAD.

For three cases where the sponsor submitted a Category 2 designation, the DRAs did not reach unanimous alignment, with one or more DRAs concluding that a 2-year rat study would not add value to the WoE assessment (Table 11). In one case (#115), the DRAs did not align on the relevance of compound-specific findings indicative of hormonal disruption and potential immunosuppression, or whether a 2-year rat study would provide adequate resolution to those concerns. The tumor outcome in this case was negative. In another case (#127), the DRAs differed on whether sufficient knowledge was available for the drug target to allow an adequate assessment of a pharmacology-based carcinogenic risk. The sponsor interpreted the 2-year rat study as being negative, whereas the DRAs interpreted the study as potentially positive for Leydig cell tumors and liver adenoma. In the final case (#121), the DRAs did not align on whether results from a 2-year rat study would adequately address the concern of immunomodulation related to the compound's pharmacological mechanism. The 2-year rat study outcome in this case was positive for Leydig cell adenoma.

Category 1

Category 1 was designated when the prospective WoE assessment supported the conclusion that the predicted carcinogenicity risk is highly likely in humans such that a product would be labeled accordingly and a 2-year rat, mouse, or transgenic mouse carcinogenicity study would not add value. The sponsors submitted 3 CADs with a Category 1 designation (Table 12), and the DRAs unanimously agreed with the sponsor's

designation in only 1 of those cases (#143, data not disclosable). In all cases, carcinogenic potential was predicted from human carcinogenicity data available from the drug class. In two cases (#s 113, 123), some DRAs concluded that the conduct of a 2-year rat study would be appropriate, based on inadequate information provided for several WoE factors and a presumption that additional data would further inform the extent of human carcinogenic risk.

The carcinogenicity study outcome of case #113 was considered positive by both the sponsor and DRAs, which was consistent with the sponsor's prediction of pilomatricoma, and with an additional observation of keratoacanthoma. For another case (#123), the rat carcinogenicity study was negative which, for some DRAs, de-risked observed proliferative findings in the stomach and renal tubules that were not considered related to potential immunosuppressive effects. The third case (#143) yielded a positive tumor outcome as determined by the sponsor and DRAs; however, additional details of this case are not disclosable.

Discussion

If a new pharmaceutical will be used as continuous therapy for 6 months or longer, or if the drug will be used intermittently for a duration of time that represents a minimum of 6 months in total, evaluation of human carcinogenic risk is recommended before licensing a marketing authorization in most cases (ICH, 1995). To this end, ICH S1B recommended that the carcinogenic potential of a pharmaceutical be evaluated in in vivo 2-year carcinogenicity studies with rats and mice. Alternatively, the 2-year mouse study can be substituted with an *in vivo* six-month study with transgenic mice. This testing strategy has been common practice since adoption of ICH S1B in 1997 and, with some exceptions, was applied to investigational pharmaceuticals regardless of drug target, compound-specific toxicology, or prior human or animal carcinogenicity data available for the drug class. Given the evolutions in understanding of potential mechanisms leading to the development of neoplasms (Hanahan and Weinberg, 2011) and the recognized limitations inherent to rodent carcinogenicity studies, during the last decades, several publications have discussed the need for refinement or alternatives to the conduct of one or both *in vivo* carcinogenicity studies (Bourcier et al., 2015; Cohen, 2004; Goodman, 2001; Reddy et al., 2010; Sistare et al., 2011; Van der Laan et al., 2017; Woutersen et al., 2016; Cohen et al., 2019).

Process-related remarks

The RND that initiated the PES included a description of WoE factors that should be addressed in a CAD (ICH, 2013). These recommendations were informed by prior retrospective studies that identified pharmacological and toxicological attributes of pharmaceuticals that correlated with a negative or positive tumor outcome in 2-year rat studies (Sistare et al., 2011; Van der Laan et al., 2016a; Van der Laan et al., 2016b). The PhRMA dataset (Sistare et al., 2011), which formed the primary basis for the prospective evaluation study, consisted of 182 compounds, and an additional 76 compounds were later included from the IARC dataset. The PhRMA dataset (without IARC data) was enlarged by data from FDA and JPMA to approximately 255 compounds (Van der Laan et al., 2016a). Another dataset of 289 compounds was analyzed later that year (Van der Laan et al., 2016b). In the study presented herein, these attributes were applied in a prospective manner to predict the outcome and potential value of 2-year rat studies that had not yet been completed. This was achieved by explicitly directing sponsors to submit CADs only for programs where the 2-year rat studies had not progressed beyond 18 months of dosing, and without including any interim information that might be available from the ongoing 2-year study. To further minimize bias, the acceptable in-life phase was reduced from 18 to 14-month for all CADs effective 1 June 2016. Sponsors were to include the date of initiation of the 2-year rat study and the date of completion of the CAD. Most (60%) CADs were prepared during months 13–18 of the 2-year rat study, while 40% were prepared during the first 12 months of dosing.

The quality of the submitted CADs was variable. In some cases, the CAD addressed all weight of evidence factors outlined in the RND with sufficient detail to enable a well-informed assessment of the potential outcome and value of the 2-year rat carcinogenicity study. In other cases, information was either insufficient or missing from the CAD. Some examples of deficiencies include:

- insufficient description of the pharmacological target, downstream pharmacological effects, and drug target biology,
- incomplete description of receptor targets in secondary pharmacology studies,
- inadequate assessment of histological findings of concern,
- margins of exposure were not discussed,
- insufficient information regarding mechanism for cited rodent-specific effects,
- lack of detail regarding metabolism of parent compound and properties of metabolites, including identification of human metabolites, and
- insufficient, incomplete, or no discussion of other compounds in the drug class.

In three cases, additional information was requested from the sponsor as the CAD lacked data to an extent that it precluded a sufficient assessment of potential outcome and value of the 2-year rat carcinogenicity study.

The 45 CADs that comprise the final dataset were self-selected by the participating sponsors. The RND called for submission of CADs for 'all investigational pharmaceuticals subject to 2-year rat carcinogenicity studies under current ICH S1A Guideline' but also emphasized that submission of CADs designated as Category 3a and 3b would be of key importance, as these cases represent the most notable departure from current carcinogenicity testing guidelines. Therefore, the PES dataset may be biased toward investigational drugs where sponsors concluded that a 2-year rat study is not warranted for assessing human carcinogenic risk. Whether these cases are representative of all investigational drugs requiring a carcinogenicity risk assessment is unknown, yet consideration of the WoE factors can be reasonably applied to

TABLE 7 Unanimous Category 3a: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	2-year rat tumor outcome	Discussion on CAD or outcome
116	3a	3a	Therapeutic indication: Insomnia Target: neuronal G-protein-coupled receptor ● Drug target is predominately expressed in brain tissue ● No cause for concern based on known drug target biology and pharmacology ● No evidence of a carcinogenic effect due to drug target inhibition in a 2-year rat study with a comparable compound ● Antagonist binding interaction identified for 1 off-target receptor. Known pharmacology of off-target receptor not associated with tumorigenesis ● Increased liver weight, hepatocellular hypertrophy, and thyroid follicular cell hypertrophy in 6-month rat study ● Observed hormonal effects due to inhibition of the drug target and were not considered a cause for concern due to margins > 60-fold human exposure ● No evidence of genotoxicity, or immunosuppressive effects	DRA: negative Sponsor: negative	Increased liver weight and thyroid follicular cell hypertrophy in the 6- month rat study suggested the potential for liver and thyroid tumors in the 2-year rat study due to adaptive changes related to hepatic enzyme induction that has limited human relevance. Data was provided to indicate that CYP1A2 and CYP 3a1 were induced in the 6- month study. While the predicted hepatocellular and thyroid follicular cell tumors did not occur, the absence of drug-related tumorigenicity in the 2-year rat study did not change the WoE assessment of low carcinogenic risk in humans, such that a 2-year study would not add value.
142	3a	3a	Therapeutic indication: Fungal infection Target: Sterol synthesis ● No cause for concern based on known drug target biology and pharmacology ● Topical application limits systemic exposure ● Major human metabolites adequately assessed ● Comprehensive secondary pharmacology screen not conducted. Drug class reported to affect steroid metabolism ● Hepatocellular adenoma and carcinoma observed in carcinogenicity studies with other compounds in the drug class ● Negative 2-year dermal mouse study and dermal rat tumor-promoter study with compound 142 were considered supportive of negligible human carcinogenic risk ● Increased liver weight and hypertrophy at 86-fold human exposure and squamous cell hyperplasia in esophagus in 6-month dermal rat study ● Potential for esophageal squamous cell papilloma and carcinoma resulting from observed esophageal squamous cell hyperplasia in the 6-month dermal rat study. Finding is likely due to local irritation attributed to oral ingestion of compound 142 during self-grooming and is not human relevant ● Inhibition of aromatase activity in vitro, slight delay in estrus cycle in pregnant rats from subcutaneous dosing ● No genotoxicity or immunosuppressive effects	DRA: negative Sponsor: negative	Liver tumors were anticipated based on 2-year rat study data with related compounds, and observed hepatocellular hypertrophy in the 6- month study at >86 times clinical exposure. The absence of drug-related tumorigenicity in the 2-year rat study did support the WoE assessment of low carcinogenic risk in humans, such that a 2-year study would not add value.
106	3a	3a	Therapeutic indication: Viral infection Target: Viral enzyme ● Non-mammalian target with no mammalian equivalent ● No off-target activity in secondary pharmacology screen ● 2-year rat study data with drugs in class support a Category 3a designation ● Negative RasH2 transgenic mouse study ● Human metabolites adequately assessed ● Potential for bladder tumors due to presence of crystalluria without histological change to bladder in 6-month rat study ● No genotoxicity, hormonal or immunosuppressive effects	DRA: negative Sponsor: negative	The presence of needle-like crystals in urine in the 6-month rat study suggested the potential for bladder tumors in the 2- year study from a crystalluria mechanism that has limited human relevance. The absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in humans, such that a 2-year study would not add value.
109	3a	3a	Therapeutic indication: Viral infection Target: Viral enzyme ● Non-mammalian target with no mammalian equivalent ● Cause for concern not identified based on the outcome of rat and mouse carcinogenicity studies conducted for other compounds in the class ● No off-target activity in secondary pharmacology screen	DRA: equivocal Granulocytic leukemia, subcutaneous fibrosarcoma (not predicted in CAD) Sponsor: negative	The presence of reactive hyperplasia in the stomach from direct drug irritation suggested the potential for squamous tumors of the stomach in the 2-year study from local irritation mechanism that has limited human relevance. The interpretation of an equivocal outcome in the 2-year rat study is based on the absence of statistical significance for both trend and pairwise tests for the numerical imbalance of granulocytic

(Continued on following page)

TABLE 7 (*Continued*) Unanimous Category 3a: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	2-year rat tumor outcome	Discussion on CAD or outcome
			• Nasal turbinate inflammation and reactive hyperplasia in the squamous mucosa of the non-glandular stomach in the 6-month rat study • No genotoxicity, hormonal or immunosuppressive effects		leukemia and fibrosarcoma. The observed tumor outcome did not impact the WoE assessment concluding the compound exhibits low carcinogenic risk in humans and the 2-year rat study would not add value.
117	3a	3a	Therapeutic indication: Type 2 diabetes Target: Renal co-transporter • No cause for concern based on known drug target biology • High target selectivity • No off-target activity in secondary pharmacology screen • Adrenal medullary, testicular Leydig, and renal tumors in 2-year rat studies observed with comparable compounds, via inhibition of related off-target co-transporter • Increased kidney weight and tubule hypertrophy, and increased adrenal weight and hypertrophy in 6-month rat study • No evidence of genotoxicity, hormonal or immunosuppressive effects	DRA: positive Sponsor: positive Adrenal medullary pheochromocytoma	Adrenal medullary, testicular Leydig, and renal tubule tumors were anticipated based on the reported tumor outcome in 2-year rat studies conducted with similar compounds in the class, and the observed increase in kidney weight and tubule hypertrophy, and increased adrenal weight and hypertrophy in the 6-month rat study. In the 2-year rat study adrenal tumors were noted which is consistent with the WoE assessment for this organ. Tumors were not observed in the testis or kidney. The proposed mode of tumorigenic action in rats for the drug class is mediated by inhibition of a related co-transporter, which would not occur at clinically relevant exposure to the test compound. Therefore, the outcome of the 2-year rat study did not impact the WoE assessment concluding the compound exhibits low carcinogenic risk in humans and the 2-year rat study would not add value.
135	3a	3a	Therapeutic indication: Hypertension Target: Lyase • No cause for concern based on known drug target biology • Negative tumor outcome in 2-year rat study with comparable compound • No relevant off-target activity in secondary pharmacology screen • Human metabolites adequately assessed • Crystalluria was identified in rat urine without a histopathological change to renal or bladder tissue in the 6-month rat study. Urinary crystals not detected in human samples • Liver hypertrophy without change in liver weight in the 6-month rat study • Diffuse adrenal hypertrophy ascribed to intended pharmacological activity in the 6-month rat study • No genotoxicity, hormonal or immunosuppressive effects	DRA: equivocal Adrenal medullary pheochromocytoma and Leydig cell adenoma (not predicted in CAD) Sponsor: negative	The presence of crystalluria in the 6-month rat study suggested the potential for renal/bladder tumors in the 2-year study from a mechanism that has limited human relevance. The interpretation of an equivocal outcome is based on the absence of statistical significance for the numerical imbalance of adrenal pheochromocytoma and testicular Leydig cell tumors. The outcome of the 2-year rat study did not impact the WoE assessment concluding the compound exhibits low carcinogenic risk in humans and the 2-year rat study would not add value.
139	3a	3a	Therapeutic indication: Insomnia Target: Neuronal G-protein coupled receptor • No cause for concern based on known drug target biology and pharmacology • No off-target activity in secondary pharmacology screen • Major human metabolites adequately assessed • Comparable compound with less receptor selectivity positive for liver and thyroid follicular tumors in 2-year rat study • Increased liver weight and hepatocellular hypertrophy, increased thyroid weight and follicular hypertrophy/hyperplasia in 6-month rat study • No genotoxicity, hormonal or immunosuppressive effects	DRA: positive Sponsor: positive Granulocytic leukemia, thyroid C-cell carcinoma (not predicted in CAD)	The presence of liver hypertrophy and thyroid follicular cell hypertrophy/hyperplasia in the 6-month rat study suggested the potential for liver and follicular thyroid tumors in the 2-year study based on a mechanism that has limited human relevance. In the 2-year study, liver and follicular thyroid tumors were not observed but granulocytic leukemia (males) and thyroid C-cell carcinoma (females) were observed at an exposure multiple of 66-times and 72-times, respectively, the anticipated clinical exposure. As tumors occurred at exposure margins that are not considered human relevant, the outcome of the 2-year study did not impact the WoE assessment concluding the compound exhibits low carcinogenic risk in humans and the 2-year rat study would not add value.

all such investigational drugs. Based on the number of cases where DRAs unanimously agreed with the sponsor's designation of a CAD as 3a, or 3b in the PES dataset, approximately 27% of 2-year rat studies could have been avoided by applying the WoE approach (12 unanimous Category 3a/3b divided by 45 CADs submitted).

Category 3b and 3a

The framework recommended in the S1B(R1) Addendum (ICH, 2022) was principally supported from evaluation of Category 3a and 3b cases in the PES. These carcinogenicity risk categories postulated that data from a 2-year rat study would not add value because the WoE assessment is sufficiently persuasive to conclude that human carcinogenicity risk is unlikely.

The presumption for Category 3b was that a 2-year rat study would yield a negative tumor outcome and therefore not contribute further to the conclusion of minimal human risk based on the WoE assessment. The negative or equivocal tumor outcomes seen for 11 of the 12 DRA-designated Category 3b cases are consistent with this presumption. Similar results were observed with the 17 Category 3b cases as designated by sponsors, wherein 15 yielded a negative tumor outcome. A review of the Category 3b cases, with a particular focus on the unanimous cases, identified common attributes that aligned with a negative 2-year rat study and are summarized in Table 13. These attributes included 1) a target biology that is well-characterized and not known to be associated with carcinogenic pathways. Often, the availability of carcinogenicity data in rats from other class members supplemented the conclusion that an investigational drug's target biology would not be of carcinogenic concern; 2) High target selectivity as assessed by sufficiently broad secondary pharmacology screens. Such screens would preferentially include targets of higher *a priori* concern, such as hormone receptors and targets with known carcinogenic liability; 3) an absence of histological changes in chronic (6-month) rat toxicology studies indicative of carcinogenic concern, notably hyperplasia, hypertrophy, atypical cellular alterations, and degenerative/regenerative findings. If such findings are present, they are demonstrated to be human irrelevant; 4) an absence of perturbation to endocrine and reproductive organs, including changes to reproductive organ weights; 5) a negative battery of genotoxicity studies based on criteria from the ICH S2 (R1) guideline (International Council for Harmonisation, 2011), and 6) no evidence of immune modulation or immunotoxicity.

As noted above, the occurrence of a negative tumor outcome for Category 3b cases was similar whether the category was designated by the sponsor or by the DRAs. However, DRAs were more likely than sponsors to designate a compound as Category 2, suggesting that DRAs were more conservative than sponsors in accepting the WoE without 2-year rat data in some cases (Table 10). A more conservative position than proposed by the sponsor was driven by at least 2 DRAs, with one exception where a single DRA took a more conservative position than the other DRAs (#112). The identity of >2 DRAs generally varied across cases. A more conservative approach was also partly due to the limited ability of both DRAs and sponsors to fully investigate signals of concern identified in the WoE assessment within the confines of the PES. For example, as seen in cases #s 102, 104, 141, and 107 of Table 10, the sponsor's WoE assessment did not provide adequate information for several WoE factors, such as target biology, general toxicity, and genetic toxicity, which could not be readily addressed by the sponsor during the PES. However, a sponsor would have greater latitude in a 'real-world' situation to clarify and supplement the WoE assessment, as needed, to address deficiencies identified by the reviewing DRA. In other cases, the issues cited by the DRAs were more substantial and difficult to resolve, and also reflect a more conservative risk tolerance relative to the sponsor (e.g., case #s 105, 140, and 148 of Table 10). For example, in one case (#140), the DRAs cited the unresolved human relevance of a known positive tumor profile for a drug class as not being consistent with a Category 3b designation, and for another case (#148) the complexity of drug pharmacology precluded confident prediction of the 2-year rat tumor outcome and value, necessitating the conduct of a 2-year rat study. Of note, the tumor outcome of these cases, both negative and positive, can be reasonably viewed as adding value to the overall WoE assessment of human risk.

Unlike Category 3b, the presumption for Category 3a was that the 2-year rat study would likely result in a positive tumor outcome through a prior established and well-recognized mechanism considered to be human irrelevant. A positive tumor outcome by a human-irrelevant pathway would therefore not contribute further to the conclusion of minimal human risk based on the WoE. The prediction of a positive, human-irrelevant tumor outcome for the 7 unanimous DRA-designated Category 3a cases was most frequently based on histological findings indicative of a hyperplastic and/or a hypertrophic response in the 6-month rat toxicology study (e.g., increased liver weight/cellular hypertrophy in cases #116, 142, and 139). In 2 cases (#106, 135), the expectation of bladder tumors was based on the presence of urinary crystals without histological changes to the urothelium. Available information on the tumor outcome for drugs with a similar pharmacological mechanism also contributed to the positive prediction in some cases (e.g., #142, 117).

The actual tumor outcome from the 2-year rat studies for these compounds indicates that predicting a positive tumor outcome with organ specificity based on 6-month toxicology data remains a challenging proposition, consistent with prior reports (Jacobs, 2005; Sistare et al., 2011). It should be noted that the absence of an anticipated tumor type from a 2-year rat study is not interpreted as being a contrary outcome, as one is predicting the probability and not the certainty of tumor emergence in a given organ. Of more concern are cases where tumor types emerged that were not anticipated from the WoE analysis in the CAD. For example, the occurrence of granulocytic leukemia and thyroid C-cell adenoma for case #139 clearly differs from the anticipated tumor types of liver and thyroid follicular tumors based on histological changes to these organs in the 6-month toxicology study. The unanticipated tumors emerged at exposure multiples of 66-times and 72-times clinical exposure, respectively, and therefore did not change the overall assessment of low human carcinogenic risk based on the prospective WoE. The tumor outcome of 2 additional unanimous Category 3a cases (#s 109, 135) was also discordant from the tumor types anticipated based on the WoE. However, the tumor signal in

TABLE 8 Non-unanimous Category 3a: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	Basis for alternative category	2-year rat tumor outcome	Discussion on CAD or outcome
145	3a	3a, 2	Therapeutic indication: Metastatic prostate cancer Target: Steroid receptor Category 3a ● Inhibition of drug target associated with reduced cell growth and increased apoptosis. Target biology involves disruption of hormonal pathway (androgen activity) leading to high sustained LHRH and LH activity ● Negative RasH2 transgenic mouse study ● Major metabolites adequately assessed ● Histological findings in chronic rat and dog studies suggest potential for tumors in liver, thyroid, bladder, renal, testicular, adrenal, pituitary, and endometrial tissues ● Tumorigenic pathway for potential liver/thyroid tumors (drug metabolism: CYP enzyme induction demonstrated) and potential renal/bladder tumors (crystalluria) considered rat-specific and human irrelevant ● Tumorigenic pathways for testicular adrenal, pituitary, and endometrial tissues relate to drug pharmacology but are not relevant to intended patient population (e.g., males on LH suppressive regimens) ● No genotoxicity or immunosuppressive effects	Category 2 ● Mechanistic link between adrenal findings and changes in LH levels not sufficiently characterized ● Compound exhibits additional mechanisms of action not observed with other compounds in the class ● Inadequate information provided to link renal/bladder histological findings to drug-related crystalluria ● Inadequate information regarding risk from potential functional interaction with secondary target (GABA-receptor) ● Margin of exposure for hypertrophic lesions difficult to establish and may be equivalent to steady state exposure at human clinical dose	DRA: positive Sponsor: positive Leydig cell adenoma, ovarian granulosa, bladder papilloma/carcinoma, pituitary pars distallis adenoma, thymoma (not predicted in CAD), mammary fibroadenoma (not predicted in CAD)	Adequacy of information regarding target biology and relevance of histological findings of concern in 6-month rat study and of potential-off target interactions varied across DRAs. The presumption of low human carcinogenic risk was driven primarily by attributes of the indicated patient population that could not be extrapolated to a different patient population. For some DRAs, the outcome of the 2-year rat study suggested that a Category 2 designation may have been more appropriate than a Category 3a designation.
125	3a	3a, 2	Therapeutic indication: Schizophrenia Target: Multiple neuronal G-protein coupled receptors Category 3a ● Tumors anticipated in mammary and pancreatic tissues of rats secondary to elevation in prolactin, considered of limited human relevance ● Hepatocellular tumors anticipated based on liver hypertrophy in 6-month study, related to rat-specific drug metabolism ● Human metabolites adequately generated and evaluated in non-clinical animal models ● Cecal tumors anticipated based on epithelial hyperplasia in 6-month study, related to direct tissue irritation or disruption to gut microflora, considered rat-specific ● No genotoxicity or immunosuppressive effects	Category 2 ● Hypertrophic / proliferative lesions observed in the mammary gland of rats in the 6-month study may be attributed to a compound-related effect on prolactin secretion ● Relevance of prolactin elevation to human risk of carcinogenicity is uncertain as epidemiological literature data indicates that drug-mediated prolactin enhancement may not be rat-specific and may pose a human cancer risk ● Results from 2-year rat study may inform relative prolactin-related tumor risk among similar compounds in class ● Inadequate characterization and relevance of cecum hyperplasia, lung phospholipidosis ● Unclear rationale for expectation of pancreatic tumors in rats	DRA: equivocal Leydig adenoma (not predicted in CAD) Sponsor: negative	Adequacy of information addressing relevance of histological findings in 6-month rat study and of prolactin elevation varied across DRAs. For DRAs selecting Category 2, the outcome of the 2-year rat study resolved uncertainties related to cecum hyperplasia in the 6-month rat toxicity study. For some DRAs, while tumors were not observed in the mammary gland (hyperplasia was noted in the chronic rat toxicity study), the outcome of the 2-year rat study did not resolve uncertainties regarding human cancer risk of drug-mediated elevated prolactin levels. Considering that epidemiological data are available, there may be alternative methods to better characterize the human relevance of this finding.
131	3a	3a, 2	Therapeutic indication: Pulmonary disorder Target: Cation channel (novel drug target) Category 3a ● Neither polymorphism nor gene mutation was associated with familial tumor susceptibility or with sporadic tumor development in humans or animals. Drug target null mice, drug target antisense oligonucleotides, assessment of the COSMIC database were included in the assessment of target biology ● Compound selective for drug target relative to receptors in the same family ● No off-target activity in secondary pharmacology screen ● Human metabolites adequately assessed ● Tumors anticipated in renal/bladder tissues based on crystalluria observed in the 6-month rat study. In dogs, crystalline material was observed in urine with no correlating histological changes. In humans, urinary crystals not observed at clinical drug exposures ● No genotoxicity, hormonal, or immunosuppressive effects	Category 2 ● Crystalluria occurs in rats and also dogs and human subjects at higher drug exposures ● Literature suggests an increased risk of urinary tract cancers following renal/ureter stones ● Crystalluria overlaps with site of pharmacological action (kidneys) ● Value of a rat study would be establishing an exposure-response relationship and for characterizing a novel drug target	DRAs: negative Sponsor: negative	Relevance of overlap between site of crystalluria and primary site of pharmacological activity varied across DRAs. For DRAs selecting Category 3a, while tumors predicted in renal and bladder tissues were not observed, the absence of drug-related tumorigenicity in the 2-year rat study supported the WoE assessment of low carcinogenic risk in humans, such that a 2-year study would not add value. For DRAs selecting Category 2, the absence of drug-related tumorigenicity in the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk.

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TABLE 8 (*Continued*) Non-unanimous Category 3a: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	Basis for alternative category	2-year rat tumor outcome	Discussion on CAD or outcome
133	3a	3a, 2	Therapeutic indication: Obesity Target: Renal co-transporters Category 3a ● Genome screens don't associate target gene mutations with human cancers ● Tumors anticipated in testicular and adrenal tissues secondary to changes in calcium balance in rats, through a mechanism reported to have minimal human relevance ● No change in urinary calcium and calcium biomarkers observed in clinical trials, further limiting relevance of findings in rats ● Negative RasH2 transgenic mouse study ● Cecal hyperplasia observed in 6-month rat study related to pharmacology and is an adaptive secondary effect ● No off-target activity in secondary pharmacology screen ● Human metabolites adequately generated and evaluated in non-clinical animal models ● No hormonal, or immunosuppressive effects ● No evidence of mutagenic activity in the Ames assay, and no increase in structural chromosome aberrations in the in vitro assay in human lymphocytes ● An increase in micronuclei formation observed in the in vitro and in vivo micronucleus test. Investigative studies indicated that the findings were likely due to interference with the spindle apparatus and consistent with an aneugenic mechanism ● Maximum clinical exposure did not exceed exposure at 1/20th the NOEL in the rat micronucleus assay	Category 2 ● Compound 133 is a mixed target inhibitor ● Different target selectivity limits extrapolation of carcinogenicity data from (selective) compounds in the class to compound 133 ● Incomplete characterization and assessment of intestinal (cecum) hyperplasia observed in the 6-month rat study	DRAs: negative Sponsor: negative	Relevance of data for related compounds with differing target selectivity varied across DRAs. For DRAs selecting Category 3a, while the predicted testicular and adrenal tumors did not occur, the absence of drug-related tumorigenicity in the 2- year rat study did not change the WoE assessment of low carcinogenic risk in humans, such that a 2-year study would not add value. For DRAs selecting Category 2, the absence of drug-related tumorigenicity in the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk.
112	3a	3a, 2	Therapeutic indication: Neurologic disorder Target: Central benzodiazepine receptor Category 3a ● Target biology not associated with tumorigenic pathways, further supported by rodent tumor profile of drug class ● Human metabolites adequately addressed ● No off-target activity in secondary pharmacology screen ● Hepatocellular and thyroid follicular tumors in rats anticipated based on increased liver/thyroid hypertrophy in 6-month and 18-month rat toxicity studies. Mechanistic studies indicated that the compound alters the pituitary-thyroid axis and increases hepatic UDPGT in rats demonstrating that the liver and thyroid findings are likely rat-specific and considered of limited human relevance ● No genotoxicity or immunosuppressive effects	Category 2 ● Previous (older) 2-year dietary study reported endometrial hyperplasia/ polyps and alterations in mammary tissue development not seen in shorter term oral gavage studies ● Added value of 2-year rat study is long-term characterization of potential hormonal perturbation	DRAs: positive Sponsor: positive Liver adenoma, thyroid follicular cell adenoma and carcinoma	Relevance of prior findings indicative of hormonal perturbation in a 2-year dietary study varied across DRAs. For DRAs selecting Category 3a, the outcome of the 2-year rat study supported the WoE of low carcinogenic risk in humans, such that a 2-year rat study would not add value. Residual uncertainty regarding hormonal perturbation was addressed from available compound-specific and drug-class specific data. For DRAs selecting Category 2, the outcome of the 2-year rat study resolved uncertainties identified in the WoE assessment and provided value to the assessment of human carcinogenic risk.

these cases was not persuasive, and the studies were interpreted as negative by the sponsors. While the outcome was interpreted as 'equivocal' by the DRAs, there was also agreement that the equivocal outcome did not change the overall assessment that human carcinogenic risk was unlikely based on the prospective WoE. That these unanticipated tumor types did not change the assessment of human carcinogenic risk is reassuring of safety for applying this WoE approach to drug candidates with similar pharmacological and toxicologic profiles. However, these cases demonstrate that positive prediction is less reliable than negative prediction of tumor outcome and, as such, may merit a more conservative evaluation of the WoE regarding the necessity of a 2-year rat study.

Category 2

Sponsors and DRAs unanimously agreed in 8 cases that the conduct of a 2-year rat study would be appropriate to address uncertainties identified in the CAD (Table 9). These unanimous

TABLE 9 Unanimous Category 2: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for category 2 agreement	Sponsor's statement of expected value (from submitted CAD)	2-year rat tumor outcome
101	2	2	Therapeutic indication: Various cancers Target: Tyrosine kinase Residual uncertainty • Potential impact of off-target kinase inhibition on tumor risk • Duodenal tumors in rats reported with similar drug in class • Histologic changes in the duodenum in 6-month rat study. Also observed in monkeys • Histologic changes in the ovary and testes indicative of potential hormonal perturbation in 6-month rat study • Changes in hematology and clinical chemistry parameters indicative of potential liver toxicity in 6-month rat study	The 2-year rat study will likely add value to the assessment of human carcinogenic risk considering the potential for chronic treatment in the adjuvant setting, and tumors identified in a 2-year rat study with a similar drug in the class	DRA: positive Sponsor: positive Duodenal adenocarcinoma, males and females at <0.4-fold clinical exposure NOAEL not identified
108	2	2	Therapeutic indication: Viral infection Target: Viral enzyme Residual uncertainty • Positive genotoxicity data (*in vivo* rat micronucleus) of uncertain human relevance • Low incidence of hemangiosarcoma in 6-month rat study • Diverse rodent tumors observed with drug class	The 2-year rat study will inform the predictive potential of the 6-month rat study for the following profile • Positive clastogenicity • No proliferative changes but observed vascular tumor • Rodent tumors in drug class There is also value in establishing a safety margin for risk assessment and human relevance based on exposure multiples	DRA: negative Sponsor: negative
111	2	2	Therapeutic indication: Hematologic disorder Target: Transcriptional regulatory complex Residual uncertainty • Inhibition of drug target increases transcription of pro-angiogenic and growth factors implicated in tumor progression • Lack of precedent for compounds of this drug class	The 2-year rat study will likely add value to the assessment of human carcinogenic risk based on • Absence of carcinogenicity data with other drugs in the class • Potential for tumors related to drug target pharmacology	DRA: negative Sponsor: negative
114	2	2	Therapeutic indication: obesity, type 2 diabetes Target: G-protein coupled receptor Residual uncertainty • Human-relevant carcinogenic hazard identified from rodent genetic models and human genetic disorders • Cellular proliferation within target tissue observed in the 3-month rat study. Also observed in mice and monkeys • Unresolved hyperplasia of intestinal crypt epithelium in small and large intestines in 3-month rat study • Lack of a 6-month rat toxicology study • Lack of precedent for compounds of this drug class	The 2-year rat study will likely add value to the assessment of human carcinogenic risk by providing information on a potential carcinogenic threshold associated with pharmacologic inhibition of the drug target.	DRA: negative Sponsor: negative

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TABLE 9 (*Continued*) Unanimous Category 2: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for category 2 agreement	Sponsor's statement of expected value (from submitted CAD)	2-year rat tumor outcome
119	2	2	Therapeutic indication: inflammatory diseases including psoriasis Target: G-protein coupled receptor (novel drug target) Residual uncertainty • Unresolved renal toxicity in 6-month rat study. Also observed in mice and monkeys • Lack of precedent for compounds of this drug class	The available set of toxicological data indicates that the carcinogenic potential for humans is uncertain and the 2-year rat study will likely add value to human carcinogenic risk assessment	DRA: negative Sponsor: negative
120	2	2	Therapeutic indication: rheumatoid arthritis Target: Lipid kinase (novel drug target) Residual uncertainty • Immunomodulatory activity with anti- and pro-tumorigenic activities • Lack of precedent for compounds of this drug class	Due to the immunosuppressive action, coupled with a lack of carcinogenicity data available for pharmaceutical compounds of this drug class, the tumorigenic potential for humans is uncertain and the 2-year rat study will likely add value to the assessment of human carcinogenic. The ability of compound 120 to increase immune surveillance may negate any tumorigenic potential arising from sustained immunosuppression	DRA: equivocal Numerical imbalance of pancreatic islet cell adenoma/carcinoma, males, at 1x clinical exposure Sponsor: negative
132	2	2	Therapeutic indication: diseases with oxidative stress and pathological inflammation Target: Serine-threonine protein kinase (novel drug target) Residual uncertainty • Carcinogenicity risk due to sustained cell survival and potential immunomodulatory activity • Tumor promotion studies in a knockout mouse model yielded mixed results • Unresolved renal, gastrointestinal, and adrenal toxicities in 6-month rat study • Lack of precedent for compounds of this drug class	The 2-year rat study will likely add value to the assessment of human carcinogenic risk based on • Absence of carcinogenicity data with other drugs in the class • Potential for tumors related to drug target pharmacology (suppressing apoptosis and/or modulation of the immune system)	DRA: positive Sponsor: positive Pituitary adenoma, males and females (reduced latency, increased incidence, and lethality). NOAEL for carcinogenicity provided ~5-fold exposure margin
138	2	2	Therapeutic indication: cholestatic disorders, non-alcoholic steatohepatitis (NASH) Target: bile acid nuclear receptor (novel drug target) Residual uncertainty • Limited information on target pharmacology • Increased liver weight in multiple species, capacity to induce CYP and bile acid transporter *in vitro* • Lack of 6-month rat toxicology study • Limited assessment on potential hormonal effects • Lack of precedent for compounds of this drug class	The 2-year rat study will likely add value to the assessment of human carcinogenic risk by identifying tumors that are potentially human relevant	DRA: positive Sponsor: positive Hepatocellular adenoma and carcinoma, hepatocholangio-cellular adenoma, males. NOAEL for carcinogenicity provided ~9-fold exposure margin

decisions aided in defining common WoE attributes that introduced significant uncertainty into predicting the outcome and/or value of a 2-year rat study. These attributes are generally captured in Figure 2 of the ICH S1B(R1) Addendum which provides guidance on integration of the key WoE factors.

For most cases, the sponsors cited drug target pharmacology and the known tumor profile from other class members as a cause for concern which merits the conduct of a 2-year rat study, rather than compound-specific toxicology findings. As captured by the sponsor's statements in the CAD, a 2-year rat study was anticipated to establish a threshold of tumorigenic activity, if

TABLE 10 DRA-designated unanimous non-concordance with sponsor's proposed Category 3a or 3b designation: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for sponsor categorization	Basis for DRA categorization	2-year rat tumor outcome	Discussion on CAD or outcome
102	3b	2	Therapeutic indication: Inflammatory diseases Target: Serine-threonine protein kinase Category 3b • Immunomodulatory agent. Negative carcinogenicity results with another compound with the same mode of action • No histological findings of concern at in 6-month rat and 9-month monkey studies • No genotoxicity • Degenerative findings in female rat reproductive tissues interpreted as not human-relevant	Category 2 • Immunomodulatory profile not sufficiently characterized to inform cancer risk • Data from similar compound could not be extrapolated due to diverse toxicity observed in class • Further immunotoxicity profiling would have given further support to the category proposed by the sponsor or may have supported Category 1 (immunosuppression) • Histological findings in female rat reproductive tissues not fully characterized	DRA: negative Sponsor: negative	The 2-year rat study was recommended by DRAs primarily due to lack of alternative proposal for assessing carcinogenicity risk of immunomodulator, and incomplete characterization of potential hormonal perturbation in female reproductive tissues
104	3b	2	Therapeutic indication: Symptomatic amyloidosis Target: Transport protein (novel drug target) • No evidence that target engages carcinogenicity pathways • No evidence of proliferative or hyperplastic changes in 6-month rat study • No genotoxicity, hormonal or immunosuppressive effects • Negative result in rasH2-Tg mouse study	Category 2 • Novel target with an insufficiently characterized mode of action • Insufficient level of information on identification and exposure to metabolites • Uncertain genotoxicity profile based on evidence suggesting possible aneugenicity and on limitations on dose selection for the genotoxicity studies performed	DRA: negative Sponsor: negative	2-year rat study recommended by DRAs due to insufficient information on pharmacological target, metabolite profile, and genotoxicity assessment
105	3b	2	Therapeutic indication: major depressive disorder Target: Ion channel Category 3b • No evidence that target engages carcinogenicity pathways • Histological findings in 6-month rat study interpreted as human irrelevant (renal necrosis/regeneration, liver hypertrophy, parotid hyperplasia, increased ovarian weight) • Negative tumor outcome in 2-year rat studies with members of class • No evidence of genotoxicity • Bladder hypertrophy without hyperplasia observed in 26-week rat study was considered to be of no relevance to cancer risk	Category 2 • Differences in selectivity and toxicity profile from drug class precludes confidence of prediction for 2-year rat study • Uncertainty or lack of mechanistic explanation underlying toxicity observed in liver, kidneys, and parotid glands • Effects on ovaries and inhibition of prolactin also limit possibility of agreeing on a Category 3	DRA: negative Sponsor: negative	The 2-year rat study was recommended by DRAs due to insufficient information regarding relevance of toxicology findings in liver, kidneys, parotid glands, and female reproductive tissues
140	3b	2	Therapeutic indication: Neuropathic pain Target: Ion channel Category 3b • Target pharmacology similar to known class profile • Erosion and/or ulceration of the forestomach and glandular stomach and thickening of the	Category 2 • Tumor profile of class is mixed, includes occurrence of pancreatic acinar cell carcinoma in male rats of uncertain human relevance • The mechanisms by which the tumors are induced are unclear	DRA: equivocal Sponsor: positive Urinary bladder papilloma	The 2-year rat study was recommended by DRAs due to unresolved human relevance of tumors reported for some compounds in the same drug class

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TABLE 10 (*Continued*) DRA-designated unanimous non-concordance with sponsor's proposed Category 3a or 3b designation: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for sponsor categorization	Basis for DRA categorization	2-year rat tumor outcome	Discussion on CAD or outcome
			forestomach mucosa were considered to be caused by chronic irritation and should not be considered relevant for human risk • Hypertrophy of hepatocytes with possible induction of liver enzyme induction were considered rat specific • No genotoxicity • Persistent estrus observed in a developmental and reproductive study was considered to be of no relevance to hormonal perturbation • No evidence of immunosuppressive effect • No histopathological changes of concern in 9-month monkey study	and cannot be the basis for considering the tumors irrelevant to humans • Literature points to carcinogenic potential for a drug of the same class		
148	3b	2	Therapeutic indication: Cancer Target: Tyrosine kinase Category 3b • Intended and off-target activities not linked to pro-tumorigenic pathways but may be anti-tumorigenic • No genotoxicity • No evidence of immunosuppressive effect • No histopathological changes of concern in 9-month dog study • Estrus/fertility findings in female rats not considered relevant to tumor risk • Negative result in rasH2-Tg mouse study	Category 2 • Multiplicity of drug targets precludes confident prediction of tumor outcome in rats • Extrapolation of findings from class not warranted based on differences in pharmacology • Potential impact of hormonal changes detected in the rats (LH/FSH) were not sufficiently addressed • In the 26-week rat study, increases in hemorrhagic cystic degeneration in the lymph nodes which might be related to hemangiosarcomas in female rats	DRA: negative Sponsor: negative	The 2-year rat study was recommended by DRAs due to uncertainty of rat tumor outcome based on complexity of drug pharmacology, and on insufficient information addressing relevance of hormonal perturbation identified in female rats
107	3a	2	Therapeutic indication: Viral infection Target: Viral polymerase Category 3a • Expectation of liver tumors in 2-year rat study based on increased liver weight/hypertrophy in 26-week rat study via a rat-specific mechanism • Gastrointestinal epithelial proliferation observed in 6-month rat study at high multiple of clinical exposure • No evidence of genotoxicity, hormonal perturbation, and immunosuppressive effects • No histopathological changes of concern in 39-week dog study	Category 2 • Hyperplastic gastrointestinal findings not sufficiently characterized to address time-dependence of exposure/response or to allow consideration of the dog study where such toxicity was not observed • Lack of precedent for compounds of this drug class • Off-target activity identified in secondary screen raised concern of product specificity • Limited information was provided on metabolites	DRA: positive Sponsor: positive Liver adenoma	The 2-year rat study was recommended by DRAs due to insufficient information regarding relevance of gastrointestinal proliferative findings, metabolite profile, and uncertainty regarding product specificity
141	3a	2	Data not disclosed	N/A	DRA: positive Sponsor: positive	Positive tumor response was partially consistent with Sponsor's expectation of outcome

present, and to identify an exposure margin that would allow an exposure-based assessment of human relevance and carcinogenic risk on a compound-specific basis. Among the 4 cases with a positive outcome as determined by the DRAs, two yielded carcinogenic exposures that were lower than clinical exposure, and two identified non-carcinogenic exposures that were 5-fold and 9-fold higher than clinical exposure. The absence of a safety margin for the former two provided the sponsor with further evidence of potential risk in addition to concerns identified with the drug class (case #101) and with the pharmacological mechanism (case #120). The presence of a safety margin for the latter two provided the sponsor with empirical evidence that mitigated carcinogenic risk raised by concerns identified in the CAD (case #s 132, 138). For other cases, studies that yielded a negative tumor outcome provided the sponsor with evidence of safety that would be integrated with other data in the overall WoE evaluation of human risk.

In their analyses of the unanimous Category 2 cases, in addition to drug target-based concerns, in some cases the DRAs cited literature reporting both pro- and anti-tumor activities of the drug target which precluded both confident prediction of human risk and rat tumor outcome. The DRAs also frequently cited more compound-specific toxicology findings with inadequate explanations of causality and human relevance as additional reasons to conduct a 2-year rat study. In practice, further investigative approaches may be applied to address the human relevance of concerns identified in the WoE assessment and, if adequately de-risked, may negate the value of conducting a 2-year rat study. The feasibility of this approach would depend on the type and number of concerns identified in the WoE assessment; for example, concerns identified for several WoE factors would be more challenging to de-risk with investigative approaches compared to a concern identified for a single WoE factor. A multiplicity of concerns was generally identified for the unanimous Category 2 cases. For such cases, the DRAs noted that a negative tumor outcome or identification of a carcinogenic threshold in a 2-year rat study can add particular value to the overall assessment of human risk.

Category 1

The current ICH S1A guideline (ICH, 1995) recommends that long term carcinogenicity studies are not needed to inform human cancer risk from compounds that exhibit unequivocal genotoxic activity. The S1A guidance, however, does not address non-genotoxic carcinogenic mechanisms that are recognized or presumed to have human relevance (Al-Zoughool et al., 2019; Krewski et al., 2019). Principal among these non-genotoxic mechanisms includes compounds that are broadly immunosuppressive, result in persistent hormonal perturbation, or otherwise engage cell growth/survival pathways that lead to persistent cell replication.

The PES dataset includes three compounds submitted as Category 1 by sponsors based on arguments related to immunosuppression for two cases and a persistent rebound proliferative response for one case. The DRAs unanimously agreed to this categorization for one case based on persuasive evidence of broad immunosuppression. For the remaining two cases, some DRAs concluded that data from a 2-year rat study would provide additional value while also acknowledging the likely human risk based on the pharmacological mechanism of each compound. In one case (#113), some DRAs were concerned that the sponsor's prediction of a benign tumor type underestimated the risk of inducing more serious malignancies, a potential outcome that could be addressed in a 2-year rat study. The tumor outcome was restricted to only benign tumor types which mitigated the concern for other malignancies and was considered an outcome of value by some DRAs. In another case (#123), some DRAs cited concerns of potential tumorigenesis arising from mechanisms unrelated to the compound's immunosuppressive activity. Specifically, observations of proliferative findings in the 6-month rat study, genetic polyploidy, and potential prolactin elevation were identified as potential tumorigenic liabilities beyond the risk from immunosuppression, which could be informed by 2-year rat data. The negative tumor outcome mitigated these concerns, although there is recognition that these concerns might have been adequately de-risked by investigative studies to reduce the need for a 2-year rat study. It is recognized that the primary human risk from immunosuppression would not be further informed by a 2-year rat study (Bugelski et al., 2010). The counterview is that the tumorigenic risk of compound #123 would be disclosed with appropriate labeling regardless of the tumor outcome from a 2-year rat study, or whether potential off-target tumorigenic risk is prospectively recognized or not.

Weight of Evidence (WoE) factors

The 2013 RND described the WoE factors that should be addressed in preparing the carcinogenicity assessment documents for the PES. These factors were in part informed by the retrospective analyses from Sistare et al. (2011) and Van der Laan et al. (2016a, 2016b), where the pharmacology, histopathology, genotoxicity, and endocrine endpoints were considered key attributes in assessing the carcinogenic potential of pharmaceuticals in rats. The RND (ICH, 2013) and the finalized S1B(R1) Addendum (ICH, 2022) incorporated these endpoints and expanded the WoE factors to include consideration of the metabolic profile, secondary pharmacology, and immunotoxicity. The WoE assessment took into account all of these factors, but the relative importance of each factor varied depending on the compound being assessed. While a low level of concern for all factors was generally considered supportive of not conducting a 2-year rat study, a clear finding of high concern for any one factor (e.g., multi-tissue hyperplasia related to pharmacology) that cannot be resolved by other investigative approaches may necessitate the need for a 2-year rat study to address that uncertainty. More commonly, cause-for-concern was identified for multiple WoE factors. The DRAs were more likely than sponsors to conclude that a 2-year rat study was appropriate in such cases (e.g., Tables 8, 10). The attributes of each WoE factor and their relative contribution to an integrated assessment of carcinogenic risk and the need for 2-year rat data is captured in the decisional framework depicted in Figure 2. This framework is incorporated into the ICH S1B(R1) addendum as an aid to determine whether the human carcinogenic potential of an

TABLE 11 DRA-designated non-unanimous Category 2: Comparison of WoE assessment to tumor outcome of the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for categorization	Basis for alternative category by DRA(s)	2-year rat tumor outcome
126	2	3a, 2	Therapeutic indication: major depressive disorder Target: Neuronal ion channel Category 2 • Non-selective nature of the compound, raising uncertainties in extrapolating carcinogenicity outcomes from others in the drug class • Evidence of hormonal disruption (literature data) • Potential immunosuppressing effects • Uncertainties in the characterization of *N*-nitroso metabolite • Exposure reached in the repeated-dose toxicity studies were not in excess of clinical exposure levels	Category 3a • Lack of proliferative findings in the systemic toxicity studies • Hormonal effects were not seen in the submission data and this overrules literature • Local exposure resulting in nasal cavity findings in animals are not reached in humans • Carcinogenicity study in rat is unlikely to add value on the definition of human risk for the above-mentioned local effect and immune suppression-related carcinogenicity	DRA: negative Sponsor: negative
127	2	3a, 2	Therapeutic indication: Alzheimer's disease Target: B-amyloid protein (novel drug target) Category 2 • Not a well understood novel target • Potential for carcinogenic risk in rat liver and possibly other organs	Category 3a • On-target pharmacology not linked in principle to anti pro-tumorigenic pathways • No genotoxicity, hormonal perturbation or immunosuppression • No evidence indicating a potential for neoplasia in the rat chronic toxicity study • Non-rodent studies in Cynomolgus monkeys did not show any toxicological findings indicating a potential for neoplastic events	DRA: equivocal Increased testicular Leydig adenoma and hepatocellular adenoma Sponsor: negative
121	2	2, 1	Therapeutic indication: Hematologic disorder Target: Tyrosine kinase and Serine-threonine kinase receptor Category 2 • Immunosuppressive activity • Uncertainties regarding action on specific target and the lack of experience regarding molecules acting on this receptor • Incomplete characterization of the major human metabolite • Heterogeneity in toxicology profile of this class of drugs limits possibility of extrapolation	Category 1 • Pharmacodynamic effects of the drug (i.e., immunosuppression) • Rodent bioassays have shown limited value in defining this type of carcinogenic risk • Malignancies observed with compounds of the same class (i.e., tofacitinib) • The lack of other risk factors such as genotoxicity, increases in neoplasia in 6-month transgenic mouse study • Difficulties in metabolite characterization, as exposure levels low in rodents	DRA: positive Sponsor: positive Testicular Leydig adenoma

investigational pharmaceutical is likely, unlikely, or uncertain. These 'risk categories' described in the addendum correspond to Categories 1, 3a/3b, and 2 as described in this report, and are accompanied by regulatory recommendations regarding the potential added value of conducting a 2-year rat study.

The availability of an established profile of other compound(s) in a drug class often contributed substantially to assessing human carcinogenic risk and was particularly relevant to informing the target biology WoE factor. Such information is limited or absent for compounds directed toward novel drug targets which presents a knowledge gap and increases uncertainty when assessing human carcinogenic risk. The PES dataset includes a total of 12 compounds with novel drug targets, of which 6 cases were designated as Category 3a or 3b (#s 103, 130, 118, 137, 136, 131), and in two cases by unanimous decision (#s 103, 130). In case #130, a cause for carcinogenic concern was not identified regarding drug target biology or compound selectivity, and no proliferative changes in any organs or tissues were observed at a high multiple of exposure in the 6-month study in rats (a pharmacologically relevant species). The high (54x) exposure multiple in this case provided additional assurance that modulation of the drug target at more clinically relevant drug concentrations would be highly unlikely to present a carcinogenic risk. In case #103, the sponsor provided results of a 2-year rat study from a comparable but discontinued compound which indicated a lack of tumorigenic potential from modulation of the pharmacological target after long-term exposure, in addition to no cause-for-concern identified from other WoE factors. The 2-year rat study yielded a negative tumor outcome for both these cases, in confirmation of the Category 3b categorization based on the WoE approach. Of note, for both these compounds, additional evidence was provided that supported a conclusion of no cause-for-concern regarding target biology, which successfully compensated for the lack of precedent for the drug class. A high exposure multiple in the 6-month toxicology study and availability of relevant 2-year rat carcinogenicity data with other compound(s) are only two examples of meeting a higher evidentiary standard that may lend further support for a using a WoE approach for compounds with a novel target. Other sources of data may also be applicable, which would likely vary by specific attributes of the compound and target, and it would be the sponsor's obligation to justify the type and scope of evidence appropriate to support a WoE approach for novel targets.

TABLE 12 Unanimous and non-unanimous Category 1: Comparison of WoE assessment to tumor outcome in the 2-year rat study.

Case ID	Sponsor category	DRA category	Basis for category 1	Basis for alternative category by DRA(s)	2-year rat tumor outcome	Discussion on CAD or outcome
113	1	1, 2	Therapeutic indication: Various types of cancer Target: Transcriptional regulatory protein Category 1 • 6-month study shows pilomatricoma • Complex pharmacology underlying Shh and catenin signaling, proposed mode of action resulting in pilomatricoma • No genotoxicity, no immunosuppressive effects	Category 2 • Complex pharmacology underlying Shh and catenin signaling and the proposed mode of action resulting in pilomatricoma raised concerns of over- or under-estimating human risk • Potential off-target effects • Potential hormonal effects (increase in FSH and LH) • Discussion on safety margins not sufficient • Experience with drug class insufficient to aid prediction for the compound	DRA: positive Sponsor: positive Pilomatricoma, keratoacanthoma	The 2-year study outcome was consistent with the sponsor's prediction of mechanism-based pilomatricoma Results of the study addressed the DRA's concern of over- or under-estimating human risk from a mechanism-based prediction, and therefore added value to the overall assessment of human carcinogenicity risk
123	1	1, 2	Therapeutic indication: Rheumatoid arthritis Target: Tyrosine kinase Category 1 • Genotoxicity findings indicate a potency to induce polyploidy • Immunosuppression in the repeated-dose toxicity study in rats • Malignancies observed with compounds of the same class (tofacitinib) • In monkeys tofacitinib induced lymphoma, related to immunosuppressive effect • Tumors reported in patients treated with JAK1/2 inhibitors, tofacitinib and ruxolitinib	Category 2 • Proliferative findings in stomach and renal tubules were considered preneoplastic changes • Hibernoma observed with tofacitinib • Address the potential for 'off target' tumors, despite the recognized malignancy risk from immunomodulation, for which rodent studies are considered poorly predictive • The effect of compound on prolactin signaling, as observed with the class, was not evaluated • Exposure associated with polyploidy at expected human therapeutic exposure unclear • Human cancer data described with tofacitinib considered not robust	DRA: negative Sponsor: negative	In this case, the 2-year rat study and transgenic mouse study are considered poor predictors for carcinogenic risk in humans due to immunosuppression Because tumors have been reported in patients treated with pharmaceutical class, the compound may exhibit tumorigenic effects in humans and could be labeled accordingly
143	1	1	Data not disclosed	N/A	DRA: positive Sponsor: positive	N/A

Conclusion

The ICH S1 PES was undertaken by the ICH S1B(R1) EWG to address the hypothesis that, for some pharmaceuticals, a WoE assessment may be sufficient to predict the outcome and value of the 2-year rat carcinogenicity study for assessing human carcinogenic risk in the absence of conducting a 2-year rat study. An additional objective of the PES was to assess the regulatory feasibility of a WoE approach by evaluating concordance among regulators from five ICH regions following independent assessment of CADs and FSR summaries, as submitted by the sponsors.

The outcome of the PES suggests that, for some investigational pharmaceuticals, a WoE approach can be used to determine if a 2-year rat study adds value to the human carcinogenic risk assessment, and the ICH S1B guideline can be expanded to include recommendations supporting a WoE approach. Based on the number of DRA-designated unanimous Category 3a and 3b cases, approximately 27% of 2-year rat studies could be omitted and a WoE approach could instead be relied upon to characterize human carcinogenic risk. The WoE attributes that define this subset of cases included target biology of the parent compound and major human metabolites that is well characterized and not associated with cellular pathways known to be involved with human cancer development, secondary pharmacology that does not identify concerns for off-target potential, chronic toxicity studies that indicate no hyperplastic, hypertrophic, atypical cellular alterations, or degenerative/regenerative changes without adequate explanation of pathogenesis or human relevance, no alterations of endocrine or reproductive organs that are not adequately explained in relation to potential human relevance, no evidence of genotoxic potential, and no evidence of immune modulation or immunotoxicity based on target biology and repeat-dose toxicology studies.

The numerous cases where the sponsor and the DRAs independently and unanimously arrived at the same CAD categorization illustrate that harmonized decisions on the necessity of a 2-year rat study are feasible. Nonetheless, conclusions can and are expected to differ on occasion given

TABLE 13 WoE attributes associated with DRA-designated unanimous Category 3a and 3b cases.

WoE factor	Attribute supportive of category 3a or 3b designation
Target Biology	Target biology is well characterized and not associated with cellular pathways known to be involved with human cancer development. Often, the pharmaceutical target was non- mammalian and carcinogenicity data were available with the pharmacologic drug class
Secondary pharmacology	No identified concerns from secondary pharmacology screens intended to inform off-target potential for the pharmaceutical
Histopathology data from chronic studies	Results from 6-month rat chronic toxicity studies indicate no hyperplasia, hypertrophy, atypical cellular alterations, or degenerative/regenerative changes without adequate explanation of pathogenesis or human relevance, indicative of no on- or off-target potential of carcinogenic concern
Hormonal effects	No perturbation of endocrine and reproductive organs observed, or endocrine findings adequately explained with respect to potential human relevance
Genotoxicity	The overall assessment of genotoxic potential is concluded to be negative
Immune modulation	No evidence of immune modulation or immunotoxicity based on target biology and repeat- dose toxicology studies in rats

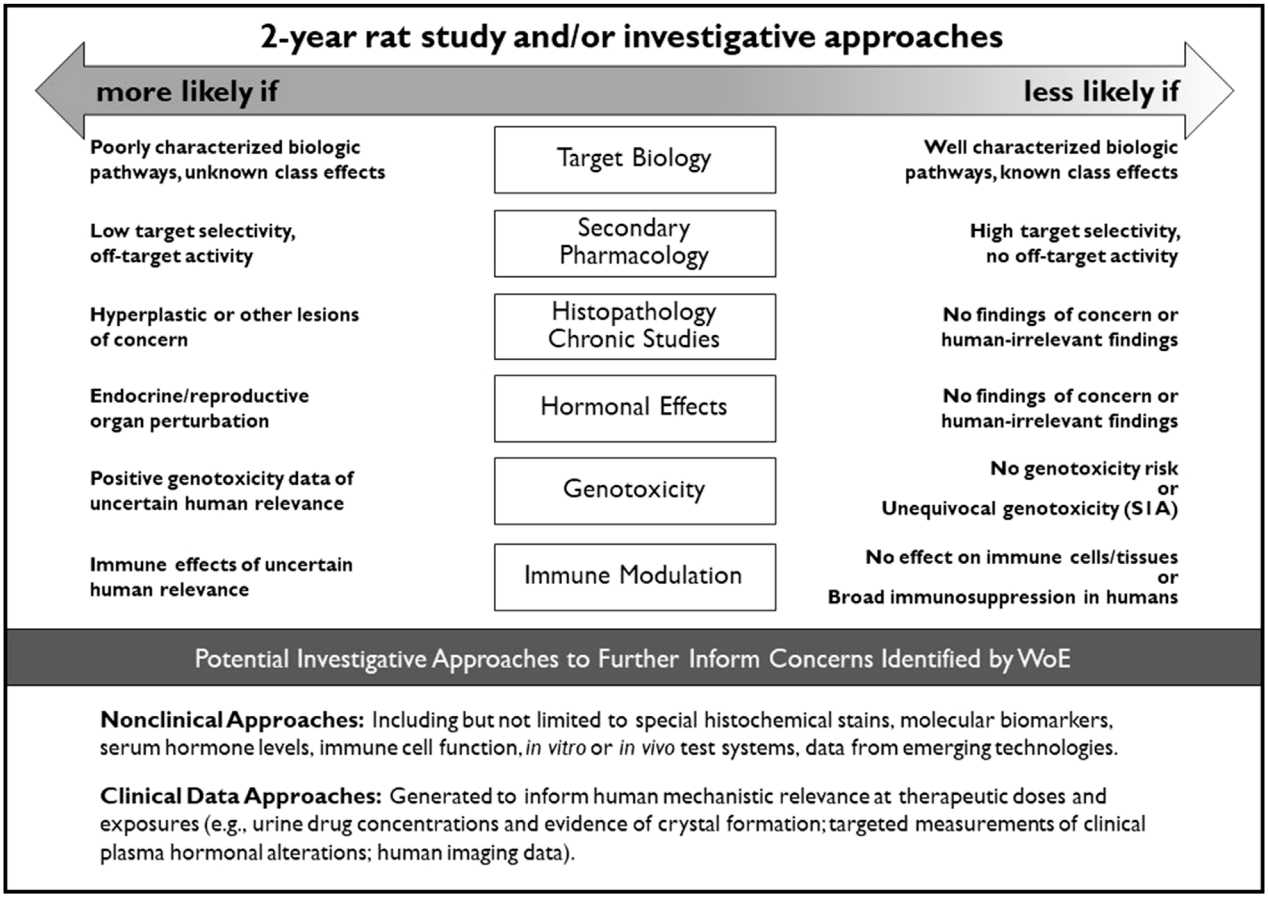

FIGURE 2
Integration of key WoE factors and potential investigative approaches to further inform on the value of conducting a 2-year rat study for assessment of human carcinogenic risk.

the complexity of integrating risk information from multiple WoE factors. As the ICH S1B(R1) Addendum is implemented across the ICH regions, it will be important to monitor how sponsors will apply the recommendations in the Addendum and track the extent of DRA alignment in their recommendations to the industry regarding the acceptance of a WoE approach *in lieu* of a 2-year rat study. Implementation of this integrative approach is anticipated to reduce the use of animals in accordance with the 3R (reduce/refine/replace) principles and ideally shift resources to focus on generating more scientific mechanism-based carcinogenicity assessments, while continuing to promote safe and ethical development of new pharmaceuticals.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the study is evaluating reports, no primary animal data.

Author contributions

TB: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. TM: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. TC: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. GE: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. AN: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. JN: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. KO: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. MP: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. AV: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing. JV: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ICH S1 prospective evaluation study and weight of evidence assessments: commentary from industry representatives

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Industry representatives on the ICH S1B(R1) Expert Working Group (EWG) worked closely with colleagues from the Drug Regulatory Authorities to develop an addendum to the ICH S1B guideline on carcinogenicity studies that allows for a weight-of-evidence (WoE) carcinogenicity assessment in some cases, rather than conducting a 2-year rat carcinogenicity study. A subgroup of the EWG composed of regulators have published in this issue a detailed analysis of the Prospective Evaluation Study (PES) conducted under the auspices of the ICH S1B(R1) EWG. Based on the experience gained through the Prospective Evaluation Study (PES) process, industry members of the EWG have prepared the following commentary to aid sponsors in assessing the standard WoE factors, considering how novel investigative approaches may be used to support a WoE assessment, and preparing appropriate documentation of the WoE assessment for presentation to regulatory authorities. The commentary also reviews some of the implementation challenges sponsors must consider in developing a carcinogenicity assessment strategy. Finally, case examples drawn from previously marketed products are provided as a supplement to this commentary to provide additional examples of how WoE criteria may be applied. The information and opinions expressed in this commentary are aimed at increasing the quality of WoE assessments to ensure the successful implementation of this approach.

KEYWORDS

carcinogenicity testing, rat carcinogenicity, rasH2-Tg mouse dose selection, regulatory toxicology, carcinogenicity weight-of-evidence criteria, best practice

1 Introduction

The paper of Bourcier et al. (2024) entitled "ICH S1 Prospective Evaluation Study: weight of evidence approach to predict outcome and value of 2-year rat carcinogenicity studies. A report from the Regulatory Authorities subgroup" that appears in this issue of *Frontiers in Toxicology*, provides a detailed analysis of the Prospective Evaluation Study (PES) conducted under the auspices of the ICH S1B(R1) Expert Working Group (EWG). As described in the paper of Bourcier et al. (2024), these data supported the first change in the

carcinogenicity assessment of small molecule pharmaceuticals since the introduction of alternative short-term mouse models in 1997. In fact, the ICH S1B(R1) Addendum (2022) could be viewed as the most notable change in carcinogenicity assessments for small molecule therapeutics since 2-year rat bioassays were first developed by the National Cancer Institute in the United States in the 1960s. The key element of the addendum is the provision of an option to conduct a weight-of-evidence (WoE) assessment of human carcinogenic risk in certain cases rather than conducting a standard 2-year rat study. While the addendum also describes a plasma exposure ratio-based approach for setting the high dose in the rasH2-Tg mouse model, the focus of this commentary is on the WoE option. For additional perspective related to dose selection for the rasH2-Tg mouse model refer to the analysis by Hisada et al. (2022).

This commentary was developed by industry members of the ICH S1B(R1) EWG and is meant to complement the information provided in the addendum to the ICH S1B(R1) guideline (2022) as well as the detailed review of the PES data by the regulatory authorities' subgroup (Bourcier et al., 2024). Toxicologists and pathologists from industry were instrumental in the origins of what evolved into the WoE option starting with the work of scientists from industry in 2010 (Reddy et al., 2010) and then a seminal paper from a collaboration of scientists from Pharmaceutical Research and Manufacturers of America (PhRMA) member companies (Sistare et al., 2011). In addition, experience using a WoE approach was accrued for biotechnology-derived pharmaceuticals following the ICH S6(R1) Addendum (2011) and this experience helped inform the ICH S1B(R1) Addendum (2022). For a review of the origins of the WoE approach, please refer to the paper of Bourcier et al. (2024).

This commentary has several objectives. First, the paper will summarize best practice principles for sponsors and regulators to consider in determining when a WoE approach is appropriate. The standard WoE factors to consider are reviewed and then the role of investigative and emerging technologies is discussed since during the PES, data gaps or the need for clarifying information emerged as a key factor of discordance either between assessments by sponsors and health authorities or among the health authority reviews. Second, the paper will provide suggestions for sponsors regarding the documentation and presentation of WoE assessments to Drug Regulatory Authorities (DRA). As expected, during the PES, variability in the quality and format of documentation was noted by regulatory members and the goal of this section is to improve the quality of WoE documents submitted to DRAs. Third, the paper will discuss challenges for sponsors in implementing a WoE approach, which are important as these logistical and procedural challenges could threaten full utilization of the new approach. Lastly, the paper will provide case examples to illustrate the principles described in the revised S1B(R1) guideline (2022). These case examples are meant to complement and extend the case studies provided in the appendix to the ICH S1B(R1) Addendum (2022). While the content of this paper was developed based on the industry EWG members' learnings and experience, to ensure industry input, this paper was reviewed by nonclinical groups within our constituent organizations (PhRMA, EFPIA, JPMA, BIO).

The ICH S1B(R1) EWG consisted of a highly engaged set of regulatory and industry members in an effort that spanned more than a decade. The authors of this paper appreciate the collaborative approach adopted by regulatory members of the EWG and acknowledge the sponsors who took the time and effort to contribute assessments and data during the PES without the possibility of direct benefit from the effort during the assessment period. We would like to thank the subset of regulatory members of EWG who analyzed and now published the key conclusions of the PES. While quite novel in the context of ICH work, the adjudication and analysis by this group of health authority scientists was essential to the success of the project.

The revision of ICH S1B (2022) to allow for a WoE option for carcinogenicity assessments in certain cases is a landmark change in carcinogenicity testing of small molecule pharmaceuticals; however, sponsors and regulatory assessors will need to effectively implement the guidance to maximize its full potential. It is important for sponsors to recognize the advantages of the WoE approach provides in assessing the safety of small molecule therapeutics. One advantage is moving from a "check the box" approach of conducting 2-year rat carcinogenicity studies to a more scientifically based approach that considers key pharmacologic and toxicologic properties for the compound. Another advantage is the opportunities for the expanded use of existing and emerging technologies to conduct more mechanism-based assessments related to assessing human carcinogenic risk. As outlined in the addendum, sponsors should rigorously assess the six primary WoE factors for all programs, not just those they consider suitable candidates for a WoE assessment. The rationale for doing the WoE assessment, even in cases where it does not result in elimination of the study, is to allow sponsors to probe potential gaps in knowledge or understand the molecules' risks prior to testing. Importantly, in those cases in which the sponsor determines a 2-year rat study is warranted, they do not need to seek input from health authorities. In those cases, where a WoE determines a 2-year rat study is not warranted, we anticipate that this will avoid some of the inherent challenges of 2-year rat carcinogenicity studies such as equivocal outcomes or a positive finding which is later shown to lack human relevance. Finally, the WoE approach provides for a substantial reduction in animal use, as the standard 2-year rat carcinogenicity studies require between 500–700 rats.

It can be expected that in the early years of implementation, both industry and regulatory scientists may be cautious in adopting a WoE approach; however, we would anticipate that as sponsors and regulators gain more experience there will be increased opportunities to utilize a WoE assessment. Our hope is that the publication of Bourcier et al. (2024) which provides details from the data gathered during the PES, as well as this commentary sharing key learnings from industry participants in the ICH process, will increase the scientific rigor and effectiveness of future WoE assessments.

2 WoE factors

a) Like other ICH guidelines, the ICH S1B(R1) Addendum is not highly detailed in terms of the data or analysis the sponsor is expected to generate for each of the WoE factors. This is due to the fact that development programs will vary significantly

TABLE 1 Examples of Information sources[a] used in assessing target-related carcinogenic risk of small molecules.

Category	Database examples	Characteristics of data source
General characteristics of target protein and related gene	www.ncbi.nlm.nih.gov/gene	Public database of National Center for Biotechnology Information
Gene function	www.geneontology.org	Public database of GO consortium
Target distribution (rat, human)	https://www.proteinatlas.org/	Public database of the Swedish Human protein atlas (Uhlén et al., 2015)
	https://gtexportal.org/home/Genotype-Tissue Expression (GTEx)	Public database of the Broad institute
	www.biogps.org	Public database of The Scripps Research Institute
	https://www.frontiersin.org/articles/10.3389/fgene.2022.1078050/full	Gene expression tissue atlas published by Abbvie scientists of nonclinical tox species - rat, mouse, dog, NHP
Signal transduction/pharmacologic pathway downstream cascade	www.reactome.org	Public database of the Reactome Team (Ontario Institute for Cancer Research, European Bioinformatics Institute, New York University Medical Center)
	Ingenuity	Commercial database of Qiagen
Genetically engineered models	www.informatics.jax.org	Public database of The Jackson Laboratory/Mouse Genome Informatics (MGI)
Human genetic association studies	https://omim.org/	Public database of the Johns Hopkins University
	https://www.disgenet.org/	Public database of the Integrative Biomedical Informatics Group
	Open Targets https://genetics.opentargets.org/	Compendium of GWAS and WES rare variant associations
Cancer gene databases	https://portal.gdc.cancer.gov/	Public database of the Cancer Genome Atlas Program (TCGA) by the National Cancer Institute
	https://cancer.sanger.ac.uk/cosmic	Catalogue of Somatic Mutations in Cancer (COSMIC) by the Wellcome Sanger Institute
	https://www.intogen.org/search	Cancer Driver Genes Mutation Browser by IntOGen
Drug Approval Information	https://www.elsevier.com/products/pharmapendium	Pharmapendium provides publicly available information on marketed pharmaceutical

[a]See also (Carss et al., 2023) "Using human genetics to improve safety assessment of therapeutics," Nat Rev Drug Discov 22:145–162.

based on the nature of the target or findings from the toxicology studies. In addition to program-specific considerations, sponsors vary in their strategy of certain aspects such as the scope of the secondary pharmacology screening data. For each of the factors listed, sponsors need to ensure they have generated a robust data set that allows them to decide on their carcinogenicity strategy and also provide regulatory scientists the data necessary to assess if a WoE approach is appropriate.

a) Target Biology

The pharmacologic activity and potency of the parent compound and major circulating metabolites should be considered for humans and in the animal species used for chronic toxicity testing. For further information on the definition of major metabolites refer to the ICH M3(R2) Guideline (2009) and its corresponding Question and Answer Document. This is frequently done by *in vitro* binding and activity assays using a recombinant cell line stably expressing the target and using inhibitory concentration (IC) or effective concentration (EC) values as an endpoint. The *in vivo* to *in vitro* ratio of these readouts serve to guide exposure targets and understanding concentrations necessary to reach full pharmacological efficacy. An additional target engagement parameter may substantiate the relevance of the *in vivo* model.

Drug target tissue distribution and pharmacological signaling cascades should be carefully assessed in rats and humans with a focus on actions relevant to carcinogenicity. This is generally done by use of open access and proprietary databases with example sources noted in Table 1, and complemented by review of relevant primary literature, which may also be referenced in databases. The general process of target safety assessment has been comprehensively described (Brennan, 2017). In addition, van der Laan et al. (2016) presented the outcome of their analysis of 298 pharmacological compounds with respect to their carcinogenic response per pharmacological class. This represents a valuable source of target-related carcinogenic risk for those established classes (van der Laan et al., 2016). Understanding interspecies differences in target distribution and pharmacologic pathways are of major relevance with respect to rat-to-human translatability. It is also important to review either publicly available or internal data on 2-year rat studies or other rodent carcinogenicity assessments conducted with other compounds in the class or compounds that have similar pharmacological properties. If such class-related carcinogenicity data are available, sponsors must consider how similar those compounds are to the molecule being

developed in terms of potency, selectivity, pharmacokinetics, and toxicity profile.

Important information sources also include phenotypic characterizations of genetically engineered animal models, human genetic association studies and cancer gene databases. Genetic variants that increase or decrease protein expression or function can inform on potential liabilities of agonistic or antagonistic target engagement, respectively. The relevance of any genetic association to pharmacological perturbation must consider multiple factors such as functional directionality, causality, penetrance, magnitude of effect, tissue distribution, etc. For a review including a listing of human genomics resources supporting human safety assessment see the manuscript by Carss et al. (2023).

Literature with relevance to target-related carcinogenic risk should be comprehensively searched in an unbiased manner, and documented as it serves as a key scientific building block to a thorough WoE assessment. Contradictory data should be mentioned with relevant context provided, as appropriate. Not all information on target biology has the same relevance for carcinogenic risk assessment, and it is important to provide an integrated analysis based on the totality of the data. Data from genetically modified animals, e.g., strains with a deleted or over-expressed target are generally considered to be of higher value than data generated *in vitro*, e.g., proliferation in a cell-based model. It is important to appreciate that homozygous gene deletion models may result in a phenotype that is more extreme and perhaps less relevant than that which would occur through pharmacologic modulation of a pathway that only partially abrogates signaling. Additionally, while cancer gene databases leverage sophisticated statistical algorithms to distinguish causal gene mutations, so called "driver" gene mutations, from "passenger" gene mutations, thresholds will vary, and inconsistencies are seen among databases. As in all scientific assessments, it is important to assess the overall quality of the publications with respect to the rigor of the model, group size, and methods of analysis with greater emphasis placed on those observations that are reproducible.

b) Secondary Pharmacology

Activity of a drug candidate and major metabolites at a pharmacological target other than the intended one, referred to as secondary pharmacology, has the potential to result in an increased carcinogenic risk. Such properties are assessed, in part, by secondary pharmacology screens, which are an integral part of drug candidate profiling (Jenkinson et al., 2020; Scott et al., 2022). However, standardization and best practices for screening methodology and targets is lacking. It is common practice to start by profiling drug candidates in an off-target *in vitro* panel in the lead optimization phase. Such early panels usually employ a limited number of targets and focus on functional effects and target organ toxicity. A commonly used panel is the one described by Bowes et al. (2012) that comprises 24 G protein-coupled receptors, 8 ion channels, 7 enzymes and 3 transporters but only 2 nuclear receptors and no kinases. Also, a recent compilation of potential adverse effects related to agonistic or antagonistic effects to 70 pharmacological targets (Lynch et al., 2017) is of limited value with respect to carcinogenic risk assessment as it focuses on common targets in pharmaceutical research and development.

Second tier screenings, conducted in a later phase of development, often as a part of the data to support Phase I clinical studies, may be more comprehensive and include targets with known carcinogenic risk, in particular kinases and nuclear hormone receptors. Examples may include the estrogen receptor (Duijndam et al., 2021), Glycogen Synthase Kinase 3 beta receptor (Heinemann et al., 2022) or the aryl hydrocarbon receptor (Podtelezhnikov et al., 2020). Under the auspices of the DruSafe leadership group of the Innovation and Quality (IQ) consortium there is ongoing work to comprehensively review current practices. Additionally, once major circulating metabolites are identified in the human ADME study, these metabolites should also be evaluated in secondary pharmacology screens.

As described above, secondary pharmacology screening strategies vary between sponsors. In fact, insufficient information on target selectivity arose as a deficiency in WoE assessment in several of the cases in the PES. As such it is important for sponsors in their WoE documentation to precisely describe the secondary pharmacology panels that were assessed and how those findings relate to carcinogenic risk. An emerging area is the inclusion of assays in off-target screening panels that specifically address the needs for a carcinogenic risk assessment. This is an area that will require additional investigation and input from the broader scientific community.

c) Histopathology

The guideline specifically emphasizes the importance of the 6-month chronic toxicity study in rats since data derived from these studies was foundational for the WoE concept. While the primary focus is on histopathology findings in rat chronic toxicity studies, results from repeat-dose toxicity studies in other species may also be helpful to assess the human relevance of a finding present in rats. For example, a finding that occurs in both rats and a nonrodent species is more likely to be of human relevance than a finding that only occurs in rats. Conversely, a finding of concern that occurred in the nonrodent only may warrant additional characterization but does not necessarily increase the need for a 2-year rat study, particularly if there are data such as species differences in potency or receptor distribution which indicate the rat is insensitive to the effect.

The ICH S1B(R1) Addendum (2022) specifies histopathology observations that are most often a risk factor including cellular hypertrophy, cellular hyperplasia, persistent tissue injury, chronic inflammation, foci of cellular alteration, preneoplastic changes and tumors. Each of these findings should be carefully considered, including their nature and magnitude. It is also important to note that some histologic findings may not have been considered adverse in the context of the repeat-dose toxicity study, but still need to be carefully considered in the WoE assessment. A low number of tumors are occasionally seen in 6-month rat studies and in many instances are spontaneous and unrelated to the test article (Son et al., 2010; Blankenship and Skaggs, 2013). For those instances where the occurrence of a tumor in a test-item treated group was considered spontaneous and this conclusion was well supported by historical control data, the data should be clearly described in the WoE documentation; however, these spontaneous tumors should not increase the need for a 2-year rat study. There are some cases in which the incidence of tumors in the 6-month study was considered

of equivocal relationship to treatment. In these cases, such tumors must be considered in the overall WoE assessment, in particular if they can plausibly be related to other test-item related pathology findings in that tissue. To better understand the potential for proliferative findings to be preneoplastic, it is recommended to refer to standard texts of toxicologic pathology, in particular the standardized toxicologic pathology nomenclature documents and publications (goRENI)[1].

The histopathologic risk factors should be considered in conjunction with any associated organ weight change. Organ weights can be a surrogate marker for hypertrophy or hyperplasia and cell proliferation if the cell compartment affected represents a major constituent of the respective organ, like hepatocytes in the liver, and there is no viable alternative explanation. On the other hand, a constant organ weight may not exclude increased proliferation: an increased cell loss by apoptosis may be counterbalanced by increased proliferation. For additional guidance on approaches to organ weight collection and interpretation refer to reviews and best practice recommendations by the Society of Toxicologic Pathology (Michael et al., 2007; Sellers et al., 2007).

For each of the histopathologic risk factors there may be cases where further investigation is warranted to assess biologic significance. For example, in some cases it may be warranted to quantitate cell proliferation or other associated parameters to aid in further understanding the nature of the finding. For further consideration on the utility of cell proliferation assessment in the context of the WoE assessment see Section 3.3.

d) Hormonal effects

Hormonal perturbation is known to represent a risk factor for nongenotoxic carcinogenesis in rodents and humans. The predominant mechanism of hormonal carcinogenesis is a sustained increase in cell proliferation induced by trophic hormones (Silva Lima and Van der Laan, 2000). The ICH S1B(R1) Addendum is comprehensive with respect to the parameters that may indicate a hormonal effect and it is important to highlight that the addendum is not suggesting that hormone levels be determined in the repeat-dose toxicity studies in rats. Changes in hormone levels are often difficult to assess in routine studies due to the interindividual variability, circadian rhythms, and analytical challenges (Stanislaus et al., 2012). Thus, even if hormone levels were evaluated in a study it may result in either a "false negative" or "false positive" with respect to an effect on circulating hormones. For evaluating potential hormonal effects, histopathology (hypertrophy and hyperplasia) and organ weights can be more robust endpoints. In cases of diffuse hypertrophy or hyperplasia, organ weight may be a more sensitive endpoint than histopathology, especially in cases of accessory male sex glands, adrenal, or pituitary. It is acknowledged that in the context of a 6-month rat study where reproductive senescence is occurring in some strains, it is important not to overinterpret a reproductive organ weight change that is not supported by corroborative findings. Sponsors should ensure that hormone-responsive organs are carefully collected and trimmed in chronic toxicity studies to minimize variability in organ weights due to tissue processing, especially if an effect on a hormonal axis is suspected.

Hormonal perturbation can be primary (e.g., direct interaction of the drug with a hormone receptor) or secondary (e.g., increased degradation of a hormone). With respect to secondary hormonal changes the addendum specifies that hormonal changes secondary to processes like stress or altered body weight are unlikely to be relevant to human risk assessment. In addition, in those cases where there has been sufficient mechanistic data that the hormonal effects in rats are a rodent-specific effect, a 2-year rat study would not be warranted based on this alone. Therefore, it is important that sponsors provide sufficient explanations of potential hormonal effects such as organ weight changes of endocrine or reproductive tissues to delineate primary vs. secondary effects. In some cases, this may warrant follow-up investigative studies that may include determination of circulating hormones on a case-by-case basis.

e) Genotoxicity

An absence of genotoxicity in a battery of tests conducted in accordance with ICH S2(R1) (2011) is an important component of a WoE assessment in concluding that a 2-year rat study is not warranted. ICH S2(R1) (2011) gives guidance on how to interpret positive or equivocal genotoxicity results from the standard test battery and suggests follow-up tests to de-risk these findings, including human relevance of the mode of action and the concentration threshold. Equivocal or positive data may require the identification of the mode of action of genotoxicity to identify if a molecule has intrinsic genotoxicity or not. For those programs where mechanistic approaches have not resolved uncertainty with respect to genotoxic potential, a 2-year rat carcinogenicity study would be warranted.

f) Immune Modulation

The addendum specifies that immune modulation, as characterized by the principles in ICH S8 (2005) on Immunotoxicity Studies, is an important WoE factor. While ICH S8 (2005) does not use the term immune modulation, it defines immunotoxicity in scope of the guideline as unintended immunosuppression or enhancement. Immunosuppression, however, is known to be associated with an increased tumor risk in animals and humans often due to reduced immune surveillance of tumorigenic viruses. Of particular note are B-cell lymphoma, squamous cell carcinoma and Kaposi sarcoma (Vial and Descotes, 2003). Building on the review of Bugelski et al. (2010), a workshop on cancer risk assessment of immunomodulators concluded that rodent carcinogenicity studies are generally not reliable predictors of human cancer risk associated with immunosuppression (Lebrec et al., 2016). Consequently, the ICH S1B(R1) Addendum (2022) states that human cancer risk assessment of a nonselective or particularly potent immunosuppressant will not be further informed by standard rat and mouse carcinogenicity studies. Examples of these types of agents include cyclosporine or tacrolimus. In such cases product labelling and post marketing surveillance will need to address the potential for increased risk for certain cancers wherein approval is otherwise warranted.

1 OECD https://www.oecd.org/chemicals

For programs where the pharmacologic intent is selective modulation of the immune system or there is an off-target effect that modulates some specific component of the immune system, sponsors should carefully assess the role the pathway plays in tumor immune surveillance to assess the potential risk along the principles outlined in the workshop report of Lebrec et al. (2016). The gradation of risk based on the specific immune pathway impacted can be illustrated in the product labelling of biotherapeutics that intend to modulate the immune system. Therapeutics that inhibit TNF (e.g., HUMIRA®) carry bolded warnings for the risk of lymphoma and other malignancies based on human data (Food and Drug Administration US, 2002), while STELARA® that binds to the p40 subunit of IL-12 and IL-23 carries a warning of potential risk of malignancy (Food and Drug Administration US, 2009), and COSENTYX® which inhibits IL-17 does not carry a warning of increased malignancy risk (Food and Drug Administration US, 2015). Despite efforts by various laboratories over the years, there are no reliable broad screening models, either *in vitro* or *in vivo*, to reliably assess malignancy risk secondary to immune modulation. As such, the sponsor should assess if there may be more targeted, hypothesis-driven experiments that would inform risk related to the pathway that is being modulated. If there are no targeted experiments that might further inform risk the sponsor should provide an integrated analysis in their WoE documents and consider what types of product labelling and post marketing surveillance might be warranted. It is the view of the industry EWG members that in those cases where the only potential risk factor is immune modulation, a 2-year rat study is generally not warranted as it neither effectively identifies nor refutes a risk.

An additional challenge is for compounds that do not intend to modulate the immune system but have clear effects on one or few associated parameters of relevance. Such first evidence for immune modulation is often derived from repeat-dose toxicity studies and may include effects on white blood cell parameters, effects on immune globulins, changes in lymphoreticular organ weight, histopathology findings in lymphoreticular/hematopoietic organs, increased incidences of infections or increased occurrence of tumors in the absence of other plausible causes as summarized in ICH S8 (2005). Accumulation of a compound in lymphoreticular organs, derived from whole body autoradiography or histopathology/mass spectrometry, should also be considered when assessing the potential to impact the immune system. In these cases, the sponsor should consider if there are investigative approaches that could inform either human relevance or potential impact on immune surveillance. As discussed above, if the only potential risk factor identified are effects on the lymphoid system, a 2-year rat study would not be informative and thus, not warranted.

3 WoE factors–role of investigative studies and emerging technology

3.1 Nonclinical data to establish a strategy for assessment of human relevance

In addition to the six primary WoE factors discussed above, ICH S1B(R1) Addendum (2022) mentions non-standard end points or techniques that may further inform human carcinogenic risk assessment on an as needed basis. These investigations may be particularly valuable when there are findings of carcinogenic concerns in the *in vivo* studies. Such end points or techniques may be applied in additional investigative studies or to specimens collected from prior studies. Techniques that may be used more frequently will include special histochemical stains, immunohistochemistry, quantification of cell proliferation, molecular pathology, additional immunotoxicity studies according to ICH S8, and the various "Omics" technologies, among others. Since unexpected findings arise during the conduct of the repeat-dose toxicity studies, sponsors may choose to prospectively bank a subset of tissues, serum, or plasma in an appropriate manner from all of their repeat-dose toxicity studies to enable potential retrospective investigations. A drug-related finding should be characterized with appropriate additional techniques applied on samples from standard toxicity studies as early as possible in development. This will enable the inclusion of suitable non-standard end points in follow-up studies and help to reduce the need for stand-alone investigative studies.

The collaborative industry data mining publication by (Sistare et al., 2011) supporting the genesis of the ICH S1 revision proposal revealed that among all rat organs, the liver was the most common organ to have histopathologic risk factors of carcinogenicity in chronic toxicity studies. Furthermore, liver findings at 6 months were closely associated not only with eventual liver tumors but also with thyroid or testicular Leydig cell tumors. Biological explanations for the causal connections between a histopathologic risk factor in one tissue with tumors seen at alternate tissue sites have emerged over decades of rodent carcinogenicity testing. Efforts have been made to systemically catalogue these findings and this can be very useful in the early stage of a WoE evaluation for carcinogenesis assessment strategies.

During ICH S1 deliberations the EWG reviewed and acknowledged the value of such historical work underlying an expansive set of such multistep rat specific tumorigenic mechanisms collected over years of mining publicly available regulatory submission documents and published manuscripts by JPMA investigators and shared by JPMA representatives with the S1 EWG. The JPMA catalogued patterns of histopathologic risk factors of rat carcinogenicity observed among similar members of numerous pharmacologic classes and explained through investigative efforts to link chronic rat study findings to tumor types in a variety of endocrine and non-endocrine organs. This JPMA data survey has been presented publicly. This summary of historical perspective has been catalogued by JPMA using publicly available regulatory submissions on investigative successes applied in drug development to provide understanding of mechanisms, reduce human safety concerns, and support marketing authorization. Patterns of risk factors and associated tumors seen among 16 organs across common members of 28 classes of pharmacologic drug action are provided in Table 2.

Under the Organisation for Economic Co-operation and Development (OECD) Adverse Outcome Pathways (AOP) Programme[1], the OECD is systematically constructing a public knowledge base by collecting AOPs on the development of human and environmental hazards on its website, the AOP Wiki[2], with the goal of developing a defined Integrated Approach to Testing and Assessment (IATA) for use in regulation, that is

2 AOP WIKI https://aopwiki.org/

TABLE 2 A summary historical perspective catalogued by JPMA from publicly available regulatory submissions on investigative successes applied in drug development to provide understanding of mechanisms of carcinogenesis, histopathologic risk factors (HPRF), and associated tumors seen among 16 organs across common members of 28 classes of pharmacologic drug action. This analysis, broken down into endocrine A) and non-endocrine B) mechanisms, was prepared as a resource for investigating tumorigenic mechanism when a positive result is obtained in a rat carcinogenicity study and/or for launching early investigations from patterns of HPRF in chronic studies and other available sources of pharmacologic and toxicologic information.

A. Endocrine tumors				
Drug–induced tumors	Drug class	MOA	HPRF	References for MOA
Pancreatic islet cell tumor	Serotonin-dopamine antagonists	Increased prolactin level	β cell hypertrophy/ hyperplasia	Mortensen (1989), Brelje et al. (1994)
Thyroid follicular cell tumor	Hepatic enzyme inducers, Antithyroid, Iodide-containing agents	Increased TSH level	Thyroid follicular cell hypertrophy/ hyperplasia	Hill et al. (1989), Thomas and Williams (1991), Hill et al., 1998; Hurley (1998)
Thyroid C cell tumor	GLP-1 agonists	Direct agonistic effects	Diffuse/focal thyroid C-cell hyperplasia	Bjerre Knudsen et al. (2010), Parks and Rosebraugh (2010), Hegedüs et al. (2011), Gier et al. (2012), Madsen et al. (2012)
Adrenal pheochromocytoma	Ca channel antagonists, Polyols, PDE3 inhibitors, Vitamin D3, Retinoids, SGLT2 inhibitors, a-glucosidase inhibitors	Sympathetic stimulation	Diffuse/nodular hyperplasia of adrenal medullary cells	Lynch et al. (1996), Tischler (1999), Greim et al. (2009)
Leydig cell tumor	Anti-androgens, 5a-reductase inhibitors, testosterone synthesis inhibitors, aromatase inhibitors, D2 agonists, PPARα agonists, polyols, a-glucosidase inhibitors, SGLT2 inhibitors, LH-RH agonists	Increased LH level	Leydig cell hyperplasia	Prentice et al. (1992), Clegg et al. (1997), Cook et al. (1999)
Mammary tumor	D2 antagonists, SDA, Estrogens, synthetic estrogens, progestogens	Increased prolactin level	Mammary gland hyperplasia (lobular, ductal)	Blum et al. (1987), Alison et al. (1994), Harvey, 2012; Vyas (2012)
Anterior Pituitary tumor	LH-RH agonists, D2 antagonists	Unknown, antagonism of inhibitory effects on proliferation	Hypertrophy/ hyperplasia, anterior pituitary	Donaubauer et al. (1987), Saiardi et al. (1997), Heaney et al. (2002), Iaccarino et al. (2002), Hnasko et al. (2007), Greaves (2012a)
Endometrial tumor	Dopamine agonists	High estrogen/progesterone	Endometrial hyperplasia	Griffith (1977), Ben-Jonathan et al. (2008), Hargreaves and Harleman (2011), Greaves (2012b)
B. Non-endocrine tumors				
Drug –induced tumors	Drug class	MOA	HPRF	References for MOA
Hepatocellular tumor	Hepatic enzyme inducers, PPARα agonists, Synthetic estrogens	Activate target molecules/ receptors: CAR, PPARα	Clonal expansion of preneoplastic foci	Kawamoto et al. (1999), Yamamoto et al. (2004), Holsapple et al. (2005), Corton et al. (2014)
Pancreatic acinar cell tumor	PPARα agonists, Trypsin inhibitors	Increased CCK levels	Pancreatic acinar cell proliferation	Douglas et al. (1989), Bourassa et al. (1999), Moore et al. (2001), Pandiri (2014)
Renal tubular tumor	α-glucosidase inhibitors, SGLT2 inhibitors, SERM	Ca imbalance by carbohydrate malabsorption	Regenerative hyperplasia of tubular cells	Hard (1998)
Hemangiosarcoma, subcutaneous sarcoma	PPARγ agonists, PPARα/γ agonists	Accelerate cell proliferation, unknown detailed mechanism	Increased epithelial proliferation in mice	Hardisty et al. (2007), Cohen et al. (2009), Criswell et al. (2012)
Urinary bladder tumor	PPARγ agonists, PPARα/γ agonists	Prolithogenic mechanism	Regenerative hyperplasia of bladder epithelium	Burin et al. (1995), Cohen and Lawson (1995), Cohen (1998), Hardisty et al. (2008)
Gastric carcinoid	Anti-secretory agents, e.g., H2-blockers and PPIs, PPAR α agonists	Hypergastrinemia	Gastric ECL cell hypertrophy/ hyperplasia	Håkanson and Sundler (1990), Robinson (1999), Lamberts et al. (2001)
Hibernoma	Nicotine receptor agonist, α receptor blocker, opioid agonist, JAK inhibitor	Sustained sympathetic stimulation	Brown adipocyte hyperplasia	Cannon and Nedergaard (2004), Sell et al. (2004), Radi et al. (2013)
Mesovarian leiomyoma	β2-agonists	Direct agonistic effects	Mesovarian smooth muscle cell hyperplasia	Poynter et al. (1978), Gopinath and Gibson (1987), Kelly et al. (1993)

HPRF, histopathologic risk factor; LH-RH, Luteinizing Hormone-Releasing Hormone; MOA, mode of action; PDE, phosphodiesterase; SDA, serotonin dopamine antagonist; SGLT2, sodium glucose co-transporter 2.

CAR, constitutive androstane receptor; CCK, cholecystokinin; HPRF, histopathologic risk factor; JAK, janus kinase; MOA, mode of action; PPAR, Peroxisome proliferator-activated receptor; PPI, proton pump inhibitor; SERM, selective estrogen receptor modulator; SGLT2, sodium glucose co-transporter 2.

becoming a similarly valuable resource for sponsors. The AOP consists of a molecular initiating event (MIE), an adverse outcome (AO), and multiple key events (KEs) in a pathway from the MIE to the AO. Measurements for each KE are described in the KE sections and the scientific and quantitative plausibility of the relationship between KEs are described in the sections of Key Event Relationships (KERs). As of April 2023, there are 23 AOPs under development on human carcinogenesis and 25 AOPs on multi-step mechanisms of rodent-specific carcinogenesis summarizing and documenting research conducted over decades.

The JPMA data survey and the AOP WIKI are extremely valuable resources to sponsors who could access such prior precedent in seeking to meet the expectation of satisfactorily addressing tumorigenic risk potential of 6-month rat study findings in accordance with the S1B(R1) Addendum (2022). Nonclinical investigative methods applied to development programs that are based on historical documentation can be useful for guiding construction of explanations for those commonly observed patterns of histopathologic risk factors associated with frequently encountered on- or off-target mechanisms involving excessive and sustained pharmacology or toxicology. It is important to remember that these historical compendia began as individual customized project-specific approaches involving unique and creative nonclinical investigative strategies. While learnings from these historical examples can be informative, it is essential to have appropriate bridging study data to support any claims of applicability to new compounds in development.

3.2 Clinical data to assess human relevance

In a similar manner it may be informative to include translational biomarkers that can inform critical aspects of tumorigenic mechanism, or specific organ safety biomarkers in clinical studies to help obtain information on the human relevance of toxicities identified in rats, when available. As pointed out in ICH S1B(R1) (2022), such human clinical trial or epidemiologic data can also be useful by providing critical human perspective to novel mechanisms underlying potential risks raised by the WoE criteria or to address findings that cannot be readily accounted for by prior established mechanisms. These may often, but not always, involve engagement of intended on-target or closely related pharmacologic targets. The initial observation of osteosarcomas in rats seen with FORTEO® first approved in the US in 2002 for treatment of osteoporosis and limited initially to use in post-menopausal women deemed at high risk for fracture, and for a limited duration of 18 months treatment, represents such an example of integrated nonclinical and clinical investigation summarized by Miller et al. (2021). In 1998 findings of osteosarcoma in the rat carcinogenicity study triggered a halt to ongoing clinical trials, and the sponsor Eli Lilly and Co., conducted long term studies in monkeys demonstrating the osteosarcoma risk to be mechanistically unique to rodents whose skeletal growth continues through life, while growth plates of primates will close (Vahle et al., 2002). While such data allowed for initial limited marketing approval, subsequent epidemiologic studies provided further confirmation of the lack of osteosarcoma risk to humans leading in 2020 to an improved benefit-risk appreciation with extension of labeled dosing duration, expansion of the indicated population, and relaxation of the carcinogenic label warnings (Krege et al., 2022).

An additional example of the need for pivotal clinical data to support marketing approval and regulatory decision making is omeprazole and other proton pump inhibitors that induce neoplasia of enterochromaffin-like cells in rats (Ekman et al., 1985; Olbe et al., 2003). These molecules indirectly lead to increased gastrin levels that, in the rat, cause hyperplasia and neoplasia of gastric enterochromaffin cells. Similar findings using clinical gastrin monitoring and endoscopic imaging are not seen in humans receiving chronic therapy with proton pump inhibitors (Massoomi et al., 1993).

It is interesting to note how the passage of time enabled accrual of pivotal clinical data allowing, in the case of Forteo, for relief of restrictive labeling, expansion of the patient population, and relaxation of rodent carcinogenicity study label warnings. And in the case of proton pump inhibitors this therapeutic class started with black box warnings for rat tumors and was eventually judged to be sufficiently safe to allow purchase without a prescription as an OTC product. The challenge for industry scientists is to be mechanistically proactive and to apply existing and emerging tools that help to resolve questions of carcinogenicity risk.

3.3 Quantification of cell proliferation

As earlier described there may be occasions when targeted investigations of cell proliferation should be considered. Increases in cell proliferation are caused either by a direct stimulus via hormonal or nuclear receptors or indirectly as a regenerative response to cell death. An increase of cell proliferation represents a key event in basically every nongenotoxic carcinogenic MoA or AOP; however, an increase in cell proliferation at a single time point does not always result in an increased tumor risk. Since tumors can originate from increased cell proliferation leading to incorporation of mutations providing cellular growth advantage, establishing the threshold dose for cell proliferation can provide a rationale for the dose-related prediction of a nongenotoxic based tumor outcome (Cohen and Ellwein, 1990) and an evidence-based assessment of the clinical relevance of increases in cell proliferation. The assessment of cell proliferation traditionally requires a dedicated study or at least dedicated investigations. Such investigations will not be conducted routinely but rather for a specific purpose, usually based on certain histopathology findings, organ weight changes, or from theoretical considerations. An increase in cell proliferation can only roughly be assessed morphologically by routine semiquantitative histopathology because of the short duration of mitosis in the cell cycle and the rarity of mitotic figures in histological slides. Regenerative cell proliferation may be indirectly assessed by evidence of sustained cell damage like single cell necrosis and associated inflammatory reactions and the morphologic appearance of some cell types (epithelial basophilia). At lower levels of injury, however, cell loss may be limited to apoptosis, which is much more difficult to assess by routine histopathology.

This indicates that assessment of cell proliferation may represent an important follow-up activity for findings in repeat-dose toxicity

testing (Wood et al., 2015). Proliferation kinetics differ based on the underlying mode of action, tissue and chemical, and need to be taken into consideration when planning for their assessment (Wood et al., 2015). Cell proliferation can be assessed by a variety of methods, and it is likely advances in digital imaging and analysis may lead to improved, more efficient methods in the future. Examples of methods currently available for use include immunohistochemistry for Ki-67 on archival sample, artificial intelligence-assisted counting of mitotic figures (Heinemann et al., 2022) or in the context of a prospective investigative studies BrdU-labelling (Nolte et al., 2005; Wood et al., 2015). Once experimental variables are optimized and sufficient data are gathered to understand normal variability in the model, cellular proliferation can be a valuable early endpoint for exploring and establishing mechanistic understanding of tumor pathogenesis. Given the criticality of experimental timing to capture a significant proliferative signal, its routine use for establishing negative predictivity can be a challenge.

3.4 Emerging role for genomic and genetic approaches

The emergence of genomic and genetic tools for predicting carcinogenicity are additional factors that can be considered when generating a WoE approach for carcinogenic risk assessment. These approaches also raise interesting questions. For example, what is necessary and sufficient to associate a well-documented mode of action with a prior established AOP to readily explain findings of concern identified in a chronic rat study as being either human relevant or irrelevant? Can genomic signatures be qualified for such an application? A collaborative approach (Corton et al., 2022) has been launched within the Health and Environmental Sciences Institute (HESI) as a direct response to begin leveraging such opportunities created by ICH S1B(R1) (2022). The initial aspect of this collaboration seeks to develop and qualify biomarker gene expression signature panels focused initially on rat liver that measure widely accepted molecular pathways linked to commonly observed tumorigenic mechanisms. Growing evidence suggests that application of such biomarker panels in short-term exposure rodent studies can readily identify both tumorigenic hazard and tumorigenic activation levels for certain chemical-induced carcinogenicity mechanisms. Success from these efforts focusing initially on rat liver is expected to help facilitate the transition from the currently heavy reliance on conventional 2-year rodent carcinogenicity studies to more rapid animal- and resource-sparing and earlier approaches for mechanism-based carcinogenicity evaluation supporting internal and regulatory decision-making.

An additional component of the HESI collaboration seeks to apply error-corrected sequencing (ECS) to identify early clonal expansion of growth advantaged cells harboring cancer driver gene mutations. While good progress has been made in demonstrating the value of ECS for identifying and examining mutations in key cancer driver gene mutation hotspots as biomarkers of *in vivo* genotoxic risk (Parsons, 2018; Merrick, 2019; Valentine et al., 2020), utility for nongenotoxic chemical tumor risk is only beginning to be explored and will require thorough validation and qualification for both sensitivity and specificity before being broadly adopted in nonclinical safety assessment. In the future, approaches such as ECS may be particularly useful for programs with novel pharmacologic targets (i.e., first-in-class molecules) by providing additional assurance that there are no molecular patterns indicative of clonal expansion in key target tissues.

3.5 Consideration of *in silico* approaches in the weight-of-evidence

Computational approaches for identifying structural alerts underlying genetic toxicology and carcinogenic risk (Smith et al., 2016) have proven to be very valuable. Early on, just prior to the initiation of the ICH S1B revision process, a proposal from FDA chemists was made for applying *in silico* tools to the 200+ compounds used in the PhRMA analyses. An analysis was conducted by the FDA chemists and no convincing argument could be made for adding this element to the WoE for carcinogenicity (Personal communication, Frank Sistare). Since so many diverse mechanisms underly the range of tumors observed, this outcome is not surprising. While *in silico* applications to carcinogenicity hazard assessment are likely to evolve (Tice et al., 2021), *in silico* predictions of carcinogenicity beyond mechanisms involving certain genotoxic mechanisms, have not been broadly accepted by the industry or DRAs and so are viewed as not presently ready as a routinely deployed WoE tool for carcinogenicity risk assessment.

3.6 Perspective on role of investigative studies

As described in Section 3.1. and Section 3.2. above, investigative strategies have long played an important role in carcinogenicity risk assessment; however, these efforts largely focused on understanding the human relevance of a rodent tumor finding that arose in standard carcinogenicity tests. Under the WoE option there now emerges the potential to leverage investigative approaches and newer technology to prospectively address potential risks. During the PES, investigative approaches to characterize potential carcinogenic risks were not common and none of the emerging genomic or *in silico* approaches described above were included in the submissions. It is critical to point out that during the PES, sponsors were still required to conduct a 2-year rat study and therefore were likely less proactive in generating data to explain the mechanism or assess human relevance of any finding that suggested a potential carcinogenic risk. During the PES an incomplete explanation of findings from the 6-month rats study findings was a common reason for disagreement between DRAs and sponsors and in some cases among or within DRAs.

In the future, strategic use of both existing models and methods as well as emerging technology will hopefully expand to provide a more mechanistic approach to carcinogenicity risk assessment as well as increase the number of programs which can utilize a WoE assessment. Investigative approaches may be particularly important to meet the higher evidentiary standard for first-in-class molecules.

As described in Section 3.4 above, ECS may emerge as a tool to support a WoE assessment for new targets. Sponsors should not forget the potential for existing models in this regard. For example, when a pharmacologic mechanism can be activated similarly in rats and mice, then one could argue that the absence of tumor findings in the 6-month rasH2-Tg mouse study are additional supportive evidence that on-target activation presents lower risk for carcinogenicity. The recent RORgT example exemplifies the value of the short-term rasH2-Tg mouse model for identifying such on-target risks of novel first-in-class therapeutics (Haggerty et al., 2021). To be clear, the guidance does not require that a rasH2-Tg mouse study be completed prior to seeking agreement on a WoE approach. The point here is that sponsors may want to consider the conduct of a rasH2-Tg study sufficiently early to support such on-target risk assessments.

4 Best practices for WOE documentation

Once a sponsor has done an integrated analysis of the WoE factors and determined that a 2-year rat study would not contribute to human risk assessment they must document their WoE assessment for review by the DRAs. As a reminder and as specified in the guidance, formal documentation, and submission to DRAs is not required in those cases where the sponsor chooses to perform a 2-year rat study. The following provides suggestions for sponsors to consider based on the authors experience during the ICH process.

Evidence sources linked to the WoE criteria for *in vivo* studies will be primarily from standard toxicology studies (e.g., the standard genetic toxicology battery, histology from subchronic and chronic rodent studies, reproductive toxicology studies, secondary pharmacology screens, etc.) to minimize the need for additional animal studies. As such, collection and documentation of data for building the WoE document can be started early in each program to enable an early decision on whether it is feasible to pursue a WoE approach and/or whether additional information that needs to be generated to support a gap in a WoE endpoint is within the constraints of the project resources and timeline (see Section 5, Implementation Challenges Section).

The summary of relevant information extracted from these studies in the WoE assessment should be focused as to how the key data from each study specifically relates to the carcinogenicity risk (e.g., what targets relevant to carcinogenicity risk were included in the *in vitro* secondary pharmacology screens to rule out secondary pharmacology as a carcinogenic risk or what clinical pathology and histology endpoints were evaluated to determine lack of immunotoxicity or hormonal effects in repeat dose toxicology studies). Figure 1 provides a pictorial overview of the process. The discussion should be balanced and indicate if gaps in data exist and the strength of the assessment of each factor in supporting the final WoE conclusion should be stated.

As part of the literature assessment, different lines of evidence can be explored, including publicly available carcinogenicity data on other chemicals within the same primary pharmacological class, the extent to which the biological pathways are well-characterized relative to potential involvement in cancer development and relevant carcinogenicity risks related to the pharmacology of any major human metabolites.

As with any regulatory submission sponsors should organize the information in a logical manner. Sponsors have the flexibility to organize the information to best suit the needs of the program. The following potential outline provides suggestions on key elements to include in their WoE submissions to DRAs.

a) Executive Summary
   - Provide a high-level yet integrated Executive Summary of the information gathered for each of the WoE factors.
   - Summarize the strategy taken to build the WoE including data sources and a discussion of which factors provide the strongest evidence to support the WoE overall conclusion with a balanced assessment of the factors that either have gaps in information or which do not clearly support the overall WoE assessment outcome.
   - Based on the overall balance of the WoE assessment, a clear assessment of whether the compound presents a high or low level of human carcinogenic risk, and how the data support the rationale for not performing the 2-year rat study should be provided. If necessary, justification for any alternative carcinogenicity assessment studies (e.g., "additional *in vivo* tests for carcinogenicity" as described in 4.2.2 of the ICH S1(R1) guidance (2022)) to complete the assessment should be discussed.

b) Materials and Methods
   - Either as a discrete section or embedded in the WoE Factor sections, sponsors should consider indicating which databases were used in their assessment and may want to elaborate on their literature search strategies.
   - An outline of *in vitro* and *in vivo* studies used to provide support for each of the WoE factors may be useful.
   - Outline any additional investigative studies or details of specific measurements taken during standard toxicology studies that were used to support the WoE conclusions.
   - Describe clinical sources of information, if applicable.
   - Hyperlinks to study reports and literature references can simplify the review process.

c) WoE Factor Subsections with Detailed Analysis
   - Each of the 6 WoE factors should have a dedicated section with a detailed discussion addressing the concepts from the ICH S1B(R1) guidance (2022). Refer to Section 2 of this commentary for specifics on each of these WoE factors. These sections should be primarily high level and strategically directed at discussing how the WoE factor contributes to the carcinogenic risk assessment with the bulk of experimental results referenced in appendices.
     - o Target Biology
     - o Secondary Pharmacology (including listing of targets screened)
     - o Histopathology from chronic studies
     - o Hormonal Effects
     - o Genetic Toxicity
     - o Immune Modulation

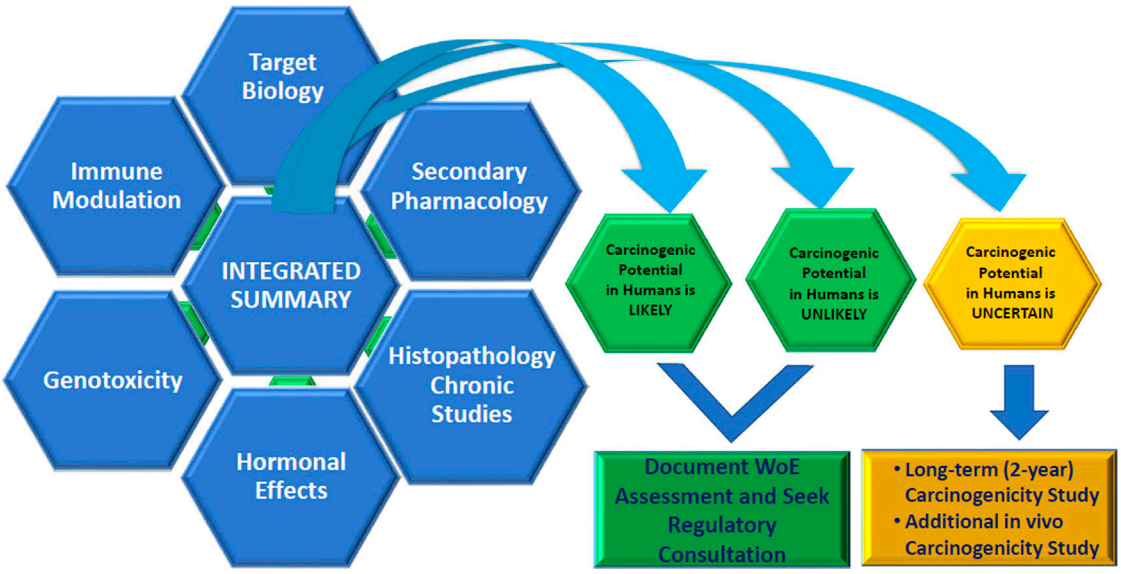

FIGURE 1
Visualization of the Integration of Weight of Evidence Factors to arrive at the conclusion of whether a 2-year rat study adds value to the human carcinogenicity risk assessment.

- Carcinogenic risk of major human metabolites should be assessed and addressed if applicable.
- Results of any investigative approaches as described in Section 3 of this commentary should be integrated into the appropriate WoE factor section.
- Assessment of pharmacokinetics, and exposures in each study used for supporting each of the WoE factors (including parent and metabolites relative to secondary pharmacology, in addition to *in vivo* studies) with exposure multiples relative to maximum human exposure should be provided. A summary table including all the studies discussed will facilitate the interpretation.

d) Integrated Risk Assessment/Conclusions
- The document should provide a conclusion section summarizing the WoE from each factor in support of the primary goal of determining whether a 2-year rat study would not add value to the human carcinogenicity risk assessment.
- Sponsors might consider placement of each WoE factor on the "sliding scale" of Figure 2 of the ICH S1B(R1) guidance (2022) to help visualize the overall "weight" of each factor.
- An overview of the proposed full carcinogenicity risk program (e.g., any additional investigative studies, additional *in vivo* carcinogenicity, etc.) should also be included.
- While the ICH S1B(R1) Addendum (2022) does not specifically address clinical safety data, sponsors should assess the clinical safety data available to date and integrate into the overall risk assessment.

Note that in some cases the use of appendices may be useful to provide details on any aspect of the WoE assessment including nonclinical study summaries, tabular data, graphical data from databases, or other information.

5 Implementation challenges

To ensure the successful implementation of the ICH S1B(R1) Addendum (2022), it is vital for industry and regulatory scientists to maintain open communication and collaboration. Under this Addendum, sponsors have the burden of proof to make the case as to whether a 2-year rat carcinogenicity study would not add value to understand human cancer risk. As described above, the WoE assessment supporting the conclusion that a 2-year rat carcinogenicity study does not add value will need to have comprehensively addressed each of the WoE factors outlined in ICH S1B(R1) (2022), in addition to other relevant information, and present a rigorous, critical, and objective science-based assessment. In addition to the scientific considerations described previously in this paper, sponsors face important regulatory and logistical challenges that are summarized below.

5.1 Need for a predictable regulatory assessment process

Among the various implementation challenges to consider, likely the most important is the need to establish a predictable regulatory assessment process that is well defined, transparent, and dependable with reasonable timelines. The reason being that planning, execution, and finalization of a 2-year rat carcinogenicity study can take up to 4 years, as detailed below.

(1) Pharmaceutical companies work with Contract Research Organizations (CROs) or internal schedulers to schedule the study. Usually, this needs to be done at least 1 year in advance, as integrating these long-term studies into the test facility schedule can be challenging. Additionally, nonclinical safety organizations within pharmaceutical companies coordinate their study planning with clinical, formulation, chemistry, and all other functional areas within their organization so that carcinogenicity study completion will not be rate limiting for filing a marketing application.
(2) For US submissions, special protocol assessments (SPA) outlining the proposed study design, final draft protocol, and dose selection rationale are generally submitted for regulatory review and concurrence (i.e., FDA Executive Carcinogenicity Assessment Committee [eCAC] process) multiple months ahead of the anticipated carcinogenicity study start. The SPA process is unique to the FDA and similar processes are not in place in other regulatory regions. These activities occur in parallel to the study scheduling described in point 1) above with adequate time to adopt revised designs that may change scheduling study start and/or reporting timelines (e.g., changes in dose selection requiring securing additional drug, recommendations for alternative/additional controls requiring additional animal rooms, staffing resources and/or ensuring timely animal orders); importantly, these are amongst the aspects managed by organizations and/or with CROs to ensure there is no impact to filing a marketing application.
(3) Once the study is initiated, the in-life study activities will require 2 years.
(4) The post-mortem activities, including histopathological evaluation of a list of >40 tissues/animal for 500–700 animals, statistical evaluation, preparation of the study report, and QA review can take a year or longer.
(5) Finally, any additional evaluations/investigations to assess the risk associated with potential observations on a 2-year rat carcinogenicity study are to be factored in the overall timelines.

Additionally, it will be important for the sponsors to factor in the availability of the 6-month rat chronic toxicity data, as this is the most critical factor in the WoE assessment, and not typically conducted until later in the drug development timelines. This is especially important when the timeline of the Phase three clinical program is relatively short, as availability of chronic toxicity studies to first registration may not allow sufficient time for seeking regulatory feedback on the WoE assessment and conduct of a 2-year carcinogenicity study if one of the DRAs were to require it. However, a draft report of this study that includes the final audited integrated data (including a signed pathology report) should typically be sufficient for the DRA review of the WoE assessment. An additional key data set that sponsors also need to factor in when planning the preparation of the WoE assessment is the human metabolite data, as potential carcinogenic risks of major circulating metabolites must be considered.

Given the potential for multiple factors that may extend the timeline for planning and execution of a 2-year rat carcinogenicity study, it is imperative that WoE assessments be integrated in the overall drug development timelines. Therefore, concurrence or feedback from DRAs on the WoE assessment is needed well in advance of the marketing application filings, so that if a 2-year rat carcinogenicity study is needed, it can be executed in a timely manner, and not delay submission of marketing authorization applications and timely access of medicines to patients. In addition, it would be important to have a predictable review process. In discussions among the industry representatives who provided input on the implementation of the S1B(R1) Addendum (2022), it was suggested that a 3–4 months review period to complete the WoE assessment would facilitate efficient and timely drug development process.

5.2 Need to seek separate input from multiple regions

As drug development is an increasingly global process, registration is most often pursued in multiple regions in parallel. As such, requests for feedback would need to overlap for each of the agencies and it would only take one DRA to indicate that a 2-year rat carcinogenicity study is considered necessary for the sponsor to have to conduct the study. As ICH does not have as a part of their remit to provide a centralized source of regulatory review, sponsors need to seek feedback on the necessity for a 2-year rat carcinogenicity study from DRAs separately in countries where marketing approval will eventually be sought. Given the approximate 4-year timeframe for planning and completing 2-year rat carcinogenicity study-related activities as described above, it becomes critical for sponsors to determine the appropriate timing for submitting a WoE assessment based on the duration of the DRA review cycles.

A key question that would need to be answered is how many agencies to seek feedback from? The answer to this will depend on the registration strategy an individual sponsor takes, but generally companies seek first approval in the three major regions (US, Europe, and Japan), before seeking approval in other countries. Although discussions will occur between the company and each of the additional countries where the marketing application will be submitted, by then input from the three major regions will be known and can help companies get a sense on whether DRAs agree that conducting a 2-year rat carcinogenicity study would not add value to the human carcinogenicity risk assessment.

5.3 Intra- and inter-DRA discussions are encouraged as well as continued dialog with industry partners

As part of the regulatory review of a WoE assessment submission, it will be important that the feedback provided to sponsors reflects an aligned, actionable perspective from within the DRA. In this respect, we encourage DRAs to establish a central expert group within their organization to provide a final recommendation that ensures intra-agency alignment. This centralized expert group could also help coordinate input to the ICH S1B(R1) Implementation Working Group (IWG) being assembled to facilitate sharing experiences among DRAs on the

outcome of the WoE assessments to help understand opportunities for improving the review process. Likewise, this IWG would allow industry members to receive feedback from DRAs on how best to improve the quality of submissions or sharing key learnings from the early stages of implementation.

The importance of such a process can be illustrated wherein the same drug and same dose are used in different patient populations, and where primary review of safety information may be undertaken by different regulatory scientists within the same agency, potentially at different points in time. As long as no substantial new scientific information relating to carcinogenic potential has become available, the central HA expert group could help maintain alignment. It would be troubling to have different conclusions on the value of the 2-year rat carcinogenicity study in contributing substantially to the human risk assessment for cancer for distinct but similar patient populations.

For transparency, individual sponsors may, as appropriate, play a proactive role in communicating to each of the DRAs when other DRAs have also received the WoE assessment submission, and if known, what the input received has been. As mentioned above, if at least one DRA asserts that a study is needed, then companies would need to trigger the conduct of the 2-year rat carcinogenicity study.

5.4 Labelling implications of not doing a 2-year rat carcinogenicity study

Currently results of rodent carcinogenicity studies are a standard part of product labeling for small molecule therapeutics. In many cases the labelling simply provides the results of those rodent studies and for those with positive rodent tumor findings often indicate that the relevance to humans is unknown. With the adoption of a WoE assessment option for some programs, it will provide regulators and sponsors an opportunity to reconsider how to make labelling of carcinogenic potential more useful for healthcare providers. The experience with labeling for biotherapeutics that have used a WoE assessment is highly variable and ranges from "carcinogenicity not assessed" to high level summaries of the WoE assessment. In the future, for programs which have used the WoE assessment option, the results of the rasH2 mouse study would likely be included in the labelling and it would be helpful if the high-level conclusion from the WoE assessment would also be included. For example, "An integrated analysis of available data suggested the potential carcinogenic risk of xxx is low".

5.5 Summary of implementation challenges

Now that the ICH S1B(R1) Addendum (2022) has been adopted, it is important that industry and DRA scientists continue to communicate and collaborate to make the implementation of this addendum successful. The S1B(R1) IWG being established by ICH should provide the right forum for industry and DRAs to have this dialog. A close partnership between DRA and industry will ultimately result in reducing animal use, in accordance with 3R's principles and an objective of the ICH S1B (R1) Addendum (2022), and optimizing resources for both Industry and DRAs without compromising the safety of medicines. It will be important, however, that industry submits only those WoE assessment with high confidence that a 2-year carcinogenicity study would not add value (either because of a high or a low carcinogenic risk); otherwise, this will end up increasing the DRA's workload rather than decreasing it.

6 Case examples

Case examples are useful tools to illustrate how WoE factors are integrated to reach a decision on the appropriate carcinogenicity approach for a particular program. The ICH S1B(R1) Addendum (2022) includes an appendix which summarizes key attributes of 4 of the cases that were submitted in the PES. In addition, the paper of Bourcier et al. (2024) describing the results of the PES provides a tabulation of key features of each of the cases submitted to the PES. These examples are instructive for both sponsors and regulators in understanding key attributes that are important in determining the appropriate carcinogenicity assessment strategy.

To further supplement available case material, industry colleagues from the JPMA retrospectively reviewed publicly available data from marketed pharmaceuticals. For this exercise the presence or absence of one of the standard WoE factors was determined and an assessment was conducted to determine if the carcinogenic potential in humans was considered likely or unlikely. The general pharmacologic class of drug, summary of the WoE assessment, and rodent tumor outcomes were summarized in a series of tables and text that are provided in the Supplementary Material.

It is important to note that the case examples presented in the ICH S1B(R1) Addendum (2022), the Supplementary Material in this commentary, or in other forms are by necessity very high-level summaries of key illustrative concepts. As described in the addendum and this commentary, the documentation of a WoE assessments requires a scientifically robust and detailed analysis of the program that goes well beyond the key points capture in case summaries.

7 Conclusion

While the value of rodent carcinogenicity studies for safety assessment in the different sectors (pharmaceuticals, chemicals, foods) will continue to be debated for years to come, the ICH S1B(R1) Addendum (2022) provides the first notable change in the paradigm for pharmaceuticals since the development and implementation of medium-term mouse models in the mid-to-late 1990s. The addendum first directs sponsors to carefully consider all elements of a program to develop a carcinogenicity assessment strategy rather than adopting a check-the-box mentality that relies solely on rodent carcinogenicity studies to assess potential risk. To aid sponsors in this process, Section 2 of this paper reviewed key WoE factors and suggested approaches for sponsors to consider in conducting their assessments and deciding if the WoE is appropriate for their program. An important component of the addendum is the acknowledgement that investigative approaches can aid in carcinogenicity risk assessment

by helping explain relevance of findings of concern observed in *in vivo* studies. To allow for sufficient flexibility and evolution of science the addendum was relatively brief and conceptual on this point, so Section 3 of this paper provides an expanded discussion of views from industry members of the EWG on the current status of these approaches. As this field of enquiry evolves it is our expectation that emerging technologies can be incorporated into the WoE paradigm on a more frequent basis without need for guidance revision. As outlined in the addendum, regulatory input is required before proceeding with registration. As highlighted in Section 4 sponsors must ensure that their WoE assessments are rigorous and carefully documented and presented to regulators in a coherent manner. In addition to the scientific considerations described in this paper, there are several implementation challenges. Section 5 reviews some of these challenges that sponsors need to carefully consider in developing a carcinogenicity strategy. Finally, based on an initiative from our colleagues in JPMA, the paper has provided additional case examples based on a retrospective review of marketed pharmaceuticals. These cases may be useful for sponsors and regulators as they consider how to apply the WoE factors.

ICH S1B(R1) (2022) provides an opportunity to move drug development and regulatory review to a more mechanistic and hypothesis-driven approach to carcinogenicity assessment that would inform both sponsor and regulatory decision-making. This shift in assessment strategies would encourage a more proactive mindset, create meaningful dialog with regulatory scientists and minimize drug development delays or discontinuations relating to carcinogenic risk.

The arc of this most recent revision to pharmaceutical carcinogenicity assessments had its origins in data gathered by industry scientists over a decade ago, evolved through multiple analyses by consortia and DRAs, and ultimately a prospective data collection and analysis that enabled the revision. Despite the substantial work and progress to date, much work remains for sponsors to effectively implement WoE approaches by conducting rigorous scientific reviews, implementing when appropriate investigational approaches, and finally presenting regulators with clear and complete dossiers to support the assessment. For DRAs, much work also remains in terms of providing consistent and timely reviews and seeking opportunities to share experiences and learning across regions so there is even greater global harmonization. Ultimately these efforts should result in a more rigorous and thoughtful approach to carcinogenicity testing that decreases animal use without compromising patient safety.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

JV: Writing–review and editing, Writing–original draft, Supervision, Project administration, Conceptualization. JD: Writing–review and editing. MG: Writing–review and editing, Conceptualization. SH: Writing–review and editing, Data curation, Conceptualization. JL: Writing–review and editing, Writing–original draft, Project administration, Conceptualization. TN: Writing–review and editing, Writing–original draft, Data curation. RS: Writing–review and editing, Writing–original draft. KT: Writing–review and editing, Writing–original draft, Project administration, Data curation. FS: Writing–review and editing, Writing–original draft, Investigation, Data curation, Conceptualization.

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Conflict of interest

Author JD was employed by the company Alnylam Pharmaceuticals. Author MG was employed by the company Organon. Author SH was employed by the company Formerly ASKA Pharmaceutical Co., Ltd. Authors JL and FS were employed by the company Merck & Co., Inc. Author TN was employed by the company Boehringer Ingelheim Pharma GmbH & Co. KG. Author RS was employed by the company Takeda Development Center Americas, Inc. Author KT was employed by the company Astellas Pharma Inc. Author JV was employed by Lilly Research Laboratories.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2024.1377990/full#supplementary-material

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Assessing human carcinogenicity risk of agrochemicals without the rodent cancer bioassay

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The rodent cancer bioassays are conducted for agrochemical safety assessment yet they often do not inform regulatory decision-making. As part of a collaborative effort, the Rethinking Carcinogenicity Assessment for Agrochemicals Project (ReCAAP) developed a reporting framework to guide a weight of evidence (WOE)-based carcinogenicity assessment that demonstrates how to fulfill the regulatory requirements for chronic risk estimation without the need to conduct lifetime rodent bioassays. The framework is the result of a multi-stakeholder collaboration that worked through an iterative process of writing case studies (in the form of waivers), technical peer reviews of waivers, and an incorporation of key learnings back into the framework to be tested in subsequent case study development. The example waivers used to develop the framework were written retrospectively for registered agrochemical active substances for which the necessary data and information could be obtained through risk assessment documents or data evaluation records from the US EPA. This exercise was critical to the development of a framework, but it lacked authenticity in that the stakeholders reviewing the waiver already knew the outcome of the rodent cancer bioassay(s). Syngenta expanded the evaluation of the ReCAAP reporting framework by writing waivers for three prospective case studies for new active substances where the data packages had not yet been submitted for registration. The prospective waivers followed the established framework considering ADME, potential exposure, subchronic toxicity, genotoxicity, immunosuppression, hormone perturbation, mode of action (MOA), and all relevant information available for read-across using a WOE assessment. The point of departure was estimated from the available data, excluding the cancer bioassay results, with a proposed use for the chronic dietary risk assessment. The read-across assessments compared data from reliable registered chemical analogues to strengthen the prediction of chronic toxicity and/or tumorigenic potential. The prospective case studies represent a range of scenarios, from a new molecule in a well-established chemical class with a known MOA to a molecule with a new pesticidal MOA (pMOA) and limited read-

Abbreviations: ACCase, Acetyl-CoA carboxylase inhibitor; ADME, Absorption, Distribution, Metabolism and Excretion; AOP, Adverse Outcome Pathway; CAR, Constitutive Androstane Receptor; cRfD, chronic reference dose; GABA-Cl, GABA-gated chloride channel allosteric modulator; LOAEL, Lowest Observed Adverse Effect Level; MOA, biological Mode of Action[1]; pMOA, pesticidal Mode of Action; NOAEL, No Observed Adverse Effect Level; POD, Point of Departure; PXR, Pregnane X receptor; ReCAAP, Rethinking Carcinogenicity Assessment for Agrochemicals Project; SDHI, Succinyl dehydrogenase inhibitor; T3, Triiodothyronine; T4, Thyroxine; TBC, Thyroxine-binding capacity; TPO, Thyroid Peroxidase inhibition; TSH, Thyroid stimulating hormone; UDPGT, Uridine diphospho-glucuronyltransferase; WOE, Weight of evidence.

across to related molecules. This effort represents an important step in establishing criteria for a WOE-based carcinogenicity assessment without the rodent cancer bioassay(s) while ensuring a health protective chronic dietary risk assessment.

KEYWORDS

new approach methods, weight of evidence, rodent cancer bioassay, carcinogenicity, risk assessment, agrochemical, regulatory toxicology

1 Introduction

As science evolves to capture a better understanding of a biological response, so too does the need to maintain adequate protection of human health and the environment against hazardous chemicals. A critical component of regulatory toxicology is the assessment of adverse health effects, and thus risks, in humans exposed to chemicals. Safety assessment of agrochemicals currently relies largely on animal-based toxicity testing to identify hazards and select reference values for human risk assessment. One concern in the current paradigm for the safety assessment of agrochemicals is the assessment of carcinogenicity. This is typically conducted on two separate species, rats, and mice (OECD, 2018a; 2018b), the conduct of which is driven by experience, historical precedence, and legislative requirements. The results of testing are used to set restrictions on the use, or method of use, for chemicals of concern; therefore, it is important that the choice of models, and the design of the studies, are truly protective of human health under a risk assessment approach.

Advancements in technologies and methods to assess systemic toxicity have led to an increased understanding of chemical carcinogenicity (Becker et al., 2017; Corvi et al., 2017; Dekant et al., 2017; Felter et al., 2022; Holsapple et al., 2006; OECD AOP-Wiki). It is now possible to evaluate the carcinogenic potential of a chemical using new approaches with improved human relevance (Wolf et al., 2019; Madia et al., 2021; Audebert et al., 2023). Such advances in the scientific understanding of chemically induced chronic toxicity, including carcinogenicity, provide an opportunity to modernize the evaluation of health risk from potential exposures to agrochemicals (Kavlock et al., 2018; Cohen et al., 2019; Wolf et al., 2019). Guidance exists to facilitate health-protective chemical evaluation while minimizing animal use, and only requires implementation (ECHA, 2017; Hartung, 2019; Stucki et al., 2022). Specifically, established regulatory guidance allows for scientific rationales to satisfy data requirements, promoting and optimizing the full use of existing information and focusing on the critical knowledge needed for risk assessment (US EPA 2013; APVMA, 2017; PMRA, 2021).

Characterizing carcinogenicity risk does not require development of new technologies or models, but rather leveraging the available understanding of carcinogenicity and applying existing tools in new ways (WHO, 2021; Stucki et al., 2022; Schmeisser et al., 2023). The ReCAAP Working Group, a group of experienced scientists from industry, non-governmental organizations, academia, and regulatory authorities with expertise in carcinogenicity testing, evaluation, and risk assessment, has developed a reporting framework for waiver rationales to rodent cancer bioassays for consideration in agrochemical safety assessment (Hilton et al., 2022).

The ReCAAP framework provides structure to support reporting of a WOE-based carcinogenicity assessment, including a comprehensive evaluation of all relevant data from the pesticidal mode-of-action (pMOA), physiochemical properties, metabolism, toxicokinetics, toxicological data including mechanistic data, and chemical read-across from similar registered agrochemicals. This assessment also includes an evaluation of data points related to well-known cancer MOAs such as genotoxicity, immunosuppression, and hormone perturbation. In addition, the use patterns, exposure scenario(s), and human exposure levels from the intended uses of chemicals are summarized to estimate the range of likely human exposures. The available data and known properties across structurally similar compounds (read-across analogues) are reviewed and considered for use to estimate appropriate departure points (POD) for chronic risk assessment of the active substance.

Hilton et al. (2022) performed a comprehensive evaluation of the framework by constructing WOE-based carcinogenicity assessments to support rodent cancer bioassay waiver rationales for registered agrochemicals, based on publicly available data. The availability of full data packages (including carcinogenicity studies) for these chemicals allowed for the waiver rationale to be compared back to the actual data, providing an important reference point for the framework. However, the exercise did not fully reflect the reality of the goal–to develop a waiver rationale based on the comprehensive data and information available, prior to the generation of carcinogenicity data. Agrochemical companies are in a unique position to construct a waiver rationale during the development of a regulatory data package for a new active substance, prior to knowing the results of a carcinogenicity study, and to have the waiver evaluated without influence of carcinogenicity results. Three prospective case studies are presented here, representing a range of scenarios, from a compound of a well-established chemical class with a known MOA to a compound with a limited chemical base for read-across. An overview of the WOE assessments is presented with key lessons learned.

This paper describes our efforts to evaluate the ability to use the ReCAAP WOE-based carcinogenicity assessment framework ("the framework") to make an informed decision in developing a waiver rationale of the chronic/carcinogenicity studies in rats and mice without having the knowledge of the outcome of the bioassays. Additionally, the framework was used to estimate the POD to adequately protect human exposures from chronic risk, including cancer. The case studies will help to familiarize the reader with the benefits of implementing this modern approach to testing and evaluation.

2 Methodological approach

The overall objective with these case studies was to provide a set of prospective WOE assessments to test the robustness of this

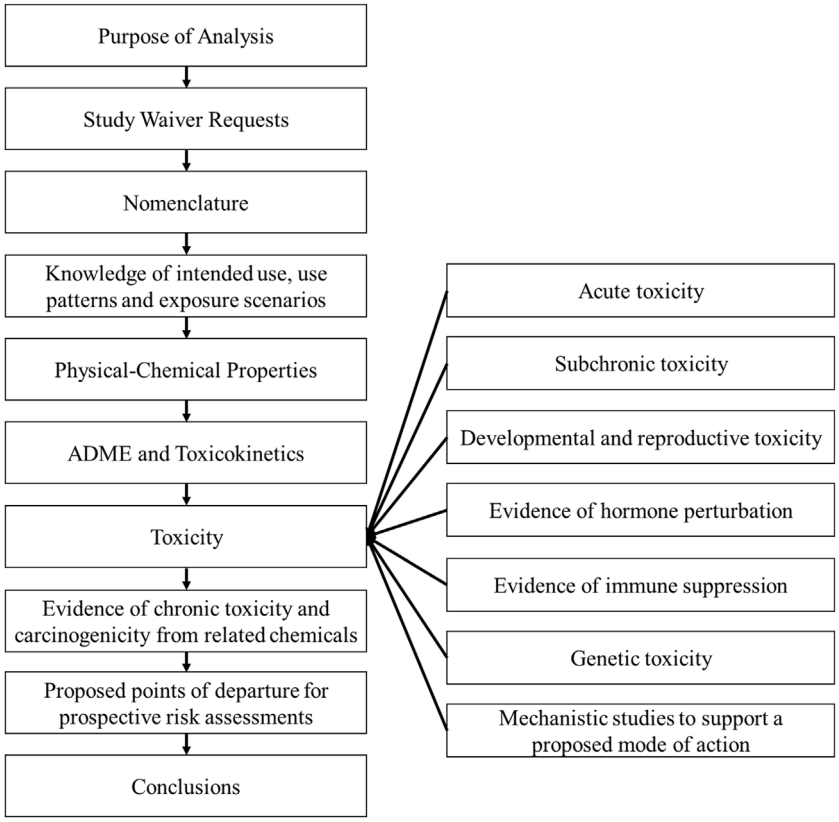

FIGURE 1
Reporting framework for the weight of evidence assessment. This workflow was used to identify and select the relevant and reliable lines of evidence used to make an informed decision in developing a waiver rationale of the chronic/carcinogenicity studies in rats and mice without having the knowledge of the outcome of the bioassays. Adapted from Hilton et al., 2022.

framework. The examples provided here were developed around the approach used by the United States Environmental Protection Agency (US EPA) to allow incorporation of a WOE-based approach into evaluating data for regulatory decisions (Craig et al., 2019). For each target compound, i.e., new active substance under development, the WOE assessment used the available data generated on the target compound with the exception of the chronic/carcinogenicity study. As the chronic/carcinogenicity studies were not complete at the time of the WOE assessment there was no influence on the interpretation of the WOE assessment and estimation of the POD for chronic dietary risk assessment. A read-across assessment was conducted with each case study, incorporating the relevant and reliable lines of evidence from read-across analogues, the source compounds, into the WOE assessment. Each case study applied the framework as it is laid out in the Hilton et al. (2022) paper. The outline of the workflow used to assess each individual chemical is shown in Figure 1.

2.1 Read-across assessment workflow

Read-across is based on the foundational principle that an association exists between structure and activity and is usually based on chemical similarity, although increasingly, also on similarities in biological effect (e.g., toxicological mode of action). Read-across seeks to inform on an endpoint outcome for an active substance (the target), where there may be a data gap, by using existing data on the same endpoint from other related substances (the sources) where a wealth of information exists (Patlewicz et al., 2013a; 2013b; 2014; 2019). In place of generating new *in vivo* toxicity testing data, read-across can be used as a line of evidence to reliably assess and address the risk, uncertainties, and deficiencies in data. The read-across assessment can be leveraged to meet regulatory data needs. The use of read-across is gaining traction as a reliable line of evidence for WOE-based safety assessments in toxicology (Low et al., 2013; Mellor et al., 2017; Alexander-White et al., 2022; Lizarraga et al., 2023). With this approach, the hazard of a target compound can be predicted from the existing toxicity data of one or many source compounds.

To identify the relevant chemical analogues for the read-across used in the case studies, structural and biological effect similarity analyses were performed. The structural similarity of these case study compounds was analyzed using an online tool ChemMine Web Tool (https://chemminetools.ucr.edu/) to examine their structural similarity to available chemistries. ChemMine is a publicly available tool available for analyzing and clustering small molecules by structural similarities, physicochemical properties or custom data types. This online tool calculates atom pair (AP) and maximum common substructure (MCS) similarities with the Tanimoto coefficient as the similarity measure, as well as

TABLE 1 Summary of case studies using the framework workflow.

Chemical/Active substance	New SDHI fungicide/ Nematicide	New ACCase inhibitor insecticide	New GABA-Cl allosteric modulator Insecticide/ Acaricide
Pesticidal Mode of Action	Disrupts cellular respiration through inhibition of mitochondrial enzyme succinate dehydrogenase	Disrupts fatty acid biosynthesis through inhibition of acetyl-CoA carboxylase	Disrupts inhibitory neurotransmitter signaling through allosteric modulation of GABA-gated chloride channels
Read-across chemicals	• Large number of chemicals available • 23 SDHI fungicides (FRAC Group 7) • 13 chemicals registered by US EPA • All 13 chemicals included in read-across	• Medium number of chemicals available • 23 ACCase inhibitor herbicides and insecticides • HRAC Group 1 and IRAC Group 23 • 14 chemicals registered by US EPA • 2 chemicals peer-reviewed by EFSA • 1 chemical included in a JMPR report • 3 of the 17 chemicals were included in the read-across based on structural similarity (TAs/TADs) and regulatory review by the same Agency (US EPA)	• Limited number of chemicals available • GABA-Cl antagonist insecticides (IRAC Group 2) registered by US EPA deemed structurally dissimilar and not appropriate analogues • 2 GABA-Cl allosteric modulators (IRAC Group 30) registered by US EPA; an isooxazoline and a meta-diamide • Considered 4 structurally similar related veterinary medicines • 6 chemicals included in read-across
Pharmacokinetics	Target substance is well absorbed and rapidly excreted. ADME properties were similar in both sexes and at all tested dose levels. There is no concern for bioaccumulation or toxic metabolites	Target substance is well absorbed and extensively metabolized, with no alerts for bioaccumulation or toxic metabolites. Excretion is rapid, with greater than 94% of the dose excreted within 48 h and essentially complete by 168 h. The predominant biotransformation pathway observed was via rapid and complete ester hydrolysis of the ethoxy carbonyl moiety to form the enol metabolite	Target substance is readily absorbed and extensively metabolized, with no alerts for bioaccumulation or toxic metabolites. ADME properties were similar in both sexes irrespective of dose levels, single or repeat dose, radiolabel, and sex
Relevant Assessment of Biological Effect and Response	Subchronic studies indicate liver and thyroid are target organs (increased weights and microscopic hypertrophy) with clear NOAELs established for all effects	Subchronic studies indicate decreased body weights in all tested species, thyroid effects in rats, liver effects in mice, and adverse clinical signs in dogs, with clear NOAELs established for all effects	Subchronic studies indicate several target organs in rats and mice, and no target organs in dogs. Clear NOAELs established for all effects
Evidence of hormone perturbation	No effects on reproductive performance or prenatal development. No evidence of estrogen, androgen, or steroid perturbation. Thyroid effects in rats considered secondary to liver enzyme induction. No evidence of direct thyroid perturbation	Based on the available data there is no toxicity via an endocrine MOA and thus not relevant for selection of endpoints for risk assessment. The lack of hormone measurement does not affect the WOE assessment or outcome because a hormonal MOA relevant to carcinogenicity was limited to thyroid, for which mechanistic data are available to address human non-relevance and/or justification for a margin of exposure-based approach for chronic risk assessment. Effects due to perturbations of reproductive hormones were considered adequately evaluated by the US EPA in the toxicological database, including the repeated dose, reproductive, developmental and ToxCast data	No effects on reproductive performance or prenatal development. No evidence of estrogen, androgen, or thyroid perturbation in subchronic studies. Unable to exclude steroid perturbation in the adrenal gland based on effects in rat and mouse subchronic studies, also observed for read-across chemicals
Evidence of immune suppression	No evidence of immune suppression in subchronic studies or with read-across chemicals	Chemicals used for read-across did not show signs of immunotoxicity in the T-cell dependent antibody response assays. In all available studies, there was no evidence of an immunosuppressive effect up to the highest dose level tested	No evidence of immune suppression in subchronic studies or with read-across chemicals
Genotoxicity	Not mutagenic, aneugenic, or clastogenic based on complete genotoxicity battery	Not mutagenic, aneugenic, or clastogenic based on complete genotoxicity battery	Not mutagenic, aneugenic, or clastogenic based on complete genotoxicity battery
Interpretation of Toxicity Profile	Investigative studies link the liver and thyroid effects to activation of CAR and induction of liver enzymes (including UDPGT), a well-established MOA common to most SDHI chemicals	Subchronic toxicity profile in line with ACCase inhibition. Investigative studies link thyroid effects in rats to induction of liver enzymes (including UDPGT). Adverse clinical signs in dogs identified as the most protective endpoint for risk assessment	Consistent testis effects in rat studies provide a protective endpoint for risk assessment. Varied effects observed in subchronic studies indicate clear thresholds but no clearly identified MOA.
Point of Departure	Lowest 90-day NOAEL (rat) with a 10X extrapolation factor for subchronic to	Lowest 90-day NOAEL (dog) with a 10X extrapolation factor for subchronic to chronic duration (1000X total uncertainty factor)	Lowest 90-day NOAEL (rat) with a 10X extrapolation factor for subchronic to

(Continued on following page)

TABLE 1 (*Continued*) Summary of case studies using the framework workflow.

Chemical/Active substance	New SDHI fungicide/ Nematicide	New ACCase inhibitor insecticide	New GABA-Cl allosteric modulator Insecticide/ Acaricide
	chronic duration (1000X total uncertainty factor)		chronic duration (1000X total uncertainty factor)
Chronic Risk Assessment	All use cases passed risk assessment based on margins of exposure	All use cases passed risk assessment based on margins of exposure	All use cases passed risk assessment based on margins of exposure
Conclusions on Weight of Evidence to Waive the Rodent Bioassays	High confidence that a chronic POD can be determined that is protective of all long-term effects, including cancer, without conducting the rodent bioassays	High confidence that a chronic POD can be determined that is protective of all long-term effects, including cancer, without conducting the rodent bioassays	Based on subchronic effects and tumor profiles of the read-across chemicals, weight of evidence considered not sufficient to waive the rodent bioassays

An illustration of the framework workflow. The left column lists the order of assessments utilized in the WOE, assessment. The results from each part of the analysis are briefly described for each case study.

identifying the largest substructure two compounds have in common. For each case study, the largest substructures in common within each class of chemistry were classified, and the AP and MCS Tanimoto scores were used to categorize the similarities. There are many software programs available for a chemical structurally-based read-across assessment, some of which are publicly available (e.g., OECD QSAR Toolbox, Morgan fingerprints, ToxPrints, US EPA's GenRA). As such, a comprehensive read-across assessment is available for determining appropriate inclusion based on structural similarities where needed. Further assessment of bioactivity similarities between the target chemical and read-across analogues was used for the potential to refine the list of relevant read-across analogues.

In addition to the structural similarity analysis, further assessments were performed to refine the selection of relevant read-across analogues including reviews of the pesticidal mode of action (i.e., the intended target mode of action), any known toxicological MOA (i.e., off-target or unintended mode of action). There may also be subcategories within a class of chemistry with distinct differences in the off-target MOA influencing the biological response to a chemical. As described in Hilton et al., 2022, this information combined with the toxicity profile of the potential analogues was considered in refining the selection of read-across analogues and strengthened the reliability of the read-across analysis in each case study.

The toxicological data available for the read-across compounds was used to further inform prospective assessments for each case study agrochemical.

3 Case studies

To illustrate the use of this framework, three case studies are presented as examples of how the framework could be applied for estimating the POD for chronic and carcinogenicity risk assessment, without life-time rodent cancer bioassays. The case studies were developed to capitalize on the fact that multiple regulatory agencies can consider the incorporation of weight of evidence-based assessments in place of guideline studies for regulatory decisions (Hilton et al., 2022).

The first case study using a succinate dehydrogenase inhibitor (SDHI) fungicide provides an example with a well-understood MOA and several relevant and reliable read-across analogues. The second case study using an ACCase inhibitor insecticide provides an example with a well-understood MOA; however, the read-across chemicals were moderate in number and, although chemically similar, did not share a similar toxicity profile to the target compound. The third case study using a GABA-gated chloride channel allosteric modulator (GABA-Cl) insecticide and acaricide provides an example with a novel MOA and limited read-across analogues. Table 1 summarizes the available information for each chemical and is organized to follow the ReCAAP framework.

Although the types of information, level of detail, and data interpretations will likely vary for different chemicals, these case studies are provided as examples to familiarize the reader with the format, information content, and level of detail that should be considered in a WOE assessment.

3.1 Succinate dehydrogenase inhibitor (SDHI)

A new agrochemical active substance has been developed which acts as an inhibitor of the mitochondrial enzyme succinate dehydrogenase; agrochemicals with a MOA involving this complex are called succinate dehydrogenase inhibitors (SDHIs). There are currently 13 SDHIs registered by the US EPA, and the data for all registered SDHIs in North America was collected for use in the read-across assessment. This target compound was selected as a case study because it has a well understood pesticidal MOA and there are several similar active substances registered, thus permitting an in-depth assessment and ability to estimate the chronic POD and cancer outcome from similar chemicals.

Short-term (28-day) and subchronic (90-day) toxicity studies in the mouse, rat and dog with this target compound primarily indicated that the liver is the target organ for toxicity. There were consistent, dose-related increases in liver weight and incidence of hepatocellular hypertrophy across the various species. Liver enzymes, including uridine diphosphate glucuronosyltransferase (UDPGT), were induced by exposure to the new SDHI. There were some noted effects on the thyroid gland (increased weight or follicular cell hypertrophy), determined to be secondary to liver effects. Additional systemic toxicity assessments demonstrated there was no evidence for genotoxicity, neurotoxicity, developmental toxicity, or reproductive toxicity for this SDHI.

The subchronic toxicity database for this new SDHI is in line with the majority of active substances in this class of chemistry. The primary target organ for SDHI inhibitors is consistently the liver across all chemicals used in the read-across assessment. The thyroid is the second most common target organ, affected by nine of the thirteen chemicals, and thyroid effects for SDHIs are considered secondary to UDPGT liver enzyme induction. The kidneys are considered a target for two of the SDHIs, and the gastrointestinal tract is considered a target for another SDHI chemical. In most cases, the rat or dog is the most sensitive species following subchronic exposure.

The read-across assessment for chronic toxicity of the presented SDHI compounds identified the same target organs observed in the subchronic toxicity studies. In general, most SDHI read-across compounds demonstrated progression of toxicity from subchronic to chronic exposure. No clear sex-specific sensitivities were identified. In the carcinogenicity assessments, treatment-related tumors were observed for nine of the thirteen compounds, as determined by the US EPA Cancer Assessment Review Committee. Consistent with the primary target organs across the class, liver and thyroid tumors were the most commonly observed. Eight SDHI compounds increased the incidence of liver and/or thyroid tumors. Two compounds increased the incidence of uterine tumors. Treatment by one compound increased incidence of brain astrocytomas, ovarian tubulostromal neoplasms, and histiocytic sarcomas of the lymphatic system. Another compound presented brain granular cell tumors in addition to thyroid tumors. For all SDHI compounds with tumors, the chronic reference dose (cRfD) was considered to provide a protective margin of exposure for carcinogenicity, with the exception of one chemical analogue that uses a q1* linear cancer risk assessment (for liver tumors). It is worth noting that a MOA framework has not been developed for this chemical, and a cancer reclassification would likely be possible if such data were generated, similar to the rest of the SDHI class. Overall, the data for SDHI chemicals indicates that liver and thyroid tumors are common and, if other tumor types do occur, a threshold-based risk assessment is considered protective of human health.

The subchronic toxicity profile of the new SDHI is consistent with the overall class of chemistry; supporting the WOE that the chronic toxicity and tumor profile will also be similar. Thus, liver, and thyroid tumors (secondary to liver enzyme induction) would be plausible for the new SDHI active substance. Considering this prediction, efforts were made to characterize the MOA, in advance of the actual observation of tumors in a carcinogenicity study. Studies demonstrated a direct activation of rat and mouse constitutive androstane nuclear receptor (CAR), increased levels of CAR-dependent gene expression, induction of liver enzymes (including UDPGT), hepatocellular hypertrophy and increased liver weight, all adding to the evidence that this new SDHI exhibits a CAR-mediated MOA (Peffer et al., 2018). It is well-established that this MOA has a clear threshold for effect, and thus does not require linear assessment of cancer risk (Meek et al., 2014). The total WOE assessment indicates that there is high certainty that a chronic POD can be determined that is protective of all long-term effects, including cancer, without conducting a chronic/carcinogenicity study.

In the absence of a chronic study for this new SDHI chemical, it is proposed to utilize the lowest 90-day no-observed-adverse-effect-level (NOAEL) 51.1 mg/kg/day and apply an additional uncertainty factor for extrapolation from subchronic to chronic duration (Figure 2A). Based on the 90-day to chronic NOAEL ratios for the 13 SDHIs used for this comparison, a 10X uncertainty factor would be conservative. The mean of the ratios is 4.2 and the median is 3.5, indicating an extrapolation factor for study duration of 3-5X would be more appropriate to represent this class of chemistry, and still provide a chronic risk assessment that is highly protective of human health, including the risk of cancer.

Uncertainty in the SDHI case study is considered low. The mammalian toxicity and tumor profiles across the SDHI class of chemistry are strikingly similar. As liver and/or thyroid tumors would be expected for an SDHI chemical, it is conservative to assume that those tumors would result from exposure and characterize the cancer MOA proactively. The key events in the CAR/PXR pathway were investigated and assessed in line with the IPCS framework, sufficient to support evaluation of cancer risk by a regulatory agency (Boobis et al., 2006; IPCS, 2007). Further, the common CAR/PXR MOA shows a clear progression of effects with increasing duration of exposure and is known to be non-linear.

3.2 ACCase inhibitor insecticide (ACCase)

A new insecticide has been developed which acts as an inhibitor of acetyl-CoA carboxylase (ACCase), disrupting fatty acid biosynthesis. The ACCase class includes both herbicides and insecticides. There are currently 14 ACCase inhibitor agrochemical active substances registered by the US EPA. The availability of information for read-across, as well as the known MOA, makes this target compound a good case study.

Although ACCase is found across species, ACCase-inhibiting herbicides/insecticides do not potently inhibit mammalian, fungal, or broadleaf plant ACCase. To assess the potential use of read-across candidates, all available 23 ACCase herbicides and insecticides were initially considered. Following the structural similarity assessment, the tetronic and tetramic acid derivatives and phenylpyrazolin compounds were most structurally similar to the new ACCase inhibitor. A review of the distinguishing factors of the different ACCase chemistries and the ACCase enzyme and its physiological function was also included to ascertain any significant changes in the subcategories of this class and assess the reliability of the potential read-across analogues (Rendina et al., 1990; Délye, 2005; Yu et al., 2010; Xia et al., 2016; Takano et al., 2021).

Read-across analogues used in this case study focused on the tetramic and tetronic acid derivatives with the greatest structural similarity within the ACCase chemistry class. The ACCase herbicides were not included as they shared less structural similarity to the new insecticide, and typically insecticides show different mammalian toxicity than herbicides.

A read-across assessment for subchronic, chronic, carcinogenicity and MOA data demonstrated weak alignment across the toxicity profiles. Common effects reported in the subchronic studies were common to only two compounds, such as findings in the adrenal glands (cytoplasmic vacuolation in the cortex) in rats, mice and/or dogs following administration of spirodiclofen or spiromesifen; however, these effects were not seen with the new ACCase insecticide. Effects were observed in the male reproductive tract;

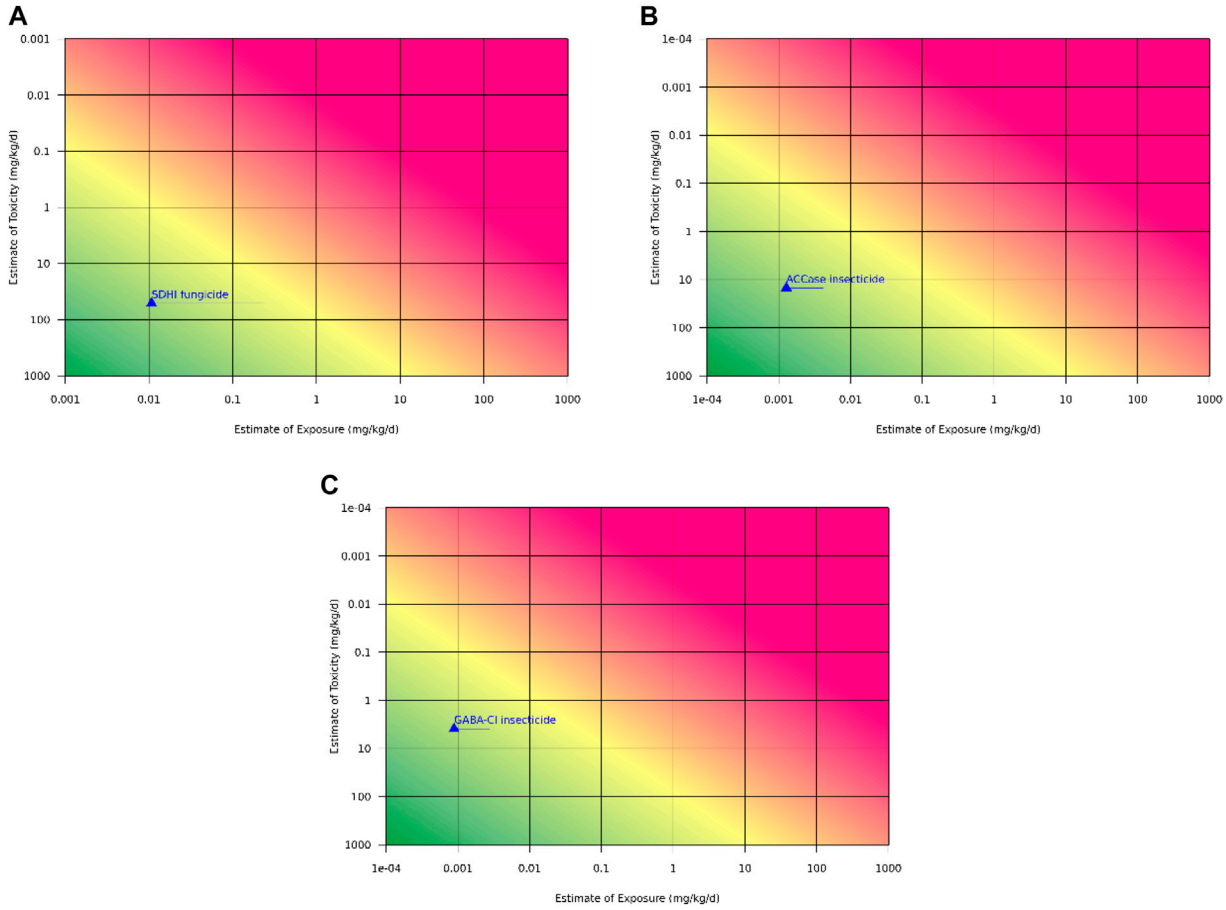

FIGURE 2
RISK21® graph for predicted chronic exposure to the new active substances. The RISK21® plots evaluating the available exposure and hazard data for the safety assessment of the **(A)**. SDHI fungicide, **(B)**. ACCase inhibitor insecticide, and **(C)**. GABA-Cl insecticide. The yellow line in the RISK21® tool represents the margin of exposure between the 90-day toxicity study NOAEL (as an estimate of toxicity) and the registrant's modeled exposure values (as estimates of exposure) generated in US EPA's DEEM dietary risk software. The Health and Environmental Science Institute (HESI) provide RISK21® tools, which are available online through the following link: https://risk21.org/webtool/.

including hypertrophic Leydig cells and histopathological findings in the testes, epididymis, and prostate following treatment with spirodiclofen, and decreased testis weight, testicular degeneration and vacuolation, hypospermia in the epididymis and abnormal spermatozoa following treatment with spirotetramat (EFSA, 2009; 2013); however, these effects were not seen with the new ACCase insecticide. In fact, the understanding of potential reproductive effects with this subcategory of ACCase inhibitors prompted additional evaluation during early research to allow selection of candidate compounds that did not inhibit testosterone production. Thymus atrophy was observed following treatment with spirodiclofen and spirotetramat in the dog; no effects on the thymus were seen with the new ACCase insecticide. Thyroid changes included colloid contraction and follicular cell hypertrophy following treatment with spiromesifen and the new ACCase insecticide; decreased T3 and T4 and increased TSH and thyroxine-binding capacity (TBC) following treatment with spiromesifen. Increased liver enzyme induction was also observed following treatment with spiromesifen and the new ACCase insecticide.

Short-term and subchronic exposures to this new ACCase insecticide indicated the target organs of toxicity were different for different species. The critical effects were loss of body weight in the mouse and rat, changes to the rat thyroid, increased liver weight in the mouse, and adverse clinical signs in the dog, such as body tremors and subdued behavior. The thyroid effects in the rat consisted of minimal to diffuse follicular cell hypertrophy and colloid contraction in the thyroid gland. Clear thresholds were established for all critical effects, and the adverse clinical observations in dogs were considered protective of other effects observed in the subchronic studies. Additional systemic toxicity assessment demonstrated there was no evidence for genotoxicity, neurotoxicity, developmental toxicity, or reproductive toxicity for this ACCase insecticide.

The subchronic toxicity profile for this target compound is in line with results indicative of effects on lipid biosynthesis which produced systemic effects such as decreased body weight, decreased cholesterol and triglycerides, adverse clinical signs, and changes to the liver. The systemic effects and NOAEL values from the subchronic studies identified the dog as the most appropriate species for estimating the POD for risk assessments if dosed over a longer time interval.

The initial strategy to include all ACCase chemistries in the read-across assessment was a conservative approach based on the

improvements made to this class of chemistry over time. Despite these improvements, the read-across assessment includes all available data on the relevant analogues to provide a thorough assessment.

Due to the liver and thyroid effects observed in the short-term and subchronic toxicity studies in the rat with the new active substance, it was investigated whether the effects were secondary to liver enzyme induction, to better understand if liver and thyroid tumors would be plausible for the new ACCase insecticide. Considering this prediction, efforts were made to proactively characterize the MOA, in advance of the actual observation of any tumors in a carcinogenicity study. Mechanistic research excluded direct effects on thyroid peroxidase (TPO) inhibition and demonstrated a dose concordance between the rat thyroid effects and induction of liver enzymes (including UDPGT activity), hepatocellular hypertrophy, and increased liver weights, providing a weight of evidence that any liver and thyroid tumor potential of this new ACCase insecticide would be secondary to liver enzyme induction. It is well-established that this MOA has a clear threshold for effect, and thus does not require linear assessment of cancer risk. A chronic POD can be determined that is protective of all long-term effects, including cancer.

In the absence of a chronic study for this new ACCase insecticide, it was proposed to utilize the lowest 90-day NOAEL 15 mg/kg/day and apply an additional uncertainty factor for extrapolation from subchronic to chronic duration (Figure 2B). Based on the 90-day to chronic NOAEL ratios for the 3 ACCase chemicals used for this comparison, a 10X uncertainty factor would be conservative. The mean of the NOAEL ratios is 5.2 and the median is 5.6, thus, an uncertainty factor of 5-6X would be more appropriate to represent this class of chemistry and still provide a chronic risk assessment that is highly protective of human health, including the risk of cancer.

Uncertainty in the ACCase case study is considered low. The mammalian toxicity profile was in line with the results indicative of effects on lipid biosynthesis which were observed in the read-across analogues. Although three structurally similar read-across compounds were identified within the pesticidal MOA ACCase inhibitors, there was no common MOA except for the UDPGT induction MOA for one analogue and the target compound. This was based on an evaluation of the publicly available toxicological datasets for all ACCase compounds, which demonstrated slightly different target organs between the chemical classes. The read-across compounds were used in this case to decrease the uncertainty of predicting chronic toxicity and carcinogenicity with the target compound. This increases confidence in a safety assessment, as effects can be observed and characterized in sub-chronic studies, without the need to progress to studies of longer duration. Defining a threshold for precursor effects in sub-chronic studies would be protective of any tumor formation or chronic toxicity, and thus form the basis for a health-protective risk assessment.

3.3 GABA-gated chloride channel allosteric modulator (GABA-Cl)

A novel agrochemical active substance has been developed which acts as a broad-spectrum insecticide and acaricide. This compound is classified as a gamma-aminobutyric acid-gated chloride channel allosteric modulator (GABA-Cl; IRAC Group 30) which acts at a site different from known conventional GABA-Cl antagonists such as fiproles and cyclodienes (IRAC Group 2; Blythe et al., 2022; Dayan, 2019). The group of chemicals from IRAC Group 2 was determined to be structurally dissimilar and not appropriate for use in the read-across evaluation. There were two other GABA-Cl allosteric modulators registered by the US EPA, fluxametamide and broflanilide. One is an isoxazoline similar to the new active substance under development and the other is a meta-diamide; both were included in the read-across evaluation. The isoxazoline chemistry has also been used in the veterinary drug industry, and therefore four analogues were selected from that chemical space. This new GABA-Cl modulator was selected as a case study because it has a novel MOA, with limited read-across analogues, and therefore estimating the chronic POD or cancer outcome based on similar chemicals was more challenging.

In the veterinary drug industry (i.e., non-food uses), carcinogenicity studies are not warranted when there is no concern for genotoxicity (EMA 2013, 2015, 2017a, 2017b). In the case of the six analogues selected for read across, all were demonstrated to be non-mutagenic and non-clastogenic. There were no structural alerts for genotoxicity, and there were no proliferative or pre-neoplastic changes in the subchronic rat toxicity studies. -Therefore, chronic toxicity and carcinogenicity data were available only for the two agrochemicals (fluxametamide and broflanilide), thus the read-across assessment analysis for long-term effects focused on the results from these two compounds. For both compounds, the rat was the most sensitive species and there was not a clear sex difference. For the dietary studies with fluxametamide, there was a common effect noted in the gastrointestinal tract which included gross pathology (increased incidence of abnormally pale color duodenum and jejunum) and an increased incidence of enterocyte epithelial vacuolation of the jejunum. Increased incidences of thyroid follicular cell adenoma in male rats and hepatocellular adenoma in male mice were observed in the carcinogenicity studies, albeit at doses approaching the limit dose; a genotoxic MOA was excluded as unlikely, and a threshold dose in the risk assessment was considered appropriate (Food Safety Commission, 2020). The US EPA determined that a non-linear approach using the chronic reference dose would account for all toxicities, including carcinogenicity (US EPA, 2020a; 2020b). Based on the overall toxicology profile for broflanilide, the target organs were the adrenal glands (rats, mice, dogs) and ovaries (rats and mice). No effects were observed in the mouse carcinogenicity study. In rats, there were testicular Leydig cell adenomas, ovarian luteomas and granulosa cell tumors, uterine adenocarcinomas, and adrenal cortex carcinomas observed in the carcinogenicity study (US EPA, 2020b).

Short-term and subchronic exposures to the new GABA-Cl modulator indicated the rat was the most sensitive species, with clear NOAELs established for all effects in all species. There was no clear consistent target organ of toxicity, as the critical effects varied across species. The target organs identified in the rat were the adrenal glands, duodenum, kidneys, liver, testes, and epididymides, while the target organs in the mouse were the adrenal glands, duodenum, liver, spleen, and thymus. There were no target organs identified in the dog. There were clear and

protective thresholds for all effects, based on dose levels with no observed effects. Additional toxicity assessment demonstrated there was no evidence for genotoxicity, neurotoxicity, developmental toxicity, and no evidence of potential carcinogenicity based on data points related to well-known cancer MOAs such as genotoxicity and immunosuppression. An effect on the hypothalamus-pituitary axis could not be completely ruled out due to effects in the adrenals. The most sensitive effect across the toxicity database was testicular tubular degeneration in rats, evident in the one-generation and two-generation reproductive studies, and the 90-day subchronic study; however, there was no effect on reproductive function for males. Consistent NOAELs for the testicular effects were observed across all studies, with no evidence of progression or lower effect levels with longer duration exposure, allowing for an estimation of a POD suitable for use in chronic risk assessment. Overall, the toxicity profile indicated this new GABA-Cl modulator would be unlikely to generate treatment-related tumors in rats or mice if a long-term set of studies were conducted. Using the lowest 90-day NOAEL 3.9 mg/kg/day as the POD and applying an additional factor of 10X for extrapolation from subchronic to chronic duration, the chronic reference value was well above the anticipated human exposures, indicating a health-protective chronic risk assessment would be possible without rodent cancer bioassays (Figure 2C).

Following the technical peer reviews, it was noted that the relevance of the histopathological findings in liver, renal cortical tubular epithelia, and adrenal zona fasciculata vacuolation could have been discussed in greater depth to better support a waiver rationale. For example, an important finding for the GABA-Cl case study was the fact that the adrenal changes were limited to zona fasciculate vacuolation and adrenal gland hypertrophy. No adrenal proliferative lesions were observed despite the presence of vacuolation. This was important as the histopathological distinction between adrenal hyperplasia and adrenal neoplastic changes in the rat is not necessarily easy to differentiate. The same feedback applied to the adrenal findings in mice. To strengthen this case study, additional data that provided more detailed understanding of the effects in the adrenal gland were recommended by the ReCAAP collaborative reviewers of the case study.

Concerning toxicokinetics, this new GABA-Cl modulator and its metabolites did not appear to bioaccumulate. In summary, the target organs for subchronic exposure included the adrenal glands (rat and mouse), liver (rat and mouse), kidneys (rat), testes (rat), duodenum (rat and mouse), spleen (mouse) and thymus (mouse). Carcinogenicity studies were only available for two of the related chemicals, and multiple tumor types were observed in the carcinogenicity studies for both chemicals. In addition, there were indications in the read-across for potential disruption of the hypothalamic-pituitary adrenal (HPA) axis, based on hypertrophy of adrenal zona fasciculata seen in mice and rats in the toxicity databases. Based on the number of organs affected in the subchronic studies and the inconsistent tumor profiles of the read-across chemicals, there was limited confidence that a waiver rationale would be acceptable for risk management, and presently, would not be sufficient to fulfill the regulatory data requirements. This case study highlights the need for additional steps to develop mechanistic cell-based assays and computational models that can acceptably address such uncertainties.

3.4 Comparison to chronic/carcinogenicity study results

As already noted, the opportunity to develop these prospective case studies for three new agrochemicals was unique, because the exercise was blinded to the results of the guideline chronic/carcinogenicity studies that were eventually conducted in both mice and rats to fulfill current data requirements for registration. For all three new agrochemicals described above, the rodent bioassays did not show any evidence of carcinogenicity not predicted by the framework assessment. For the SDHI chemical, mouse liver tumors were observed (although at a low incidence considered marginally treatment-related). As the CAR-mediated MOA had been demonstrated through mechanistic studies, a threshold-based endpoint for chronic toxicity was considered protective of all long-term effects in humans, including cancer. No treatment-related tumors were observed in either rats or mice for the ACCase inhibitor or the GABA-Cl modulator. The application of the ReCAAP framework to these chemicals resulted in waiver rationales and estimated chronic PODs that were equally or more conservative than the actual results of the rodent bioassays, illustrating that human health-protective chronic and carcinogenicity risk assessments do not necessarily require long-term animal data.

4 Key learnings

The intended value of these Syngenta case studies was to provide an opportunity for reviewers from the ReCAAP Work Group to test the application of the ReCAAP framework for three new agrochemicals, without any knowledge of the rodent bioassay outcomes. The aggregated reviewer feedback from this exercise underlined the core strengths of the framework to support a WOE-based assessment of chronic and carcinogenicity risk without the rodent bioassays. Through this exercise, several key learnings were identified, including the advantage of using read-across and mode-of-action information to support the WOE, as well as the need for transparency in the selection and justification of information used in the WOE. In the following, we summarize some of the key learnings and recommendations from this exercise, to support confidence in using the ReCAAP framework for future application.

4.1 Read-across

One of the lessons learned with these case studies included the benefits of using a consistent approach in read-across to available guideline studies and research models and strengthening the reliability of comparing findings in known toxicological profiles. A thorough discussion of the information considered in the read-across approach was important to support the selection of chemical analogues. Read-across assessments for these case studies were conducted by evaluating structural similarity, which is a common approach for analogue selection for industrial and cosmetic chemicals. Agrochemicals, unlike industrial chemicals, typically have a well-characterized toxicity profile, as well as known on-

target mode of action (i.e., pesticidal mode of action). For agrochemicals, similarity of biological effects (off-target mode of action), in addition to similarity of known on-target pesticidal mode of action, is considered an important consideration in selecting analogues for read-across evaluation. Biological similarity among chemicals is a scientifically acceptable concept, but its application requires robust justification (Escher et al., 2019; Rovida et al., 2020). Given the breadth of information supporting the read-across, it is useful to provide data tables that show a normalized magnitude of change (e.g., percent change relative to control) for similar critical effects, to aid interpretation of biological significance across toxicity studies and databases for a new active substance and read-across chemicals.

One of the challenges that arose was data availability for structurally similar chemicals. Without publicly-available data, it may not be possible to include all relevant chemicals in the read-across exercise. Likewise, reliable regulatory reviews may not be available for all chemicals, or reviews may be available from different agencies with differing interpretations. In the case of differing regulatory conclusions, choices must be made as to which positions to use in the read-across, and these decision points should be transparent and documented in the read-across assessment. While there may have been a larger library of structurally similar chemicals available for each of the case studies, only a subset was available in the public domain and in regulatory reviews. One weakness of limiting the chemical analogues (source chemicals) in this way is the potential to introduce bias for compounds with lower toxicity (i.e., chemicals that have successfully achieved development and registration). It was also recommended to apply a structured evaluation approach, a globally harmonized approach for consistency in assessing the relevance and reliability of a read-across analogue (Boobis et al., 2006; Moustakas et al., 2022).

It was noted in the reviewers' feedback that mechanistic understanding of the treatment-related effects of an active substance and structurally similar chemicals, and the ability to compare the toxicity profiles in terms of dose-response and duration, provided the strongest read-across evidence to estimate a protective POD for human health risk assessment.

4.2 Mode of action

Another key strength of the framework was the emphasis on using mechanistic understanding of carcinogenicity (such as mode of action, adverse outcome pathways, and human relevance) to evaluate human risk, including targeted investigative studies if necessary. Specifically, mode of action research supports a better understanding of the biological response; through such understanding, the human relevance, and health-protective thresholds (e.g., occurrence of key events) can be identified.

To bring increased consistency to MOA evaluation it was recommended during the technical peer reviews that possible MOAs and/or AOPs be considered systematically during the WOE assessment. and included in the framework to prompt the registrants to include this in the assessment. To develop a sustainable framework, relevant MOAs and/or AOPs to evaluate would be helpful to streamline the WOE and should be adaptable to evolve over time. It is important to consider the relevant MOA that may drive the chronic toxicity risk of a chemical; however, this framework is not designed to be prescriptive on which tools or studies must be used. As highlighted in this ReCAAP framework, each weight of evidence assessment should be evaluated on a case-by-case basis, using scientifically sound relevant MOA based on the available data. Additional feedback on the MOA research for each case study highlighted the strength of the available data. Some potential MOAs were accepted as adequately addressed (e.g., rat thyroid effects secondary to liver enzyme induction) while certain alternative MOAs were considered only partially addressed (e.g., receptor-mediated MOA) or not sufficiently addressed (e.g., altered apoptosis). Further research may be required to strengthen the MOA assessment of certain effects of concern, and iterative engagement with the Regulatory Agencies could help to identify database uncertainties and ensure an acceptable MOA assessment strategy.

4.3 Transparency

Another key learning from the case studies was the need for transparency in the rationale used to assess the safety of the target compound with the available data and read-across analogues. Depending on the individual case study, various lines of evidence may be deemed more or less informative and relevant to the overall WOE. For instance, read-across may be highly useful in some cases (e.g., in the SDHI case study), but in other cases may not be strong enough to predict chronic and carcinogenicity risk for certain endpoints (e.g., in the GABA-Cl case study). While there may be a common pesticidal MOA across the chemicals used in the read-across, the off-target effects and biological response may be different. Thus, the selection of read-across analogs must be adequately justified.

The MOA data is generally expected to be an impactful line of evidence. The use of GLP OECD guideline studies (i.e., regulatory approved study design and quality), as well as any publicly available relevant information on the chemical analogues, strengthened these assessments.

The Syngenta prospective case study reviews provided useful feedback and guidance on options to improve and increase the acceptance of the ReCAAP framework. Recommendations from the ReCAAP Work Group technical reviews included the provision of a consistent and structured approach in the methodology used for read-across, including well-articulated criteria and a transparent decision tree used for selecting read-across chemicals. Additionally, presenting more information on similarity grouping, mechanistic data, and mammalian mode of action research to support the read-across rationale would increase the confidence and strengthen the ability to compare toxicity profiles, and thus inform on an endpoint outcome for a new target active substance.

5 Next steps for this framework

The WOE-based ReCAAP framework is designed to integrate several different types of toxicological evidence, which can include regulatory-required guideline toxicity studies, chemical read-across, and mechanistic new approach methods (e.g., *in vitro* assays,

toxicogenomics). In some cases, the selection of and confidence in each line of evidence will have addressed the outcomes of concern in all data streams. In others, there may only be data available. In each case, the data integration process considers the findings described in the qualitative and/or quantitative data selection and the certainty of the evidence for each outcome, to determine conclusions that directly address the human health and safety of the target compound. Looking forward, as the guidance for this approach is further developed, it may be useful to involve a matrix-based approach (e.g., a matrix describing how the confidence in the lines of evidence are combined to reach different hazard conclusions, or techniques for eliciting expert knowledge) to support the needs for regulatory risk assessment. In general, higher confidence in the lines of evidence results in stronger conclusions. The use of mechanistic data is particularly valuable to support evaluation of biological plausibility with hazard conclusions or extrapolation approaches in dose-response assessments.

The three Syngenta prospective case studies presented here demonstrate the utility of the developed ReCAAP framework to a) assess confidence in evaluating potential for carcinogenicity without the conduct of the rodent cancer bioassays, b) estimate a POD for chronic risk assessment, and c) assess the relevance and reliability of the lines of evidence identified and selected for the read-across and WOE analysis. This modern approach can be applied to a range of different endpoints that are of common concern for safety assessment. Moreover, the framework is demonstrated to be transparent and scientifically sound, such that it is ready to implement into human health risk assessment. Further, with the key learnings during the WOE assessment, feedback and learnings from the technical reviews, and recommendations presented herein, this approach can be refined further to address all uncertainties and facilitate the development of guidance for more efficient, fit-for-purpose, human-relevant and equally health-protective safety, and risk assessment of chemicals. Efforts are now underway to establish a decision-making framework in the form of an Integrated Approach to Testing and Assessment (IATA) for guiding data collection, evaluating reliable and relevant information, and the decision-making process. As registrants and regulators continue to gain experience with the application of this framework to new chemicals, similar to our experience through these case studies, we anticipate further progress and acceptance of WOE rationales to support regulatory decision-making and protection of human health, without requiring long-term animal testing to evaluate chronic and carcinogenicity risk.

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Conflict of interest

Authors AG, NR, AS-V, CL, and DW were employed by Syngenta Crop Protection LLC.

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An insight into carcinogenic activity and molecular mechanisms of Bis(2-ethylhexyl) phthalate

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Di(ethylhexyl) phthalate (DEHP) is a ubiquitous environmental contaminant to which humans are exposed via multiple routes. Human health risk assessments for this substance have recently been updated, focusing on reproductive toxicity, including DEHP, in the list of chemicals classified as carcinogenic, mutagenic, or toxic to reproduction (CMR). Moreover, DEHP has also been defined as probably and possibly carcinogenic to humans based on its carcinogenicity in rodents. However, the mechanism of action of DEHP and its relevance in humans remain unclear. Rodent data suggests that DEHP induces cancer through non-genotoxic mechanisms related to multiple molecular signals, including PPARα activation, perturbation of fatty acid metabolism, induction of cell proliferation, decreased apoptosis, production of reactive oxygen species, and oxidative stress. According to the DEHP toxicological dataset, several *in vitro* cell transformation assays have been performed using different protocols and cellular models to produce different results. This study aimed to evaluate the carcinogenic potential of DEHP by using the A31-1-1 BALB/c-3T3 cell line in a standard cell transformation assay. Additionally, transcriptomic analysis was performed to explore the molecular responses and identify the affected toxicological pathways. Although DEHP treatment did not induce transformation in BALB/c-3T3 cells, the transcriptomic results revealed significant modulation of several pathways associated with DEHP metabolism, tissue-specific functions related to systemic metabolism, and basal cellular signaling with pleiotropic outcomes. Among these signaling pathways, modulation of cell-regulating signaling pathways, such as Notch, Wnt, and TGF-β, can be highlighted. More specific modulation of such genes and pathways with double functions in metabolism and neurophysiology underlies the well-known crosstalk that may be crucial for the mechanism of action of DEHP. Our findings offer evidence to support the notion that these models are effective in minimizing the use of animal testing for toxicity assessment.

KEYWORDS

bis(2-ethylhexyl) phthalate, non-genotoxic carcinogens, alternative methods, transcriptomics, toxicogenomics, cell transformation assay, cytotoxicity, transformics

1 Introduction

Di(2-ethylhexyl) phthalate (DEHP; CAS No. 117-81-7), a chemical belonging to the phthalate family, is a synthetic substance that is commonly incorporated into plastics to increase their flexibility. DEHP is particularly noteworthy as it is the index compound of the class for group-tolerable daily intake (TDI) calculations because it possesses the most extensive toxicological dataset among its counterparts.

Phthalates are widely used in various commercial products and as packaging materials. Because they are non-covalently bonded to polyvinyl chloride (PVC), they can be easily released by plastics in the surrounding matrices, generating widespread pollution that affects the environment worldwide and poses a greater exposure risk to the general population (Rowdhwal and Chen, 2018). DEHP metabolites have been detected in human bodily fluids (Wang et al., 2019). DEHP can be absorbed via the dermal, inhalation, and oral routes. Once ingested, DEHP is rapidly metabolized in the liver, producing approximately 30 different metabolites that are promptly excreted in the urine as glucuronide conjugates (Hauser and Calafat, 2005). DEHP is first hydrolyzed to mono(2-ethylhexyl) phthalate (MEHP). Subsequently, MEHP is metabolized by cytochrome P450 enzymes, specifically human CYP2C9(*)1, CYP2C9(*)2, CYP2C19, and rat CYP2C6 (Choi et al., 2012) to generate oxidative and dealkylated metabolites. The most common metabolites of MEHP are mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP). These metabolites have been frequently detected in biological samples (Koch et al., 2006).

Over time, research has hinted at the potential toxicological and carcinogenic effects of phthalates in humans, prompting regulatory measures in the European Union to limit their use. However, the evidence remains suggestive rather than conclusive. The carcinogenic potential of DEHP has been assessed by various regulatory authorities, and conclusions have changed over time.

DEHP causes cancer and reproductive, developmental, nerve, immune, and endocrine disruptions in rodents (Rowdhwal and Chen, 2018). After much debate, 11 types of phthalates, including BBP, DBP, and DEHP, have been classified as reproductive toxicants in category 1 B (suspected reproductive toxicants) according to the carcinogenic, mutagenic, or toxic to reproduction (CMR) classification (SCHEER, 2019).

The overall weight of evidence suggests that DEHP is not genotoxic, but can induce hepatic tumors in mice and rats, with some inconclusive evidence of testicular and pancreatic tumors (Madia et al., 2020; NTP, 2021) (Table 1). However, extrapolation of these results to humans has not yet been proven.

The main mechanism involved in rodent hepatotoxicity and hepatocarcinogencity of DEHP is transactivation of peroxisome proliferator-activated receptor alpha (PPARα) signaling, which is physiologically involved in the regulation of lipid metabolism and glucose homeostasis. Perturbation of this signaling pathway is thought to have little or no relevance in humans (Hasmall et al., 2000; Isenberg et al., 2000; Colacci et al., 2023).

The current body of evidence does not conclusively establish a causal relationship between DEHP exposure and cancer development. Although many scientists acknowledge that the lack of carcinogenicity of DEHP in humans is primarily based on indirect evidence and peroxisome proliferation cannot be definitively identified as the sole mechanism of DEHP carcinogenicity, the possibility of DEHP tumorigenesis via non-PPARα pathways, such as nuclear factor kappa B (NFκB), androstane receptor (CAR), and pregnant X receptor (PXR), remains unclear. *In vivo* studies employing PPARα-null mice and PPARα-humanized mouse carcinogenicity tests have yielded conflicting results, with some evidence of DEHP hepatocarcinogenesis in both genotypes; however, these findings remain controversial (Ito et al., 2007; Corton et al., 2018; Foreman et al., 2021a; Foreman et al., 2021b; Colacci et al., 2023).

Additionally, the tumor-promoting activity of DEHP has been investigated, and research points to its potential to promote the progression of hormone-related lesions and increase the risk of

TABLE 1 Comprehensive genotoxicity and carcinogenicity assessment results of DEHP from the EURL ECVAM genotoxicity and carcinogenicity consolidated database of Ames-negative chemicals (Koch et al., 2006).

Genotoxicity and carcinogenicity assay	Overall result[a]
AMES Tests (OECD 471 TG): both in the presence and absence of an exogenous source of metabolic activation	Negative
***In vitro* Mammalian Cell Gene Mutation (MCGM) assays:** mouse lymphoma Tk+/− mutation assay, Hprt mutation assay and human TK6 cells mutation assay	Negative
***In vitro* Mammalian Cell Micronucleus Test**	Negative
***In vitro* Comet Assay**	Negative
***In vivo* Mammalian Cell Micronucleus Test**	Negative
***In vivo* Comet Assay**	Negative
Transgenic rodent gene mutation assays (TGR)	Equivocal
***In vivo* unscheduled DNA synthesis**	Negative
***In vivo* Comet Assay:** in stomach, colon, liver, kidney, bladder, lung, brain and bone marrow of male mice via gavage; in stomach, liver and bone marrow of male rats via gavage	Negative
Rodent Carcinogenicity	Positive

[a]Overall result refers to the final call provided by EURL ECVAM applying specific criteria, including quality and the robustness of the study.

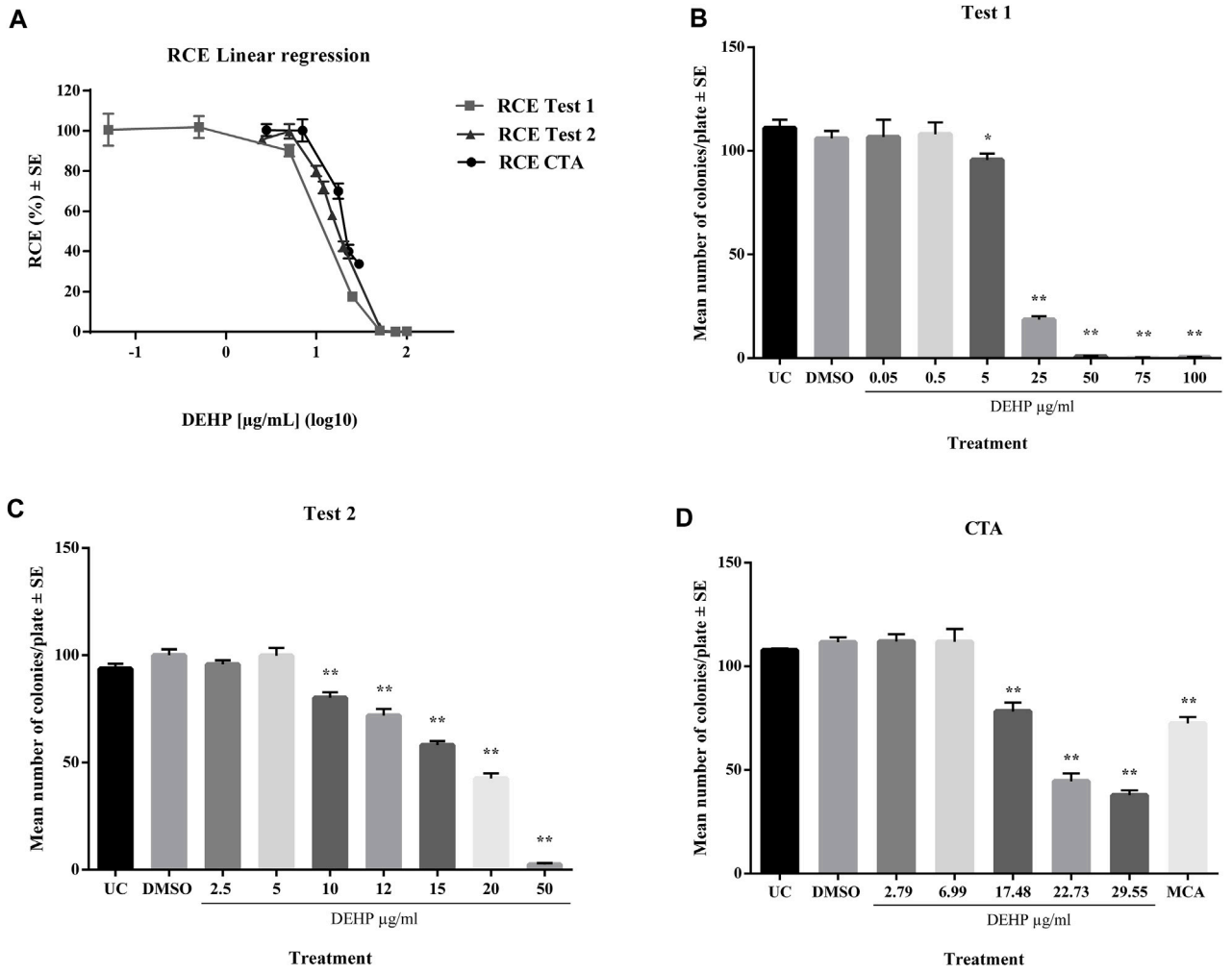

FIGURE 1
(A) Comparison of cytotoxicity assay results expressed as RCE linear regression (χ2-test). **(B)** Preliminary cytotoxicity assay 1 results expressed as the mean number of colonies ±SE. *$p \leq 0.05$ vs. vehicle control, t-test. **$p \leq 0.01$ vs. vehicle control, t-test. **(C)** Preliminary cytotoxicity assay 2 results expressed as the mean number of colonies ±SE. *$p \leq 0.05$ vs. vehicle control, t-test. **$p \leq 0.01$ vs. vehicle control, t-test. **(D)** Cell Transformation assay (CTA) concurrent cytotoxicity (CC) results expressed as the mean number of colonies ±SE. *$p \leq 0.05$ vs. vehicle control, t-test. **$p \leq 0.01$ vs. vehicle control, t-test. Abbreviations: SE: standard error; DMSO: Dimethyl sulfoxide 0.5%; MCA: 3-Methylcholanthrene 4 µg/µL

various cancers, including breast (Wu et al., 2021; Mukherjee Das et al., 2022), thyroid (Marotta et al., 2019; Liu et al., 2020), ovarian (Leng et al., 2021), and prostate (Chuang et al., 2020; Colacci et al., 2023; Guo et al., 2023) cancers.

Based on these results, the International Agency for International Research on Cancer (IARC) and US-EPA classified DEHP as a possible carcinogen (2 B substance suspected of causing cancer), subject to multiple mechanisms and pathways simultaneously involved, related to a non-genotoxic Mode of Action (MoA) (IARC, 2013). However, the European Chemicals Agency (ECHA) does not warrant classification for carcinogenicity, as the risk assessment conducted under Regulation (EC) N° 1907-2006 (REACH) does not consider these data owing to the derivation of the Dose-Response for Exposure Assessment (DNELs) for DEHP from reproductive toxicity data.

In addition to *in vivo* carcinogenicity data, controversial results were obtained by testing DEHP in cell transformation assays (CTAs) (Supplementary Table S1). CTA is a valuable *in vitro* test used to assess the carcinogenic potential of both genotoxic and non-genotoxic chemicals as well as environmental agents. CTAs use cultured mammalian cells to measure their ability to undergo malignant transformation in response to a test substance (Colacci et al., 2023). All CTAs provide an easily detectable endpoint for morphological transformation, anchoring chemical exposure to the acquisition of the malignant phenotype. Moreover, the application of transcriptomic approaches to CTAs offers a powerful means to elucidate the mechanisms underlying the carcinogenic potential of the tested substances (Mascolo et al., 2018; Pillo et al., 2022; Colacci et al., 2023).

Although CTA is considered insufficient for classifying chemicals as carcinogens on its own, it is a crucial component integrated approach to testing and assessment (IATA) for non-genotoxic carcinogens (NGTxC) based on leveraging omics technology, particularly transcriptomics, to gain a more nuanced mechanistic understanding of the behavior exhibited by the tested chemical (Jacobs et al., 2016; Jacobs et al., 2020; Oku et al., 2022).

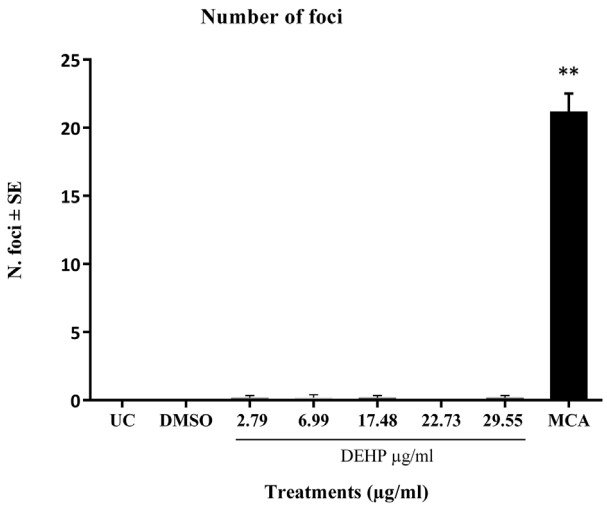

FIGURE 2
Cell Transformation assay (CTA) results expressed as the mean number of foci ±SE. **$p \leq 0.01$ vs. vehicle control, t-test. Abbreviations: SE: standard error; DMSO: Dimethyl sulfoxide 0.5%; MCA: 3-Methylcholanthrene 4 µg/µL. Cells in the logarithmic growth phase were seeded at a density of 1 × 104 cells/60 mm dish, with 10 dishes per treatment, in M10F culture media. After 24 h, the cells were exposed to the test item for 72 h. Untreated cells and solvent-treated cells served as the negative controls, while cells treated with MCA represented the positive controls. From day 8 post-seeding, the culture medium was replaced twice a week with DF2I2F containing a low concentration of FBS (2%) and insulin (final concentration 2 µg/mL). At 24 days post-seeding, no further medium changes were observed. At day 31-32 post-seeding, the plates were fixed with methanol and stained with 0.04% Giemsa. The occurrence of transformed Type III foci was assessed using an optical microscope, characterized by deep basophilic staining, random cell orientation, dense multilayering of cells, and invasion into the surrounding contact-inhibited monolayer (Sasaki et al., 2012b).

There are currently three widely used *in vitro* models for testing chemically induced transformations, which have been considered for inclusion in the IATA for NGTxC: the SHE model, BALB/c 3T3 model, and Bhas42 CTA, differing in the degree of cell progression towards transformation.

There is still an ongoing debate on whether the three CTA models are interchangeable or whether there should be criteria guiding the choice of one over the other based on their peculiarities and the characteristics of the tested chemicals (Colacci et al., 2023), as there are still some critical issues related to the use of the current experimental protocols of CTA.

In the absence of approved test guidelines, the OECD issued two guidance documents endorsing the use of CTA based on SHE and Bhas 42 cells. Additionally, ECVAM recommended a protocol for BALB/c 3T3 CTA following a pre-validation study (Tanaka et al., 2012), aiming to encourage feedback from further studies exploring the transforming abilities of chemicals to enhance the experimental protocols (Colacci et al., 2023).

In this context, DEHP is a paradigmatic compound that can be used to address critical issues.

DEHP has been listed as a potential non-genotoxic carcinogen and has been tested in rodents and two CTA models, yielding varied and inconclusive results, demonstrating primarily positive results in the SHE CTA but producing negative outcomes in the BALB/c 3T3 CTA (Colacci et al., 2023). The mechanisms underlying DEHP toxicity, including the initiating molecular event and the type of receptor involved, have not yet been fully elucidated. Furthermore, DEHP is a prototype chemical compound whose low solubility may lead to procedural issues in *in vitro* tests in cell cultures, according to good *in vitro* practices for the development and implementation of *in vitro* methods for regulatory use in human safety assessments (OECD, 2018).

Therefore, to enhance the full utilization of CTA in IATA for NGTxC, we conducted a study on DEHP to understand the reasons for the discrepancies observed in various CTA tests and to identify its mechanism of action as a possible non-genotoxic carcinogen.

To achieve our objective, a standard CTA protocol using A-31-1-1 BALB/c-3T3 cells (Sasaki et al., 2012a; Corvi et al., 2012; Tanaka et al., 2012) was conducted, followed by transcriptomic analysis using the so-called transformics assay.

Transformics provides a comprehensive view of the entire process from chemical exposure to the final outcome, thereby elucidating the molecular mechanisms underlying oncotransformation. Gene modulation data were collected at key time points throughout the experimental protocol, allowing for detailed analysis of the molecular events driving the transformation process.

The transformics approach was developed to bridge gaps in mechanistic knowledge related to *in vitro* cell transformation, reconciling apparently conflicting data from CTA studies, supporting the integration of CTA within the IATA for NGTxC, and serving as a foundation for refining thresholds derived from *in vitro* experiments.

Indeed, the application of transcriptomic analysis to CTA has highlighted a cascade of key molecular events underlying *in vitro* oncotransformation, mirroring critical steps observed in human cancer progression. This comprehensive understanding, extensively discussed previously (Colacci et al., 2023), underscores the relevance of CTA results in human cancer pathogenesis and affirms the translational potential of these findings (Colacci et al., 2023).

Furthermore, transcriptomic analysis applied to CTA revealed the activation of receptor-mediated pathways crucial for metabolic processes, facilitating both bioactivation and detoxification of chemicals. This approach also provides insights into the molecular initiating events that drive chemically induced toxicity.

This investigation was intended to provide essential information for evaluating the feasibility of the proposed method for fulfilling the criteria for regulatory toxicology. These results are critical for endorsing the potential incorporation of this method into an integrated approach to testing and assessment (IATA) designed for NGTxC (Jacobs et al., 2020; Oku et al., 2022; Pillo et al., 2022). In addition, this study aimed to elucidate the toxicological behavior of DEHP.

2 Materials and methods

2.1 Cells

Mouse embryo BALB/c 3T3 fibroblasts (clone A31-1-1) were obtained from the Health Science Research Resource Bank and were stored in liquid nitrogen. Cells at passage three were used for the

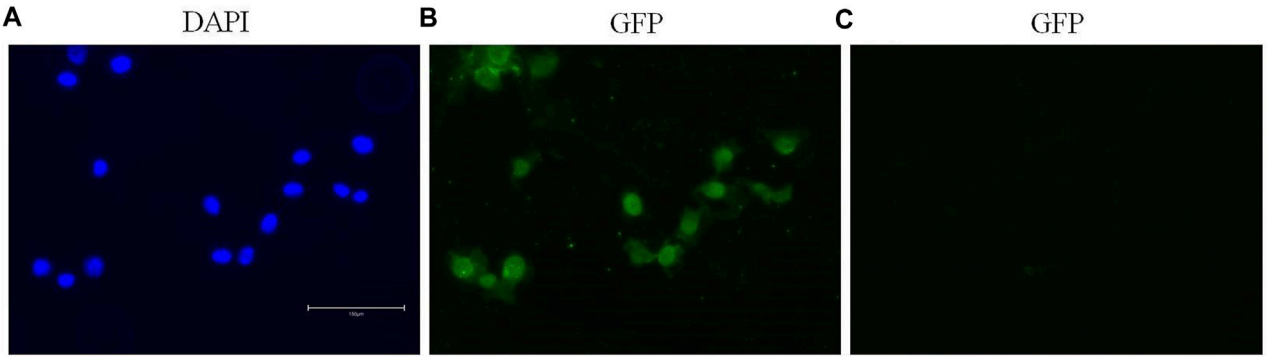

FIGURE 3
The first ten pathway maps with a False Discovery Rate (FDR) less than 0.05, as identified using the "Filter Pathway Maps by Category" function in MetaCore, categories: "Tox processes," modulated by DEHP treatment in the BALB/c 3T3 A31-1-1 cell model. Produced with MetaCore.

preliminary cytotoxicity assay, whereas cells at passage one were used for CTA. Cells were seeded at a density of 125,000 cells/T75 flasks. Cells were cultured until they reached 70% confluence in M10F medium, which consisted of Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS; Gibco BRL) and 1% 10,000 U/mL penicillin–10 mg/mL streptomycin.

2.2 Chemicals

Bis(2-ethylhexyl) phthalate (PESTANAL®), an analytical standard (DEHP, CAS No: 117-81-7, ≥98.0% purity, SIAL 36735), was used. Dimethyl Sulfoxide (DMSO, CAS number 67-68-5, Hybri-max Sterile, Sigma/D2650) was employed as the vehicle and solvent for the tested chemicals. Several studies have identified challenges in conducting assays for DEHP, particularly related to its poor miscibility and solubility in polar solvents despite the use of organic solvents as vehicles. In our literature review, we observed that several DEHP CTAs were performed at high concentrations, and many studies used 0.1% DMSO or other solvents as vehicles (Supplementary Table S1). Therefore, in this study, particular attention was paid to the dissolution of DEHP in cell media, leading to the use of a final concentration of 0.5% DMSO.

A concentrated solution of the chemicals in DMSO was prepared and serial dilutions were prepared from this solution. Vigorous vortexing was performed for approximately 5 min to ensure complete solubilization of the test items. During this experiment, DEHP was readily dissolved in DMSO without any increase in the turbidity.

The solubility of DEHP in water is 0.00003% (23.8°C); therefore, its solubility might decrease as the volume of DMSO decreases and the volume of cell culture medium increases.

The dissolution behavior of DEHP in DMSO and the stability of the stock solutions in cell medium were evaluated using a simple test and direct visual observation. The working solutions were incubated under test conditions (37°C, 5% CO2, and 90% relative humidity) for 72 h, and periodic checks were conducted to detect the presence of precipitates.

The working solutions were obtained by two groups of dilutions of the DMSO stock solutions in M10F: 1:1,000 and 1:200, resulting in final DEHP concentrations of 100, 75, and 25 μg/mL for each group. At the end of the procedure, solutions with a final DMSO concentration of 0.1% exhibited turbidity, and small oil droplets formed in the suspension were faintly visible to the naked eye.

Therefore, the final DMSO concentration of 0.5% was deemed more suitable for this experiment.

2.3 Transformics experimental protocol

The experimental protocol included a preliminary cytotoxicity assay, cell transformation assay including a concurrent cytotoxicity test, and transcriptomic experiment.

2.3.1 Preliminary cytotoxicity assays

Two preliminary cytotoxicity assays were performed, covering a concentration range of 0.05–100 μg/mL, corresponding to 0.05 μL/mL to 102 μL/mL, in order to identify the range of DEHP concentrations to be tested in further experiments. Based on the results of the preliminary cytotoxicity assay, the following concentrations were used in the cell transformation assay: 2.79 μg/mL, 6.99 μg/mL, 17.48 μg/mL, 22.73 μg/mL, and 29.55 μg/mL. Transcriptomic experiments were conducted using cells treated with a cytotoxic concentration of DEHP 19.7 μg/mL for 24 h and 72 h.

2.3.2 Cell transformation assay

The transformation assay was performed by applying the standard BALB/c-3T3 A-31-1-1 CTA ECVAM DB-ALM Protocol N. 137 (Sasaki et al., 2021a; Corvi et al., 2012; Tanaka et al., 2012; IARC, 2013; Mascolo et al., 2018). Cells in the logarithmic growth phase were seeded at a density of 1×10^4 cells/60 mm dish, with 10 dishes per treatment, in M10F culture media. After 24 h, the cells were exposed to the test compounds for 72 h. Untreated and solvent-treated cells served as negative controls, whereas MCA-treated cells were used as positive controls.

From day 8 post-seeding, the culture medium was replaced twice a week with DF2I2F, containing a low concentration of FBS (2%) and insulin (final concentration, 2 μg/mL). After 24 days post-seeding, no further medium changes were undertaken on day 31-32 post-seeding, and the plates were fixed with methanol and stained

TABLE 2 Differentially expressed genes within the pathway "PXR-mediated direct regulation of xenobiotic metabolizing enzymes/Rodent version," are involved in the regulation of lipid homeostasis.

Gene symbol	FC	Enzymatic activity
CYP11A1	1.61	Cytochrome P450 (Phase 1 metabolic enzyme)
CYP27A1	1.61	Cytochrome P450 (Phase 1 metabolic enzyme)
CYP2C19	1.58	Cytochrome P450 (Phase 1 metabolic enzyme)
CYP2C8	23.92	Cytochrome P450 (Phase 1 metabolic enzyme)
CYP2C9	4.21	Cytochrome P450 (Phase 1 metabolic enzyme)
CYP3A5	1.60	Cytochrome P450 (Phase 1 metabolic enzyme)
CYP3A7	1.53	Cytochrome P450 (Phase 1 metabolic enzyme)
ELOVL6	−1.56	Elongation of very long chain fatty acids protein 6 (Lipid metabolism)
MDR1	3.17	Multidrug resistance protein 3 Protein (Renal secretion)
SLC21A7	−2.48	Solute carrier organic anion transporter family member 1A5 Protein (Cholehepatic circulation of bile acids)
UGT1A1	2.58	UDP-glucuronosyltransferase (Phase 2 metabolic enzyme)
UGT1A6	5.40	UDP-glucuronosyltransferase (Phase 2 metabolic enzyme)

with 0.04% Giemsa stain. The occurrence of transformed Type III foci, characterized by deep basophilic staining, random cell orientation, dense multilayering of cells, and invasion into the surrounding contact-inhibited monolayer, was assessed using an optical microscope (Sasaki et al., 2012b).

2.3.3 Transcriptomics experiment

2.3.3.1 Total RNA extraction

Cells in the logarithmic phase of growth were seeded at a density of 1×10^4 cells per 60 mm diameter dish using the CTA culture protocol. Twenty-four hours after seeding, cells were treated with 19.70 μg/mL DEHP or 0.5% DMSO as a control. Total RNA was isolated after 24 h and 72 h of exposure using TRIzol Reagent (Invitrogen, San Diego, CA, United States), followed by purification with an RNeasy affinity column (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. RNA quality was assessed using an Agilent 4200 TapeStation system (Agilent RNA ScreenTape Analysis Kit) and NanoDrop OneC. Four type 1 biological replicates were obtained for each treatment (19.70 μg/mL DEHP and 0.5% DMSO).

2.3.3.2 Total RNA labeling and hybridization

cRNA was labeled, purified, and hybridized on oligonucleotide slides (SurePrint G3 Mouse Gene Expression v2 8 × 60 K Microarray Kit) using the Low Input Quick Amp Labeling Kit, version 6.9.1, December 2015 (Agilent Technologies, Santa Clara, CA, United States) (www.genomics.agilent.com HYPERLINK http://www.chem.agilent.com/, accessed on 13 Oct 2023). Four arrays were hybridized with the treated cell lysate, and four with the control lysate, for each time points. Slides were scanned using an Agilent SureScan Microarray Scanner G2600D.

2.3.3.3 Statistical analysis of microarray data

The image data were extracted using Feature Extraction Project software and analyzed using Agilent GeneSpring 14.9.1. For this study, differentially expressed genes were identified according to the following criteria: unpaired t-test p (Corr) cut-off = 0.05, with Benjamini Hochberg False Discovery Rate correction. In addition, a t-test unpaired p (Corr) cut-off = 0.05 adjusted by Bonferroni was also performed in order to make a comparison.

2.3.3.4 Tools of biological interpretation

The lists of differentially expressed genes were imported into MetaCore software V6.34 (Clarivate Analytics (https://portal.genego.com/, accessed on 15 Oct 2023). Enrichment analysis was performed using the Analyze Single Experiment workflow with a fold-change cutoff of 1.5.

2.4 Immunofluorescence staining

2.5×10^4 BALB/c 3T3 fibroblasts, clone A31-1-1 were cultured in ibidi μ-Slide 8 Wells, fixed in 4% paraformaldehyde for 30 min and then permeabilized with 0.2% Triton X-100. The cells were then treated with blocking solution (dPBS + 2.5% BSA) for 20 min at room temperature. The cells were then incubated with the primary antibodies (ab61182; Abcam, Shanghai, China) at room temperature for 1 h. The cells were then incubated with a secondary fluorescent-conjugated IgG (Alexa Fluor 488- IgG) (ab150077, Abcam) at room temperature for 1 h. The primary antibody dilution was 1:200, and the secondary antibody dilution was 1:500. After 1 h, the cells were washed thrice with dPBS. Hoechst staining was used to counterstain the nuclei. An inverted fluorescence microscope was used to capture the images.

3 Results

3.1 Cytotoxicity assay

In a preliminary cytotoxicity study, 13 concentrations ranging from 0.05 to 100 μg/mL, were explored through two clonal efficiency

TABLE 3 Pathway map ontology enrichment analysis: Top 10 statistically significant pathway maps modulated by DEHP treatment (19.7 μg/mL) for 72 h in BALB/c 3T3 A31-1-1 cells.

PathwayMap	Regulated objecks	*p*-value	FDR	Biological interpretation	Upregulated genes	Downregulate genesd
Apoptosis and survival_Granzyme A signaling	18/41	3.971E-10	3.953E-07	Apoptosis	IL-6, Collagen IV, Fibronectin, Histone H1, Histone H2B	APEX, HSP70, IFN-alpha, Lamin B1, HMGB2, NDPK A, Histone H3, hnRNP A2, PARP-1, TLR4, SET, hnRNP C, hnRNP A1
Oxidative stress_ROS signaling	30/108	5.306E-10	3.953E-07	Oxidative stress	PTEN, TfR1, p300, HES1, ADAM17, VEGF-ATXNIP (VDUP1), ATM, NOTCH1 (NICD), c-Abl, PLK3 (CNK), GADD45 alpha, p38 MAPK, MDM2, PKC, IL-6, NRF2, COX-2 (PTGS2), ERK1/2	ACACA, APEX, Bax, Cyclin B1, E2I, FASN, iNOS, GRP75, PUMA, PKA-reg (cAMP-dependent), SCD
Immune response_IL-6 signaling via JAK/STAT	21/71	6.281E-08	3.119E-05	Immune response inflammation	TEC, CDK4, Rac1, p300, ADAM17, VEGF-A, c-Fos, AP-1, IL-6 receptor, IL6RA, sIL6-RA, SHP-2, ADAM10, JunB, gp130, IL-6, CDK6, COX-2 (PTGS2)	p18, iNOS, RacGAP1
Signal transduction_RANKL-dependent osteoclast differentiation	21/81	7.329E-07	2.730E-04	Immune response	TEC, NF-AT1(NFATC2), PI3K reg class IA, TCIRG1 (Atp6i), OSCAR, Syndecan-4, IFRD1, c-Fos, AP-1, MITFFra-1, p38 MAPK, CDK6, Calcineurin A (catalytic)	ATF-4, Calmodulin, iNOS, CREB1, MMP-9, PPA5, Rac1
DNA damage_ATM/ATR regulation of G2/M checkpoint: cytoplasmic signaling	16/51	9.653E-07	2.877E-04	DNA damage	MLCP (cat), PP1-cat, Cul1/Rbx1 E3 ligase, PP2A regulatory, Brca1, beta-TrCP, ATM, c-Abl, ATR, GADD45 alpha, p38 MAPK, p38gamma (MAPK12), ERK2 (MAPK1)	Cyclin B1, Histone H3, UBE2C
Signal transduction_Calcium-mediated signaling	19/72	1.838E-06	4.564E-04	cytoskeleton remodelling	MLCP (cat), p300, Tiam1, c-Fos, ACTA2, p38 MAPK, PKC, CaMKK, CaMKK2, MUNC13, Calcineurin A (catalytic), COX-2 (PTGS2), ERK1/2	MMP-9, NURR1, Calmodulin, CREB1, PPA5, Rac1
Signal transduction_mTORC1 downstream signaling	17/60	2.191E-06	4.664E-04	Metabolism Autophagy	Rictor, ATG13, eIF4A, VEGF-A, PPARγ, eEF2K, PDIP46, MDM2	ACSL3, ATF-4, CBP80, eIF4B, MTHFD2, YY1, MVK, RPS6, SCD
Immune response_IL-6 signaling via MEK/ERK and PI3K/AKT cascades	19/74	2.875E-06	4.760E-04	Immune response inflammation	TEC, PI3K reg class IA (p85), PI3K reg class IA, ADAM17, Proepithelin, c-Fos, IL-6 receptor, IL6RA, sIL6-RA, SHP-2, PLC-beta1, ADAM10, JunB, gp130, IL-6, ERK1/2	Bax, RPS6, CREB1
G-protein signaling_Rac1 activation	19/74	2.875E-06	4.760E-04	cytoskeleton remodelling	PI3K reg class IA, Rho GTPase, DOCK4, Tiam1, KIDINS220, DOCK7, PI3K reg class IB (p101), Dcc, CaMK II alpha, ALS2, Semaphorin 3A, AF-6, CaMKK2, EPS8	G-protein beta/gamma, Rac1-related, Rac1, SHANK, TrkB

(Continued on following page)

TABLE 3 (*Continued*) Pathway map ontology enrichment analysis: Top 10 statistically significant pathway maps modulated by DEHP treatment (19.7 μg/mL) for 72 h in BALB/c 3T3 A31-1-1 cells.

PathwayMap	Regulated objecks	*p*-value	FDR	Biological interpretation	Upregulated genes	Downregulate genesd
Eosinophil adhesion and transendothelial migration in asthma	18/68	3.259E-06	4.855E-04	Adhesion Inflammation	P-selectin, MGF, alpha-1/beta-1 integrin, C3aR, PLAU (UPA), Histamine H4 receptor, CD67, Collagen IV, Fibronectin, alpha-6/beta-1 integrin, p38 MAPK, PKC, ERK1/2	CCL5, MMP-9, C3a, Calmodulin, Eotaxin

The analysis was conducted using the Metacore™ software V6.34 (Clarivate Analytics; https://portal.genego.com).

tests. Cells were treated for 24 h after seeding and exposed for 72 h (Figure 1). The tested chemicals exhibited toxic effects in the concentration range 10–100 μg/mL. The cells treated with higher concentrations exhibited extremely low colony-forming activity. These results were confirmed by CTA (Figure 1). The IC50 value was calculated through interpolation and estimated to be approximately 17 μg/mL.

3.2 Cell transformation assay

The effect of DEHP on the transformation frequency of BALB/c 3T3 A31-1-1 cells was assessed according to the protocol recommended by ECVAM (Sasaki et al., 2021a; Corvi et al., 2012; Tanaka et al., 2012; IARC, 2013; Mascolo et al., 2018).

The positive control MCA (4 μg/mL) induced a statistically significant increase in the number of transformed type III foci, which were almost absent in untreated and solvent-treated cells (Figure 2).

DEHP treatment did not significantly increase the formation of malignant foci in BALB/c 3T3 A31-1-1 cells (Figure 2).

Based on these criteria, DEHP can be classified as negative on CTA.

3.3 Molecular data analysis

Based on GeneSpring analysis using the unpaired t-test ($p < 0.05$) and Benjamini-Hochberg multiple test correction, 13.164 genes were identified after the analyses, of which 7.870 were upregulated and 5.294 were downregulated. Next, using the unpaired t-test ($p < 0.05$) and Bonferroni multiple test correction, 334 differentially expressed genes were identified, of which 240 were upregulated and 94 were downregulated. The latter gene list constitutes a subset of the former because all genes are common (data not shown; available at https://www.ebi.ac.uk/biostudies/arrayexpress).

The first differentially expressed transcriptome dataset (n = 13.164 genes) was imported into the MetaCore™ integrated software suite and functionally processed for functional enrichment by "Pathway Map" ontologies using the Functional Ontology Enrichment tool. A fold-change threshold of ±1.5 was applied.

Pathway enrichment analysis helps highlight mechanistic insights into gene lists generated from genome-wide transcriptomic experiments. The Pathway map Ontology Enrichment Analysis scored and sorted 5,573 network objects and more than 200 perturbed pathway maps with false discovery rate (FDR) < 0.05 (Supplementary Table S2).

The filter Pathway Maps using the category MetaCore option were used to split the maps into four categories: metabolic maps, regulatory maps, toxicity processes, and disease maps (Supplementary Table S2; Supplementary Figures S1–S4). Each pathway map could be related to more than one category.

Regulatory maps resulted in the most represented category, which was analyzed in the discussion with particular attention to the top most significant pathways (Supplementary Figures S1–S3; Supplementary Table S2).

A focus on the Tox process-modulated map was proposed to analyze the dataset in view of toxicogenomics and the modulation of drug-metabolizing nuclear receptors and enzymes (Figure 3; Table 2).

The gene modulation observed after 72 h of exposure is reported in Table 3, where the top 10 modulated gene pathways and the genes involved in the modulation of each pathway are shown.

3.4 Immunofluorescence staining

Immunofluorescence staining permitted the detection and visualization of PPARα protein in the nuclear compartment of BALB/c-3T3 A31-1-1 cells (Figure 4).

4 Discussion

The primary objective of this study was to evaluate the potential of DEHP in standard CTA, using A31-1-1 BALB/c-3T3 cells.

Early attempts to develop omics-based CTA models revealed that most, if not all, key events and biological processes leading to oncotransformation are common to all the three current models of CTA. However, the gene transcript enrichment for each process highlights the ability of each model to emphasize different aspects of the process (Colacci et al., 2023). Primary SHE cells allow the identification of several gene signatures related to cytoskeleton remodeling, the first necessary condition for malignant changes, and events related to cell cycle control and senescence bypassing. Bhas 42 CTA is better suited for investigating mitogenic signals downstream of the activation of key oncogenes associated with RAS gene activation. BALB/c 3T3 CTA is an excellent model for

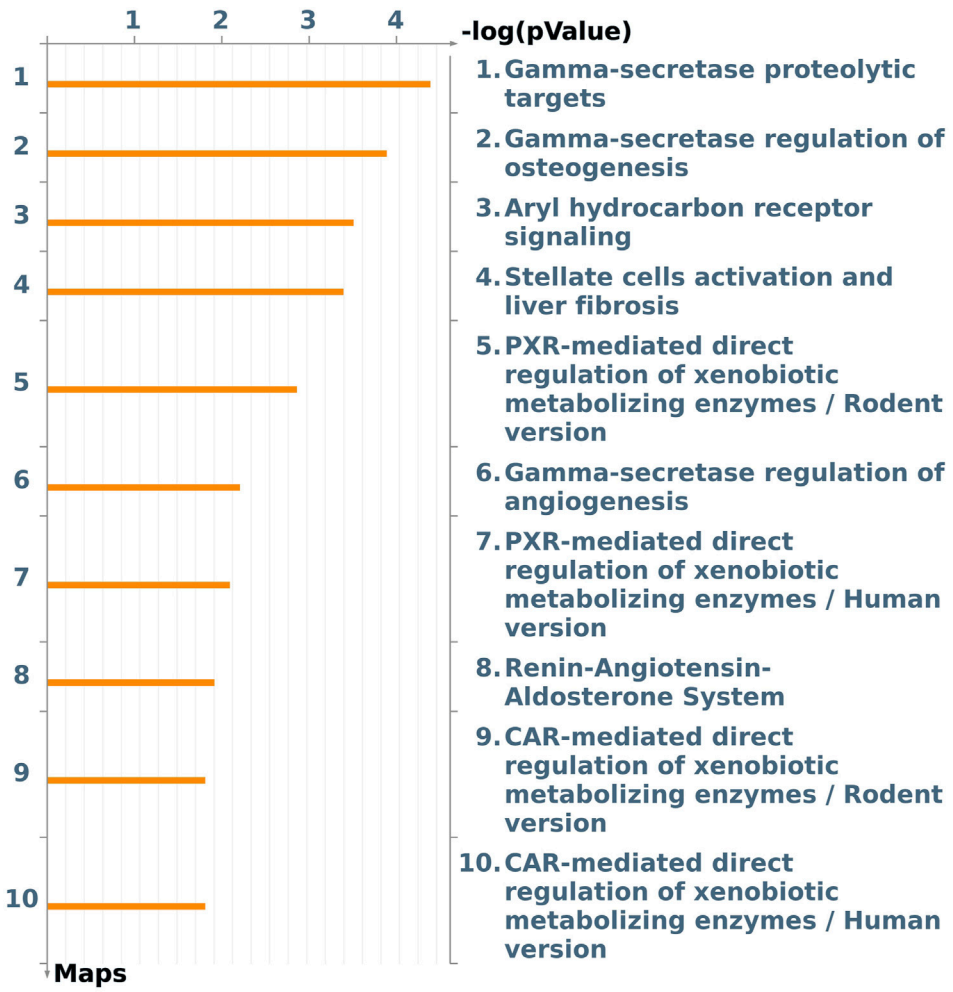

FIGURE 4
Expression of PPARα in BALB/c 3T3 clone A31-1-1 cells. Images were captured using the EVOS M5000 imaging system ×20 objective. **(A)** DAPI filter for the visualization of Hoechst staining. **(B)** GFP filter for the visualization of Alexa Fluor® 488 (anti-PPAR alpha antibody, Abcam ab61182). **(C)** GFP filter: negative control, cells treated with the secondary antibody only.

investigating the role of inflammasomes and immune-mediated inflammation in malignancy through epithelial-mesenchymal transition, which is recognized as the committed step at the tissue level that marks dysplasia progression and acquisition of invasive properties. Moreover, transcriptomic analysis applied to CTA revealed the activation of receptor-mediated pathways involved in metabolic processes that are crucial for both the bioactivation and detoxification of chemicals. Specifically, the BALB/c 3T3 CTA has been reported to be a suitable model for elucidating the role of the aryl hydrocarbon receptor (AhR) in the activation and detoxification of xenobiotics. Therefore, we selected this model to investigate the molecular initiating events that drive DEHP-induced toxicity, and the early molecular events that are possibly related to DEHP toxicity.

DEHP has been extensively tested in CTAs using various protocols and cell models over time (Supplementary Table S1), revealing predominantly positive outcomes in SHE CTA and negative results in BALB/c 3T3 CTA (Supplementary Table S1). Notably, the conventional SHE CTA protocol employed 0.2% (v/v) DMSO as the vehicle, and the exposure duration for the cells in this assay was 7 days. It is important to acknowledge that many BALB/c 3T3 assay studies have been conducted more than 20 years ago, exhibiting significant variability in the experimental conditions and vehicles used. Furthermore, numerous studies have been conducted at DEHP concentrations that surpass their solubility limits, complicating their interpretation. Additionally, despite the use of organic solvents such as DMSO, F68 Pluronic, and acetone as vehicles, several studies have emphasized the poor miscibility and solubility of the test item. Finally, the original CTA protocols have undergone substantial modifications and amendments over the years, potentially influencing observed outcomes (Colacci et al., 2023).

Therefore, one of the objectives of this study was to explore key factors concerning experimental conditions that may influence the ultimate outcome when working with poorly soluble chemicals in order to refine the CTA experimental protocols.

In this study, a particular focus was placed on the dissolution of DEHP in the cell media. The test chemical stock solution was prepared by dissolving DEHP in DMSO, and the stock solution was diluted in the culture medium at various concentrations. The

final concentration of DMSO in the cell medium was 0.5% (v/v), which was preferred over the more typical 0.1% (v/v) concentration to ensure homogeneous distribution of DEHP, as previously recommended (Sasaki et al., 2012a).

The results of clonal efficiency tests revealed a greater cytotoxic effect of DEHP in BALB/c 3T3 A31-1-1 cells than in previous *in vitro* studies (Supplementary Table S1). A concentration-dependent reduction in colony formation was observed at relatively low concentrations, beginning at 10 μg/mL, corresponds to 10.20 μL/mL.

We hypothesized that the higher concentration of DMSO used in this experiment would enhance the bioavailability of DEHP in cells, resulting in a more significant effect.

Notably, no increase in the cell transformation rate associated with DEHP exposure was observed in this study, which is consistent with the findings of previous studies that used the same CTA model.

It is widely recognized that results obtained from SHE and BALB/c 3T3 cell transformation assays can vary significantly, and various key events and biomarkers have been identified for each model (Colacci et al., 2023; Benigni et al., 2012). Moreover, it has been suggested that SHE may be more sensitive to a broader range of carcinogenic types than other cell transformation assays, as it detects more basic and nonspecific mechanisms and earlier stages of cell transformation (Colacci et al., 2023).

However, the precise mechanism by which DEHP induces malignant transformation in SHE cells remains unclear, despite evidence suggesting that it proceeds independent of PPAR activation (Tsutsui et al., 1993; Landkocz et al., 2011; Colacci et al., 2023).

More specifically, DEHP, MEHP, clofibrate, or WY-14,643 did not induce peroxisome proliferation in the SHE model when treated in the absence of exogenous metabolic activation, but DEHP was still able to induce cell transformation (Isenberg et al., 2000; Isenberg et al., 2001).

Furthermore, it should be noted that the inhibition of gap junctions intercellular communication (GJIC), peroxisomal β-oxidation and enhanced cell replication in rodent livers following DEHP, feeding have been identified as reversible effects. These effects persisted throughout the treatment period but were reversed upon discontinuation of the treatment. (Isenberg et al., 2000; Isenberg et al., 2001). Additionally, the inhibition of GJIC has been described as a transient effect in the SHE cell model (Cruciani et al., 1997).

It is reasonable to hypothesize that the unfavorable results observed in the BALB/c-3T3 CTA could be attributed to the shorter duration of chemical exposure compared to the standard 7 days exposure required in the SHE CTA. Indeed, DEHP failed to induce SHE cell transformation after a 24 h period (LeBoeuf et al., 1996). This difference may be noteworthy, because the mechanisms involved may be transient. It is important to mention that we carried out transcriptomic experiments on cells that had been treated with a toxic concentration of DEHP (19.7 μg/mL for 24 h and 72 h). This concentration is close to the half-maximal inhibitory concentration (IC50) value.

4.1 Regulation pathway maps

The pathway map with the lowest False Discovery Rate (FDR) is the "Protein folding and maturation_Amyloid precursor protein processing" pathway (pathway #1; FDR 7.303e-8, 25 modulated network objects out of 50). This pathway involves the amyloid precursor protein (APP) processing scheme, with APP mRNA being the primary gene involved, exhibiting a Fold Change (FC) of 1.81. Other genes involved in this pathway include matrix metalloproteinase 9 (MMP9; FC −3.14) and beta-secretase 2 (BACE2; FC 1.58) (Supplementary Table S2).

4.1.1 APP pathway

APP is a type 1 transmembrane glycoprotein that plays a critical role in neural transmission, neuronal homeostasis, and development. Alternative splicing generates APP mRNAs that encode several isoforms with tissue-specific and physiological functions. APP has been extensively studied as a precursor of amyloid β neurotoxic peptides in Alzheimer's disease. APP is particularly expressed in neuronal tissues and its expression is upregulated following brain injury (Liang et al., 2020).

Exposure to DEHP during early life or pregnancy has been linked to increased amyloid-β toxicity in *Caenorhabditis elegans* (Yen et al., 2021). Furthermore, animal and epidemiological studies have demonstrated a positive correlation between DEHP exposure in early childhood or maternal exposure during pregnancy and various neuropathologies and neurobehavioral diseases, suggesting a neurotoxic action of DEHP. This neurotoxicity has primarily been attributed to cellular oxidative damage, apoptosis, and ion channel imbalance (Liu et al., 2023).

APP has been found to be expressed in non-neuronal tissues and overexpressed in several types of cancer (Lee et al., 2021). Additionally, multiple fragments generated by the proteolytic processing of APP have been implicated in the regulation of cholesterol metabolism and may directly influence Low Density Lipoprotein Receptor (LDLR) expression (Wang et al., 2014).

Despite conducting an enrichment analysis, we did not identify any significantly modulated pathways specifically related to lipid metabolism and trafficking. However, we observed modulation of several genes related to these cellular processes, which will be discussed later. For example, we noted modulation of the LDLR (FC −1.79) and LDLR-related protein 1 (LRP1, FC −2.01).

BACE2, along with BACE1, has been extensively studied in the context of Alzheimer's disease, as both enzymes are responsible for processing APP into neurotoxic Aβ peptides. Conversely, BACE2 is ubiquitously expressed and can cleave APP at a site different from that of BACE1, producing non-neurotoxic peptides. BACE2 has also been linked to type 2 diabetes and tumor progression (Farris et al., 2021).

Interestingly, abnormal APP metabolism in the pancreas has been linked to type 2 diabetes, and recent epidemiological evidence suggests a strong association between diabetes and Alzheimer's disease (Hamzé et al., 2022).

4.1.2 POMC pathway

The second pathway map pertains to a distinct peptide processing mechanism, specifically, "Protein folding and maturation_POMC (pro-opiomelanocortin protein) (pathway #2) (FDR 2.518e-5)". POMC is a prohormone found in various tissues and undergoes extensive post-translational modifications, resulting in the generation of diverse sets of tissue-specific peptides that perform various biological functions (Raffin-Sanson et al.,

2003). The most well-studied POMC polypeptide is the 29-Kd polypeptide, which is post-translationally processed in the pituitary gland to form biologically active peptides, such as adrenocorticotropin (ACTH), endorphins (α-, β-, γ-EP), and melanotropins (α-, β-, γ-MSH). These peptides are involved in the regulation of the melanocortin pathway in response to leptin and insulin. The central melanocortin system plays a key role in regulating energy metabolism and body weight homeostasis, as evidenced in numerous recent studies (Li et al., 2023).

The "POMC, alpha-MSH, and AGRP in the regulation of food intake and energy expenditure in obesity in the hypothalamus" pathway map (pathway #149; FDR 1.118e-2; 13 modulated network objects out of 43) is highlighted for significant modulation. This pathway includes the overexpression of melanocortin receptor 4 (MCR-4, FC 1.69) and agouti-related neuropeptide (Agrp, FC 1.67), which act as antagonists of melanocortin receptor signaling, as well as the downregulation of brain-derived neurotrophic factor (BDNF, FC −1.62) (Supplementary Table S2).

These findings support the effects of neurotoxic and endocrine disruptors such as DEHP at the hypothalamic level in rodents (Lv et al., 2016). Wang et al. (2014) Lv et al. focused on the mechanisms underlying the impact of DEHP on the pathogenesis of obesity and hypothyroidism as well as the relationship between the two conditions, supported by the downregulation of thyroid hormone receptor beta (TR-beta) and Retinoid X receptors (RXR) genes in DEHP-treated C3H/He mice.

Our data revealed the modulation of two receptors for thyroid hormones: TR-beta and thyroid hormone receptor alpha (TR-alpha) [FC 2.95 for Thrb(A_51_P388835) and 1.58 for Thrb(A_52_P532559)] for TR-beta, and FC 1.66 for TR-alpha). It is important to note that, in the first two pathway maps, several network objects were derived from only one or a few modulated transcripts. Both pathways are characterized by upregulation of peptides, which have been studied for their potential "bridging roles" in metabolic regulation and neurophysiological implications.

4.1.3 FGFR pathway

The third pathway map in the list is "The Signal transduction_ Nuclear FGFR1 signaling" pathway #3; FDR 2.418e-5. Fibroblast growth factor (FGF) family signaling through the receptor tyrosine kinase FGF receptors (FGFR) regulates many cellular processes and plays essential roles in the early stages of embryonic development. In contrast to the first two pathway maps, this map consisted of 88 genes, 30 of which were modulated by DEHP treatment at 19.7 μg/mL for 24 h. FGF1 has emerged as a key regulator of bile acid, lipid, and carbohydrate metabolism, and in this pathway, FGFR1 is upregulated (FC 1.5). (Supplementary Table S2).

It is important to mention that FGFR1 has been proposed as a potential regulator of adipogenesis and may contribute to obesity by modulating the number of fat cells.

Although there was a slight upregulation of FGFR1, the overall trend of this molecular signaling appeared to be inhibited, as several downstream target genes were downregulated, whereas diverse downstream FGF-inhibited targets were upregulated.

Previous studies have shown that two sets of growth factors are necessary for efficient stimulation of DNA synthesis in murine BALB/c 3T3 fibroblasts. The first set includes platelet-derived growth factor (PDGF) and FGF, rendering the cells "competent" to enter the S phase. Competent cells respond to a second set of growth factors, including epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1), which allows the "progression" of cells into the cell cycle (Jones and Kazlauskas, 2001).

In our dataset, some FGF-related transcripts resulted in upregulation, as well as the EGFR ligand EGF, which showed an increase of 1.59 for Egf (A_55_P2733187) and 1.64 for Egf (A_55_P2822952). On the other hand, some PDGF-related transcripts resulted in downregulation, including PDGF-B, which showed a decrease of FC −2.43 for Pdgfb (A_55_P2047310) and FC −2.32 for Pdgfb (A_55_P2733467), and PDGF receptor subunit (PDGF-R-alpha), which decreased by FC −2.00 for Pdgfra (A_51_P345649), −1.71 for Pdgfra (A_55_P2734892), and −2.03 for Pdgfra (A_55_P2735715). Similarly, the insulin-related transcripts, Insulin substrate receptor-1 (ISR-1), and the Insulin-like growth factor 1 receptor Protein (IGF-1 Receptor), decreased by FC −2.21 for ISR-1, and FC −1.69 for Igf1r (A_52_P668647) and −1.72 for Igf1r (A_55_P2804885).

Notably, FGF1 plays a role in adaptive adipose remodeling (Wang et al., 2020; Sancar et al., 2022; Hamzé et al., 2022). FGF1 expression in adipose tissue is regulated by PPARγ and mice lacking FGF1 develop a more aggressive diabetic phenotype in response to dietary challenges (Sancar et al., 2022).

Additionally, among the extensively modulated "regulatory pathway maps," the regulation of metabolic pathways was highlighted using a MetaCore filter. Interestingly, among the last category of pathway maps, three significantly modulated pathways were highlighted: 1) "signal transduction_ WNT/β-catenin signaling in tissue homeostasis" (pathway #19) (FDR 2.010e-4; 17 modulated network objects out of 42); 2) "regulation of metabolism of GLP-1 signaling in beta cells" (pathway #34); (FDR6.727 e-4; 26 modulated network objects out of 91); and 3) "regulation of metabolism: glucocorticoid receptor signaling in glucose and lipid metabolism" (FDR 5.027e-2; 17 modulated network objects out of 80).

Overall, these transcriptome results support the toxic action of DEHP on cell metabolism, leading to impaired insulin signal transduction and the deregulation of glucose utilization and lipid synthesis.

DEHP causes obesity and hypothyroidism in both humans and rodents and induces lipid metabolism disorders, liver toxicity, and adrenocortical dysfunction (Tickner et al., 2001; Lv et al., 2016; Zhang et al., 2023). Evidence has shown that exposure to DEHP increases blood glucose levels, impairs energy metabolic balance, induces insulin resistance, and leads to prediabetes (Dales et al., 2018).

4.1.4 Possible involvement of PPAR regulation

Although the MetaCore pathway map "Regulation of lipid metabolism_PPAR regulation of lipid metabolism" did not exhibit significant modulation in this enrichment analysis (FDR 4.901e-1), several genes associated with PPARα signaling, fatty acid metabolism, and beta-oxidation were modulated in this experiment.

Fatty acid-binding protein 1 (Fabpl; FC 1.61) is upregulated, potentially facilitating fatty acid delivery to the nucleus and enhancing ligand-mediated transactivation of PPARα by directly binding to PPAR agonists (Hughes et al., 2015).

Additionally, the long-chain fatty acid transporter (Slc27a1, also known as FAT1; FC 2.32) was upregulated, suggesting the involvement of fatty acid transmembrane transporter activity, long-chain fatty acid import into cells, and the positive regulation of triglyceride biosynthetic processes.

L-bifunctional enzyme (Ehhadh; FC 1.59), also known as peroxisomal bifunctional enzyme protein, is part of the classical peroxisomal fatty acid β-oxidation pathway and is induced by PPARα activation. Long-chain-fatty-acid-CoA ligase 1 [ACLS1; FC 1.58 for Acsl1(A_51_P496432) and 1.51 for Acsl1(A_52_P597618)] was observed to convert long-chain fatty acids to acyl-CoA products via an ATP-dependent pathway and could be induced by both PPARα and PPARγ. Furthermore, the 3-ketoacyl-CoA thiolase peroxisomal protein [FC 1.94, Acaa1a (A_52_P155990) and 1.74 for Acaa1a (A_55_P2076580)], located upstream of or within fatty acid beta-oxidation and found in the mitochondria, exhibited modulation. The peroxisomal acyl-coenzyme A oxidase 3 protein (FC 1.81) has been implicated in the desaturation of 2-methyl-branched fatty acids in peroxisomes. Additionally, the carnitine palmitoyltransferase 1A (CPT-1A; FC 2.00), a key enzyme in the positive regulation of fatty acid beta-oxidation and insulin secretion regulation, and acyl-CoA synthetase long-chain family member 1 (ACSL1; FC 1.58 for A_51_P496432 and 1.51 for A_52_P597618) has been identified. CPT-1 was identified as a PPARα activation marker in DEHP-exposed mice (Lv et al., 2016).

PPARα-related toxicity induced by DEHP has been described as a series of events starting with receptor activation, resulting in peroxisome proliferation, induction of peroxisomal proteins, elevated fatty acid metabolism, increased cell proliferation and decreased apoptosis, production of reactive oxygen species, oxidative DNA damage, and inhibition of gap junctional intercellular communication. These events are associated with DEHP-induced hepatocarcinogenesis in rodents (Ito et al., 2007; Corton et al., 2014; Rajesh and Balasubramanian, 2014). It has been suggested that DEHP can stimulate the activation of PPARγ, leading to oxidative stress, downregulation of insulin receptor and GLUT4 protein expression, and disruption of insulin signaling (Mariana and Cairrao, 2023).

Initially, we confirmed PPARα expression in our cellular model to rule out the possibility that the negative outcome was due to the absence of what is commonly regarded as the primary receptor of DEHP.

In this study, we discovered the activation of certain peroxisomal proteins and regulation of genes involved in fatty acid metabolism. Furthermore, there was an indication of reduced insulin signaling, with an intriguing increase in the mRNA of the insulin-regulated glucose transporter GLUT4 (Slc2a4, Solute carrier family 2, facilitated glucose transporter member 4 Protein, FC 2.04) compared with the control.

However, the precise mechanisms governing cell survival and proliferation remain unclear. In the subsequent pathway map analysis, we highlighted the regulation of proliferation and extracellular matrix reorganization signaling. The pathway map "TGF-beta signaling via SMADs in breast cancer" (pathway #4) (FDR 4.910e-5) pertains to TGF-beta signaling and its role in breast cancer and its associated metastases. We observed a general reduction in the expression levels of these factors.

This finding suggests the potential inhibition of TGF-β signaling. Additionally, we identified other pathway maps connected to TGF-β signaling, including maps #8, #12, #17, and #31. Several transcripts associated with proteins involved in extracellular matrix reorganization were found to be downregulated, such as MMP-2 (FC −1.55), MMP-9 (FC −3.14), Stromelysin-1 (FC −9.44), and MMP-13 (FC −5.34). Notably, some transcription factors that regulate the transcription of these proteases were found to be downregulated in our study. Moreover, the gene network can be analyzed using the pathway "Signal transduction_PDGF signaling via MAPK cascades" (pathway #5) (FDR 5.293e-5), which appears to be inhibited because the upstream factors PDGF-B (FC −2.43) and PDGFR-alpha (FC −2.03) were both downregulated. Additionally, Hyaluronan synthase 2 (HAS2) and hyaluronan synthase 1 (HAS1) were modulated, HAS2 was downregulated (FC −3.44), and HAS1 was slightly upregulated (FC 1.58).

The relationship between LAMA3 (Epiligrin, FC −2.27) and the extracellular matrix remodeling process was confirmed. Thrombospondin 1 [FC −3.67 for Thbs1 (A_55_P2746459) and −3.88 for Thbs1 (A_65_P13588)] is also in agreement with this process, as observed in the modulated pathway map "CHDI_Correlations from Discovery data_Causal network (positive)" (pathway #6) (FDR 5.293e-5). In this map, ephrin signaling was also modulated. Ephrins and their receptors play important roles in regulating cell migration and adhesion, with Ephrin-B receptors and Ephrin-B being downregulated (respectively FC −1.80 for the receptor and FC −1.76 for Efnb1, -2.78 for Efnb2, and −1.74 for Efnb3 ligands) (pathways #6, #14, #20, #28, #54, and #104). The modulation of "cytoskeleton remodeling and regulation of actin cytoskeleton organization by the kinase effectors of Rho GTPases" (pathway #18) (FDR 1.337e-4) is related to this issue.

The downregulation of these metalloproteinases can also be observed within the pathway map "Immune response,_IL-17 signaling" (pathway #10) (FDR 9.344e-5), which shows an upregulation of the cytokines IL-21, IL-17, and IL-17R, which are involved in the differentiation, maintenance, and expansion of Th17 cells, and play an important role in regulating oxidative stress and inflammation (Supplementary Table S2).

Based on these findings, we conclude that DEHP treatment affects cell-cell adhesion and cell-matrix adhesion. Treatment appears to increase cell-cell contact and cell-matrix adhesion. Moreover, the extracellular matrix is reinforced through the overexpression of Col1A2, which increases collagen and E-cadherin, which act as cell–cell adhesion molecules by connecting with cytoplasmic β-catenin to form cadherin/catenin complexes. Recently, it was shown that IGF-1 is inversely associated with E-cadherin expression in various types of cancers (Zeljkovic et al., 2020).

In addition, gene expression analysis in SHE cells exposed to DEHP revealed an unexpected outcome regarding the cell-matrix adhesion processes. Specifically, a temporary increase in cell adhesion was observed after 5 h of exposure to all the tested doses (Landkocz et al., 2011). It has also been proposed that TGF-β signaling is regulated (Landkocz et al., 2011).

Utilizing the Pathway filter option and focusing on regulatory pathways pertaining to apoptosis and survival, it was noted that modulation of "signal transduction_WNT/β-catenin signaling in tissue homeostasis" (pathway #42) (FDR = 2.010e-4) occurred.

Several other significantly modulated pathway maps further support the modulation of WNT signaling. Activation of the canonical Wnt/β-catenin signaling pathway is influenced by both ligand and receptor contexts. In the current experiment, several WNT ligands and receptors were scored as deregulated: 1.60 for Wnt3a (A_51_P210970), −2.46 for Wnt5b (A_55_P1984976), −1.54 for Wnt7b (A_52_P231691), and 2.39 for Wnt9a (A_55_P2032147). WNT ligands bind to Frizzled receptors [FC 2.25 for Fzd4 (A_51_P361220) and 2.38 for Fzd4 (A_66_P132734)], which activate signaling via β-catenin and SNAIL1 (FC −1.75). After translocation to the nucleus, β-catenin regulates target gene expression via activation of several gene targets, including Lef-1(FC 2.54), TCF7 (FC 1.58), and TCF7L2 (FC −1.60).

It is essential to emphasize that the transcriptional profile described so far reflects gene modulation at 24 h. It may be necessary to expose cells for an extended period to identify genes associated with more significant cellular disruptions, including perturbation of metabolic pathways and cellular stress (Poitou et al., 2022).

4.2 Inflammation and immune responses

The significantly altered pathway maps included several pathways related to cytokine production, inflammation, and immune response. These pathways include "Immune response_Histamine H1 receptor signaling in immune response" (pathway #9) (FDR 9.344e-5), "Immune response_IL-17 signaling" (pathway #10) (FDR 9.344e-5), and "Immune response_IL-6 signaling via JAK/STAT" (pathway #15) (FDR 1.168e-4). Additionally, pathways such as "Th2 cytokine- and TNF-alpha-induced profibrotic response in asthmatic airway fibroblasts/myofibroblasts" (pathway #13) (FDR 1.000e-4) and "TNF-alpha and IL-1 beta-mediated regulation of contraction and secretion of inflammatory factors in normal and asthmatic airway smooth muscle" (pathway #21) (FDR 2.361e-4) were also altered.

Within these pathways, several cytokine signaling factors, including IL-21, IL-17, IL-17R, IL-6R, INF-alpha, and IL-8RA, were upregulated. Notably, the transcription factor NFAT is overexpressed, which can induce the expression of several pro-inflammatory genes. The upregulation of transcripts may be linked to the activation of IP3 receptor signaling in the mitochondria where the IP3 receptor is upregulated. Activation of the IP3 receptor triggers the release of calcium from the endoplasmic reticulum into the cytosol, thereby activating calmodulin. Calmodulin activates Calcineurin A leading to NFAT activation. Additionally, upregulation of transcription factor Nuclear factor (Erythroid-derived 2)-like 2 (Nrf2), was observed. This gene encodes a transcription factor that regulates genes containing antioxidant response elements (ARE) in their promoters, many of which encode proteins involved in the response to injury and inflammation, including the production of free radicals (Saha et al., 2020). The overexpression of heme oxygenase supports the overexpression of Nrf2 as an anti-oxidative response.

Inflammation-related pathways are modulated by several downregulations, such as Nf-Kb (FC −1.65), COX-2 (FC −2.68), CCL20 (FC −3.43), VCAM [FC −2.41 for Vcam1(A_51_P210956) and −1.91 for Vcam1 (A_52_P520495)], IGF-1 receptor, and MMP-2 (FC −1.55) and MMP-9. Despite the upregulation of IL-6R, several downstream factors of this signaling pathway were downregulated. IL-6 activation can induce STAT3, leading to the initiation of the expression of activator protein 1 (AP-1, a complex of several subunits: FC −1.51 for Fos, FC −2.27 for Fosl2, FC −2.31 for Fosl2, FC −1.79 for Jun, FC −1.56 for Junb, and FC −1.54 for Junb) RUNX2, IL-RAP, c-Jun, and c-Fos factors, all related to inflammation and immune responses. On the other hand, IL-6 can also promote the activation of Mucin 4 and the angiogenic factor VEGF-A, which are both upregulated (FC 2.43 and 1.61, respectively).

These results support the activation of antioxidant and inflammatory signaling pathways in response to DEHP. The downregulation of related genes suggested downregulation of the NF-κB/AP-1 signaling pathway, which was supported by the upregulation of inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (IKK-gamma, FC 1.51). This inhibition of signaling could be related to previously documented negative interference with PPARα activation. Indeed, PPARα activation can inhibit the nuclear translocation of the NF-κB/p65 subunit and reduce the phosphorylation of nuclear c-Jun/AP-1, thereby inhibiting the production of pro-inflammatory cytokines such as TNFα, IL-1β, Cox-2, and iNOS (Delerive et al., 1999; Xu et al., 2001; Korbecki et al., 2019).

4.3 Effects of DEHP on toxic pathways

An image was generated utilizing the "filter by Map Categories: Tox processes" function to display the top ten pathways in order of significance for toxic processes that may be induced by DEHP treatment in the BALB/c 3T3 A31-1-1 cell model (Figure 3).

This list of pathways focuses on the significant modulation of toxic processes.

The first three pathways in the list are all related to the gamma-secretase complex, which is involved in critical cellular processes through the cleavage of type I transmembrane proteins, such as Notch family proteins (FC 1.59) and APP (FC 1.88). The regulation and function of these proteins have been previously described. Additionally, presenilin mRNA, which is upregulated (FC 1.99), encodes a catalytic component of the gamma-secretase complex, and its essential functions in calcium homeostasis have been well-documented.

The Notch signaling pathway is involved in various processes including immune cell development, epithelial-to-mesenchymal transition, angiogenesis, mammary gland development, osteogenesis, and gastrointestinal cell differentiation. It also plays a crucial role in the regulation of the development of different tissues. In the context of this study, it was expected that the PPARα pathway would be modulated given that DEHP is a PPARα agonist. However, enrichment analysis revealed that Aryl hydrocarbon receptor (AhR) and Pregnane X Receptor (PXR) signaling pathways were also affected by DEHP treatment. Specifically, the "Aryl hydrocarbon receptor signaling pathway" (pathway #103) (FDR 4.720e-3) was the third most perturbed toxicity pathway in this study when considering the tox process pathway map list (Figure 3). This pathway includes 19 modulated genes out of a total of 53 modulated genes. The BALB/c 3T3 A31-1-1 cell model was found to have an active AhR signaling pathway, and

immunofluorescence staining performed in the study showed that the cells were capable of expressing PPARα without any treatment, primarily in the nuclear compartment.

Some studies have suggested that DEHP may act as a weak agonist of AhR in human and rodent cell types, activating AhR signaling (Villard et al., 2007; Ernst et al., 2014; Zou et al., 2020; Ge et al., 2022; Hsieh et al., 2022).

Crosstalk between PPAR and AhR suggests that PPAR signaling regulates and activates AhR expression, ultimately downregulating estrogen synthesis by upregulating CYP1B1 and downregulating CYP19 signaling (Villard et al., 2007; Ernst et al., 2014). Both PPARα and PPARγ bind to estrogen response elements and act as competitive inhibitors, thereby affecting estradiol synthesis (Mu et al., 2000; Yanase et al., 2001; Fan et al., 2005; Benigni et al., 2012).

Phthalates also exhibit estrogen-like functions by binding to estrogen receptors and increasing estrogen synthesis by inducing aromatase expression (Chen et al., 2016; Zheng et al., 2023). In theory, AhR suppresses estrogen receptor 1 (ESR1, nuclear) signaling, recruiting both ESR1 and proteasomes, leading to ubiquitination and degradation of both AhR and ESR1 (Wormke et al., 2003). Additionally, AhR promotes the transcription of nuclear receptor-interacting protein 1 (RIP140), which inhibits ESR1 signaling (Augereau et al., 2006). Hsieh et al. (2022) also reported that DEHP mediates ER degradation via the AhR.

Notably, the 24 h exposure cells profile demonstrated an upregulation of ESR1 under treatment with 19.7 μg/mL DEHP (pathways #70, #103, #113, #167 FDR <0.05), along with gonadotropin-releasing hormone receptor (GnRH) (pathway #65) (FDR 1.879e-3). The altered expression of gonadotropin has also been linked to the disruption of AhR signaling by TCDD (Horling et al., 2011).

It is also intriguing to observe the modulation of pathway maps related to PXR signaling (Rodent/human version) (pathway #172) (FDR 1.291e-2). PXR is a nuclear receptor subfamily 1 group I member 2, pregnane X Receptor that is activated by a wide range of drugs, xenobiotics, and endogenous metabolites including steroids and bile acids. In specific cell types such as the liver and intestine, it serves as a "xenosensor" by regulating the expression of a network of genes involved in xenobiotic clearance. PXR is sequestered in the cytoplasm and translocates to the nucleus, where it forms a PXR/RXR-alpha complex with Retinoid X receptor alpha (RXRA) and binds to target gene promoters. Many plastic-associated endocrine-disrupting chemicals, such as BPA, BPB, and phthalates, have been reported to be potent agonists of the PXR (DeKeyser et al., 2011; Sui et al., 2012; Zhou, 2016; Helsley and Zhou, 2017; Sui et al., 2018).

Several studies have identified PXR as playing a role in maintaining lipid homeostasis and atherogenesis (de Haan et al., 2009; Cheng et al., 2012; Sui et al., 2012; Zhou, 2016; Helsley and Zhou, 2017; Sui et al., 2018; Gwag et al., 2019; Meng et al., 2019). For example, activating PXR through ligand-mediated means has been shown to raise plasma total cholesterol and atherogenic LDL levels in mice (Gwag et al., 2019; Meng et al., 2019).

In this context, the upregulation of the transcription factor hepatocyte nuclear factor 4 alpha (HNF4-alpha, FC 1.61) is highlighted as a key component of this pathway. HNF4-alpha is a crucial master transcription factor for the hepatic fat and bile acid metabolic pathways.

Notably, PXR and CAR regulate overlapping sets of genes encoding phase I- and II-metabolizing enzymes and transporters that are involved in xenobiotic detoxification and elimination. The DEGs associated with this pathway are listed in Table 2. Notably, the CAR pathway maps were not significantly modulated with an FDR of 6.229e-2 (Figure 3).

Additionally, the xenobiotic metabolizing systems induced by AhR, PXR, and CAR are involved in the metabolism of endogenous molecules such as steroids and thyroid hormones, including CYP3A. Induction of these systems may contribute to the endocrine disruptive activity of DEHP.

4.4 Sustained molecular signals after extended DEHP exposure and final remarks

We conducted a comprehensive analysis of the molecular signals after 24 h of DEHP exposure to identify the initial molecular events. Additionally, the transformation assay provided insights at the end of the 72 h exposure period (Table 3). Analysis of the results at this juncture revealed the amplification of signals observed at 24 h, confirming the involvement of the AhR receptor and the innate immune-mediated response initiated by IL-17 signaling and supported by IL-6, a pivotal interleukin in the inflammation pathway. Furthermore, signals indicative of PPARγ activation were observed (Table 3). Conversely, signals related to PPARα were diminished. A comparison of the top 10 modulated pathways at 24 h and 72 h is presented in Table 4.

The activation of AhR signaling pathways in the BALB/c 3T3 CTA model was not unexpected, as previously reported (Colacci et al., 2023). The canonical AhR pathway plays a role in both bioactivation and detoxification, potentially leading to or preventing oncotransformation *in vitro* (Mascolo et al., 2018; Pillo et al., 2022). Even in the absence of a recognizable formation of malignant foci, AhR is activated, indicating the modulation of several pathways associated with various potential adverse outcomes resulting from sustained inflammation. The upregulation of Cyp1A1 observed after 24 h of exposure and Cyp1B1 at 72 h confirmed the activation of the AhR canonical pathway.

The upregulation of Cyp2C enzymes, specifically Cyp2C9 and Cyp2C19, which are involved in human DEHP metabolism, confirmed the activation of PPARα. Indeed, CYP epoxygenases, including CYP2C and CYP2J, are affected by PPARα ligands (Cizkova et al., 2012). A fascinating notion is that Cyp2C enzymes are key molecules in the defensive response of embryonic and tumor cells, a phenomenon that translates into the mechanisms of multidrug resistance (MDR) in human pathophysiology. Upregulation of multidrug resistance protein 3 (MRP3), which is responsible for the transport of glucuronide conjugates and bile salts from the cell, can also confer resistance to several anticancer drugs (Aleo et al., 2017), further confirming that a series of key molecules in the cellular response to DEHP exposure move in unison in a string of genes correlated with the PXR pathway involved in the regulation of xenobiotic metabolism. Certain ligands or activators of PPARs affect the expression or activity of PXR and *vice versa*. The cooperative response observed when both RXR and partner receptor ligands are present highlights

TABLE 4 Comparison of molecular pathway modulation at 24 hours and 72 hours of DEHP exposure[a].

#	Modulated pathway map at 24 h	FDR	Modulated pathway map at 72 h	FDR
1	Protein folding and maturation_Amyloid precursor protein processing (schema)	7.137E-08	Apoptosis and survival_Granzyme A signaling	3.953E-07
2	Protein folding and maturation_POMC processing	2.361E-05	Oxidative stress_ROS signaling	3.953E-07
3	Signal transduction_Nuclear FGFR1 signaling	2.361E-05	Immune response_IL-6 signaling via JAK/STAT	3.119E-05
4	TGF-beta signaling via SMADs in breast cancer	4.825E-05	Signal transduction_RANKL-dependent osteoclast differentiation	2.730E-04
5	Signal transduction_PDGF signaling via MAPK cascades	5.180E-05	DNA damage_ATM/ATR regulation of G2/M checkpoint: cytoplasmic signaling	2.877E-04
6	CHDI_Correlations from Replication data_Causal network (positive correlations)	5.180E-05	Signal transduction_Calcium-mediated signaling	4.564E-04
7	Signal transduction_CXCR4 signaling via MAPKs cascades	5.823E-05	Signal transduction_mTORC1 downstream signaling	4.664E-04
8	Development_Regulation of epithelial-to-mesenchymal transition (EMT)	9.200E-05	Immune response_IL-6 signaling via MEK/ERK and PI3K/AKT cascades	4.760E-04
9	Immune response_Histamine H1 receptor signaling in immune response	9.200E-05	G-protein signaling_Rac1 activation	4.760E-04
10	Immune response_IL-17 signaling	9.200E-05	Eosinophil adhesion and transendothelial migration in asthma	4.855E-04

the regulatory interplay between permissive receptor partners, such as PPARs, PXR, and CAR (Evans and Mangelsdorf, 2014).

The high upregulation of Cyp2C8 highlights the interesting crosstalk between PPAR and AhR in our model. DEHP induces Cyp2C8 expression through the AhR genomic pathway, which is typically independent of ligand binding, and can interact with other transcription factors (Hsieh et al., 2022). The induction of Cyp2C8 increases epithelial-mesenchymal transition (EMT) sustained by the AhR/ERK signaling pathway (Hsieh et al., 2022). EMT plays a role in various biological processes under normal conditions such as embryogenesis and wound healing in adults (Colacci et al., 2023). However, they also contribute to the development of tissue fibrosis and cancer. In human cancers, EMT is considered a pivotal stage at the tissue level, signifying the progression of dysplasia and the acquisition of invasive characteristics (Colacci et al., 2023). It has previously been reported that CTAs, especially the BALB/c 3T3 model, offer the possibility of identifying critical steps related to EMT, a process that starts with cytoskeleton modifications as an adaptive response to chemical exposure and proceeds according to chemical concentration and exposure duration to extensive morphological changes, sustaining the acquisition of fully malignant characteristics (Colacci et al., 2023).

Based on our findings, we can infer that the molecular initiating event in our model involves the binding of DEHP to PPARα, which triggers a cascade of molecular events supporting DEHP metabolism and the AhR-mediated immune response. These pivotal molecular events are detectable only after 24 h of exposure, indicating that this timeframe allows for the early detection of chemical responses and the identification of molecular initiating events in *in vitro* oncogenesis.

At the 24 h mark, we also observed the activation of Ah-dependent detoxifying enzymes, specifically UDP-glucuronosyltransferase (phase 2 metabolic enzymes), Ugt1a1 and Ugt1a6. These enzymes facilitate glucuronidation, enabling the covalent attachment of glucuronic acid derived from the cofactor UDP-glucuronic acid to substrates containing appropriate acceptor functional groups. The upregulation of these enzymes confirms that DEHP metabolites are actively detoxified via glucuronidation, which is the primary route for the elimination of DEHP metabolites in humans.

Previous studies have indicated that the activation of UDP-glucuronosyltransferase plays a crucial role in detoxifying carcinogens in CTAs (Mascolo et al., 2018), and this serves as a unique signature of BALB/c 3T3 CTA (Colacci et al., 2023). Therefore, we can deduce that the negative results observed in the BALB/c 3T3 CTA compared with the SHE model can be attributed to the active detoxification of DEHP metabolites driven by a robust AhR-mediated response characteristic of the BALB/c 3T3 model.

BALB/c 3T3 cells, specifically the A31-1-1 clone, exhibit notable metabolic competence encompassing both phase-1 and phase-2 enzymes (Mascolo et al., 2018). This clone was deliberately selected for its superior metabolic capabilities compared with the A31-1-1 clone (Colacci et al., 2011; Colacci et al., 2023). Although initially believed to be derived from the A31 clone, subsequent characterization has revealed that it originated from a distinct mouse strain (Colacci et al., 2023). This discrepancy in lineage sheds light on the observed variations between the two clones and potentially explains the inconsistencies among studies that have utilized different clones. Moreover, these findings underscore the importance of considering the genetic background and metabolic characteristics of cell lines when interpreting toxicity data and highlight the need for continued research to elucidate the mechanistic basis of these differences.

After 72 h, all the genes associated with the AhR canonical pathway, including Cyp1A1, Cyp1B1, and UDP-

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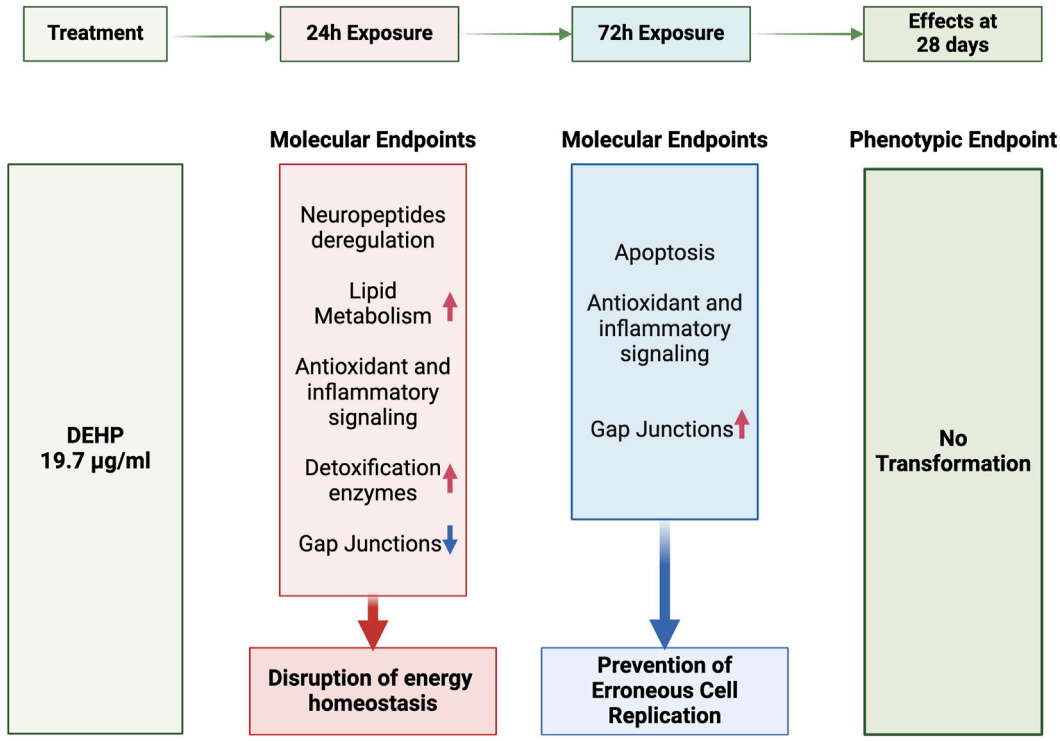

FIGURE 5
Temporal dynamics of key molecular endpoints during chemical treatment.

glucuronosyltransferases, remained modulated, whereas the expression of genes linked to PPARα was negligible. Notably, the gene profiles associated with PPARγ were discernible at this juncture, which was surprising (Table 3).

5 Conclusion

The primary goal of this study was to evaluate the carcinogenic potential of DEHP using the A31-1-1 BALB/c-3T3 cell line in a standard CTA according to the ECVAM DB-ALM protocol No. 137 (Sasaki et al., 2012a; Corvi et al., 2012; Tanaka et al., 2012). Our investigation extended beyond CTA by incorporating a transcriptomic analysis to explore molecular responses. In this study, we examined the effects of DEHP exposure on various toxicological pathways. The results revealed significant modulation of several pathways associated with tissue-specific functions related to systemic metabolic and basal cellular signaling with pleiotropic outcomes. Among these signaling pathways, modulation of cell-regulating signaling pathways, such as Notch, Wnt, and TGF-β, can be highlighted. More specific modulation of such genes and pathways with double functions in metabolism and neurophysiology underlies a well-known crosstalk that may be crucial in the mechanism of action of DEHP. It is intriguing to note that such tissue-specific molecular signaling, which is known to be perturbed by DEHP, was scored in this enrichment analysis using mouse embryonic fibroblasts. Fibroblasts play a crucial role in tumor progression through their interactions within the tumor microenvironment. Once viewed solely as supportive cells that provide structural integrity, fibroblasts are now recognized as active participants in malignancy. Fibroblasts undergo activation to acquire the cancer-associated fibroblast (CAFs) phenotype. These CAFs secrete growth factors, cytokines, and extracellular matrix proteins that contribute to tumor growth, angiogenesis, invasion, and metastasis. The tumor microenvironment, characterized by dynamic and reciprocal interactions between cancer cells and the surrounding stromal cells, including fibroblasts, has emerged as a hallmark of cancer (Casey et al., 2015; Goodson et al., 2015). The ability of BALB/c 3T3 CTA to recapitulate key aspects of tumor progression, including the involvement of fibroblasts and key molecular events related to the tumor microenvironment, has been previously reported (Colacci et al., 2023) further emphasizing the relevance of CTA in delineating the multifaceted mechanisms underlying carcinogenesis.

In this study, the mode of action of DEHP related to the disruption of energy homeostasis, which has been previously described in mice, was similarly observed in the molecular toxicity data of 3T3 cells. The influence of PPARα molecular signaling on the modulated pathway was not statistically significant, despite the presence of several gene modulations associated with its signaling process. However, the upregulation of Cyp2C enzymes, integral to DEHP metabolism, directly regulated by PPARs, either independently or via crosstalk with AhR, strongly indicates that PPARα activation serves as the initiating event in our model. Moreover, persistent activation of the AhR canonical pathway throughout the exposure of cells to DEHP ensures sustained upregulation of detoxifying enzymes, thereby

mitigating potential adverse effects induced by the chemical and preventing cell transformation.

While the negative CTA result indicated that DEHP did not induce malignant transformation in cultured cells under specific experimental conditions, transcriptomic analysis provided deeper insights into the molecular responses elicited by DEHP. Indeed, transcriptomic data can offer a critical context for interpreting CTA results, aiding in the identification of the underlying molecular pathways associated with carcinogenicity. As previously documented (Colacci et al., 2023), the modulation of gene pathways supporting cell proliferation is anticipated to lead to oncotransformation *in vitro* (Colacci et al., 2023). Sustained cell proliferation can be regarded as a necessary hallmark, albeit not sufficient, for progression towards malignancy in CTA models as well as *in vivo* cancer processes (Colacci et al., 2023). The absence of clear signals related to sustained proliferation, particularly those supporting cytoskeleton remodeling-related EMT at 72 h, suggests interruption of the process leading to oncotransformation through the induction of apoptosis to prevent the replication of faulty cells (Figure 5).

According to our data, up to 24 h of exposure, the initiating event signals were still visible and prevailed in the cytotoxic and apoptotic signaling, which manifested at 72 h. This extended analysis aimed to delve deeper into the molecular mechanisms of action and their temporal evolution, thereby contributing to a more nuanced assessment of the toxicological implications of DEHP.

Therefore, our findings underscore the effectiveness of an integrated approach combining CTA with transcriptomics. This integration not only aids in interpreting results when CTA is employed as a standalone assay but also enhances the sensitivity and specificity of the test. In the context of a battery of tests such as the IATA, the likelihood of encountering false negatives (or false positives) is mitigated by the inclusion of multiple endpoints from diverse assays. Moreover, although CTA may serve as a component of the battery, integration of transcriptomic analysis can further enhance the predictive power of IATA.

In conclusion, the results of our study demonstrated that the BALB/c 3T3 A31-1-1 cell line does not exhibit a transformative effect in response to DEHP exposure. Nevertheless, our data revealed a nuanced molecular response to DEHP after 24 h of exposure, shedding light on the mechanisms underlying the metabolic activation and detoxification in our model. This underscores the potential contribution of AhR-mediated pathways to negative results in BALB/c 3T3 CTA. The identification of relevant metabolic pathways associated with human DEHP exposure further provides compelling evidence supporting the predictive capabilities of CTA models in assessing chemical toxicity in humans, thus offering promising avenues to reduce reliance on animal testing for toxicity assessments (Huang et al., 2017; Hansen and Piorczynski, 2019).

Data availability statement

The datasets presented in the study are deposited in the repository Array Express—Genomic Collection (https://www.ebi.ac.uk/biostudies/arrayexpress), accession number E-MTAB-13716.

Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

GP: Writing–review and editing, Writing–original draft, Visualization, Validation, Methodology, Investigation, Formal Analysis, Data curation, Conceptualization. FA: Writing–original draft, Investigation, Formal Analysis. AM: Writing–review and editing, Investigation. GM: Writing–review and editing, Investigation. MM: Writing–review and editing, Validation, Supervision, Methodology. MV: Writing–review and editing, Supervision, Resources. AC: Writing–original draft, Writing–review and editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2024.1389160/full#supplementary-material

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A new database contains 520 studies investigating the carcinogenicity data of 238 pharmaceuticals across 14 ATC classifications

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1 Introduction

Recently, we compiled a new database with toxicity data from non-clinical animal studies along with human information for 528 approved drugs (Evangelisti et al., 2023). The database contains non-clinical studies for repeat-dose, carcinogenicity, developmental toxicity, and reproductive toxicity. It is made available free of charge at https://iuclid6.echa.europa.eu/us-fda-toxicity-data. The terminology used within this database has been harmonized to support further analyses, such as correlation and concordance studies. The corresponding ontology is accessible at https://github.com/innovamol/PaCCO.

The database can be used for correlation and concordance analyses (Baan et al., 2019), as well as offering insights into the tumorigenic potential of structural analogues (Alden et al., 2011) by including structural information as the original source only contained textual identifiers such as an international non-proprietary name. It facilitates the examination of species and strain sensitivities and aids in the adoption of new approach methodologies (NAMs) (Van Oosterhout et al., 1997; Reddy et al., 2010) in alignment with regulatory standards (Contrera et al., 1997).

In this research brief, our goal is to enhance data presentation for practical usage within the cancer research community. To support this, the carcinogenicity study information has been made available as Excel file (Supplementary Material).

Abbreviations: ATC, Anatomical therapeutic chemical classification system; ID, Identifier; IUCLID, International uniform chemical information database; IUPAC, International Union of Pure and Applied Chemistry; LOAEL, Lowest observed adverse effect level; NDA, New drug application; NAM, New approach methodologies; NOAEL, No observed adverse effect level; US FDA, US Food and Drug Administration; UUID, Universally unique identifier.

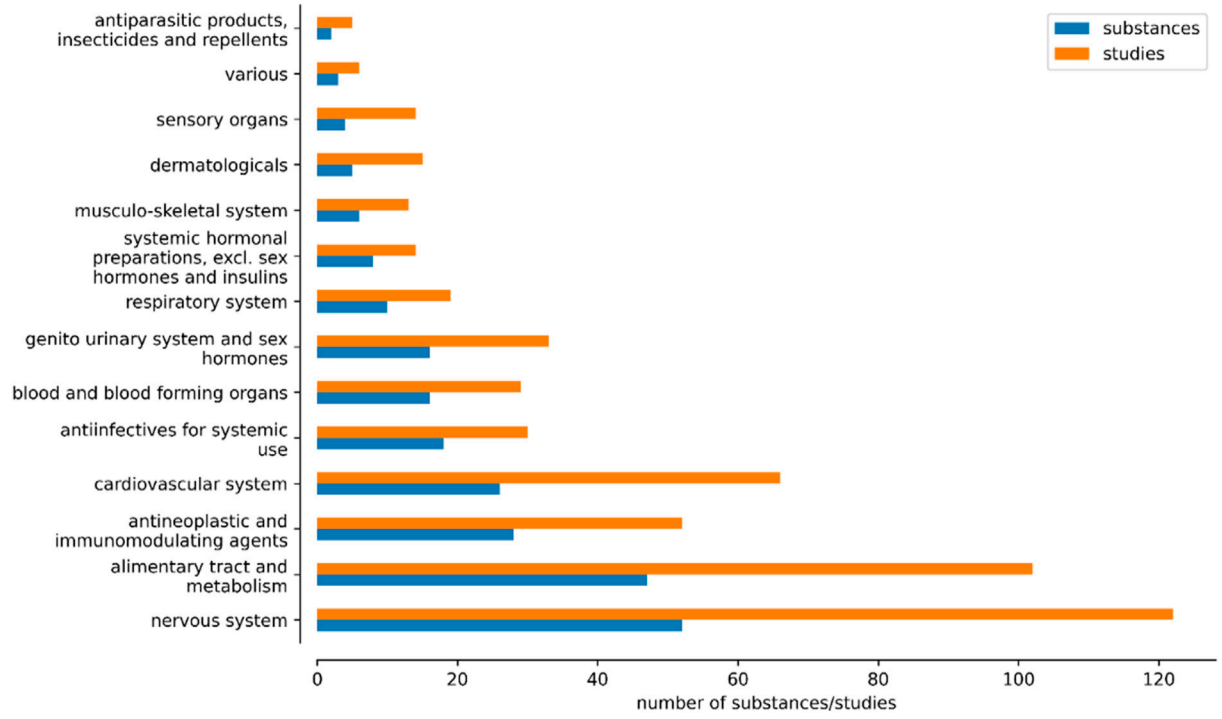

FIGURE 1
Number of substances and studies across the Anatomical Therapeutic Chemical (ATC) classifications.

2 Results and discussions

2.1 Data density

The database (Evangelisti et al., 2023) contains 520 studies investigating the tumorigenic potential of 238 pharmaceuticals, meaning a density of approximately two studies per drug. This finding is in line with the regulatory requirement to test in two species, usually rat and mouse.

The database includes approved medical drugs of 14 ATC classifications (Figure 1): 46 drugs targeting the alimentary tract and metabolism (102 studies), 18 anti-infectives for systemic use (30 studies), 28 anti-neoplastic and immunomodulating agents (54 studies), 2 antiparasitic products (5 studies), 15 drugs targeting blood and blood forming organs (27 studies), 25 cardiovascular drugs (64 studies), 5 dermatologicals (15 studies), 16 drugs targeting the genitourinary system and sex hormones (33 studies), 6 drugs acting on the musculo-skeletal system (13 studies), 50 drugs targeting the nervous system (119 studies), 12 drugs acting on the respiratory system (24 studies), 4 drugs targeting the sensory system (14 studies), 8 systemic hormonal preparations excluding sex hormones and insulins (14 studies), and 3 various drugs (6 studies).

2.2 Species, strain, sex specificity, and affected organs/tissues

2.2.1 Species and strains

Tumorigenic potential was primarily investigated in two species: 253 studies using mice, 263 studies using rats and 4 studies reporting both species. The Chinese hamster was used in only one study, and the New Zealand White rabbit in another.

The following mouse strains were used in the indicated percentages of mouse studies: CD-1 (ca 60%), B6C3F1 (ca 10%), CB6F1 (ca 5%), other Tg (ca 4%), and NMRI (ca 4%). Other strains used in a very few studies included C57BL, SWISS, Balb/c, ICR, and specific knockout strains. In around 8% of studies, the mouse strain was not specified.

Of the 263 rat studies, 56% were conducted with Sprague-Dawley, 22% with Wistar, and 5% with Fischer 344 rats. A few other studies were performed with other albino rat strains or Long Evans rats in two studies. In approximately 7% of studies the rat strain was not specified.

The distribution of species and strains in the carcinogenicity studies of our database is consistent with the use of mouse and rat strains typically encountered when testing for tumorigenic potential for regulatory needs (Organisation for Economic Co-operation and Development, 2012).

2.2.2 Sex specificity

Figure 2 illustrates the sex specificity of tumours targeting specific organs/tissues across the predominantly used mouse and rat strains. The figure was constructed by filtering the provided dataset so that the basis for effect corresponds to neoplastic histopathological findings, in rats or mice, and for which the sex for which the effect was reported has been provided. We then identified the two most common strains for each species and mapped all other strains to "other". For each species and strain combination we counted the number of studies for the eight most common effects after mapping the ontology. Table 1 provides a

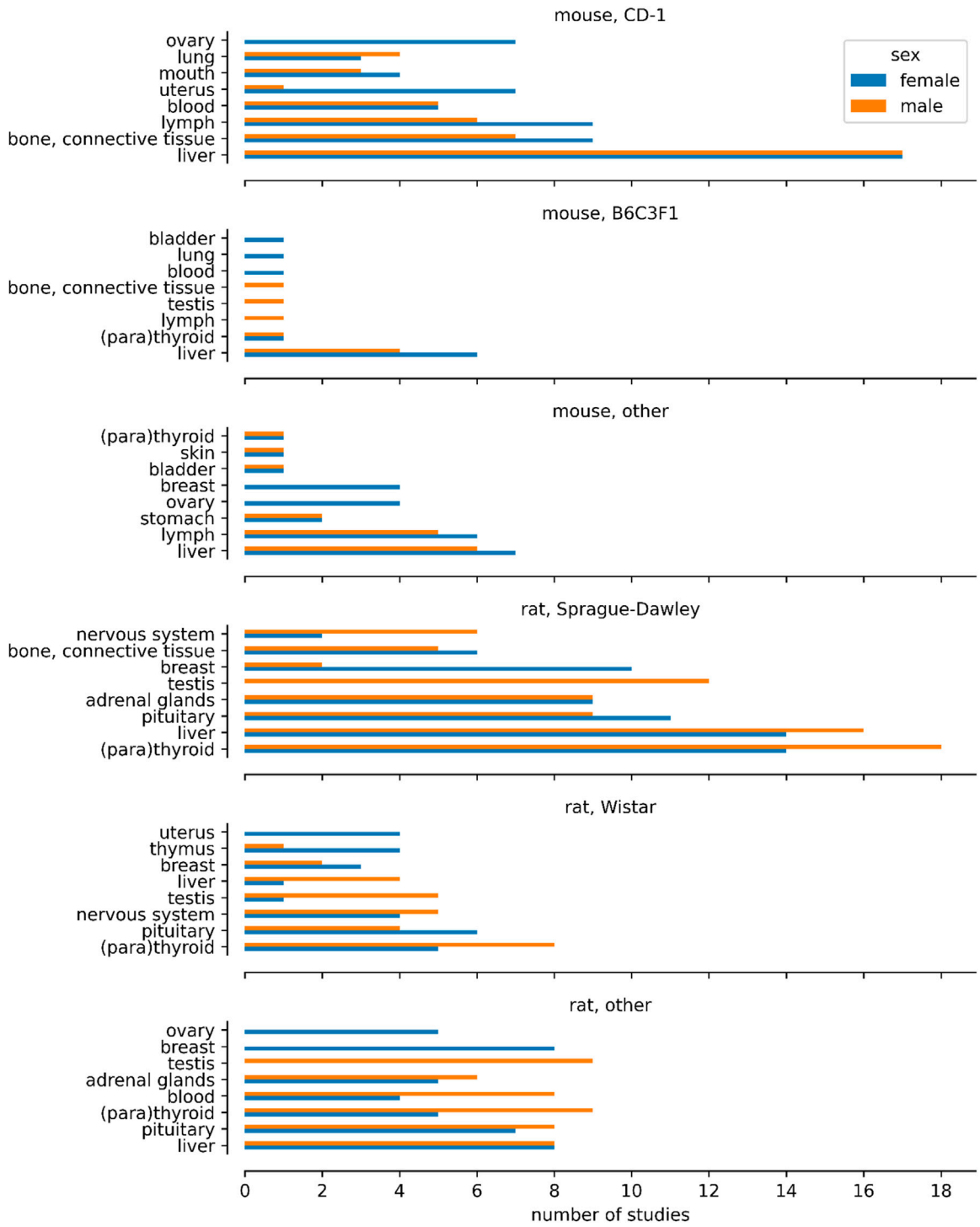

FIGURE 2
Number of studies showing affected organs and tissues in the mouse and rat strains mostly used in carcinogenicity studies of the database. All other mouse and rat strains are included in the histograms "mouse, other" and "rat, other". In CD-1 mouse and Wistar rat, for one case the finding of tumour growth in uterus and testis was erroneously assigned to male mice and female rats, respectively. Such errors are common in large inventories of substances despite curation efforts.

detailed analysis, showing the incidence of various tumours by sex: in males only, females only, or in both sexes. It is important to note that not all studies were conducted in both sexes, and this is noted to ensure transparency regarding the scope of our data and to help set realistic expectations for its use.

2.2.3 Affected organs and tissues

Figure 3 shows the ontology terms associated with the effect levels plotted in Figure 2. We note that the same ontology term can appear in different hierarchies in the ontology, in which case it is shown only once.

TABLE 1 Substances with tumorigenic potential by affected organs/tissues and sex (No.: number, NA: not applicable).

Organ/tissue	No. Substances with findings	Males only	Both sexes	Females only
adrenal glands	26	9	10	7
blood, blood forming tissues	25	13	6	6
bone, connective tissue	28	12	5	11
Breast	27	0	4	23
intestine	3	1	0	2
Kidney	7	2	4	1
liver	59	17	27	15
thyroid + liver[a]	23	12	7	4
lung	8	4	1	3
lymph	21	3	12	6
mouth	6	1	3	2
nervous system	15	7	7	1
ovaries	15	NA	NA	15
pancreas	8	6	2	0
pituitary	32	4	17	11
skin	14	10	3	1
stomach	9	1	6	2
testes	31	31	NA	NA
thymus	7	1	2	4
thyroid/parathyroid[b]	44	17	21	6
urinary bladder	2	0	2	0
uterus	16	NA	NA	16

[a]Observed thyroid hyperplasia, adenoma, and/or carcinoma in presence of liver findings. All findings in the 23 substances are from rat studies. No such overlap was observed in mouse studies because the database only contains 3 mouse studies with neoplastic changes in the thyroid in the absence of liver effects.
[b]Tumorigenic potential in parathyroid was observed for 11 substances (2 in males only, 9 in both sexes).

In the genitourinary system, 7 pharmaceuticals showed tumorigenic potential in the kidneys (2 in males only, 4 in both sexes, and 1 in females only). Regarding hepatobiliary organs and tissues, in the liver, 59 pharmaceuticals induced tumour growth (17 in males only, 27 in both sexes, 15 in females only).

In reproductive and endocrine organs/tissues, 26 drugs impacted the adrenal glands (9 in males only, 10 in both sexes, 7 in females only), while 27 influenced mammary tissues (none in males only, 4 in both sexes, 23 in females only). In addition, 44 substances affected the thyroid/parathyroid (17 in males only, 21 in both sexes, and 6 in females only), with parathyroid effects observed in 11 substances (2 in males only and 9 in both sexes). Tumours also developed in the ovaries (15 substances) and the uterus (16 substances). The pituitary was affected by 32 pharmaceuticals (4 in males only, 17 in both sexes, 11 in females only), and 31 substances impacted the testes.

For the immune system, 21 pharmaceuticals promoted growth in lymphatic tissues (3 in males only, 12 in both sexes, 6 in females only), and 7 affected the thymus (1 in males only, 2 in both sexes, 4 in females only).

Table 1 includes additional data on pharmaceuticals with tumorigenic potential affecting blood constituents, blood-forming tissues, bone, connective tissues, intestine, lungs, pancreas, skin, stomach, and urinary bladder.

For 23 NDAs, tumorigenic potential in rat thyroid glands (hyperplasia, adenoma, and/or carcinoma) was observed in conjunction with liver effects (e.g., increased liver weight, liver hypertrophy, and/or hyperplasia). This finding aligns with the established understanding that neoplastic changes in the thyroid of rodents can occur as a secondary consequence of liver effects altering the metabolism of thyroid hormones (Bartsch et al., 2018).

2.2.4 Concern for tumorigenic potential stemming from repeatdose toxicity studies

Our database (Evangelisti et al., 2023) includes information from repeat-dose toxicity studies, which are valuable for identifying potential concerns regarding tumorigenic potential (the original source files are attached to the IUCLID dossiers of the database (Evangelisti et al., 2023; IUCLIDa, 2024); IUCLID stands for International Uniform ChemicaL Information Database (IUCLIDb, 2024)). The distribution of species and strains in our database does not necessarily reflect ICH guidance (S1B) (European Medicines Agency, 2022), as many data are linked to experimental studies performed before these guidelines were

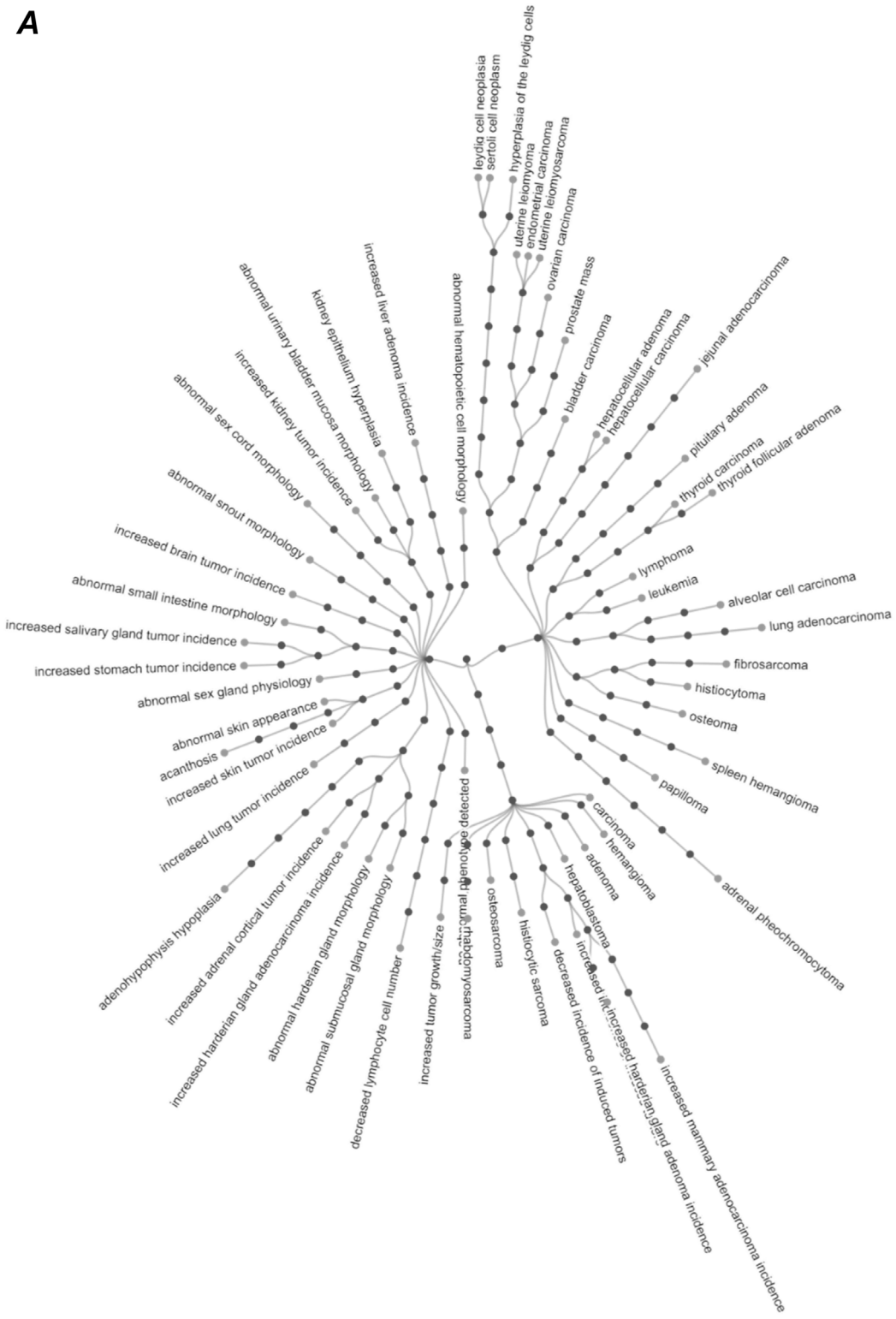

FIGURE 3
(Continued).

established. However, it is important to consider these guidelines as published by the EMA can also be effectively used for comparison and context (European Medicines Agency, 2022). Indeed, sub-acute, sub-chronic and chronic repeat-dose toxicity studies reveal morphological changes, such as the presence of poorly differentiated or undifferentiated cells and hyperplasia in tissues and organs, signalling

FIGURE 3
(Continued). Visualization of the ontology terms associated with neoplastic histopathological effects in in mice **(A)** and rats **(B)**. Only the leaf ontology terms that have been associated with the effect level are labelled. The remaining of the ontology terms in the ontology hierarchy are only shown as filled circles to illustrate the ontology architecture. Both visualizations are provided as high-resolution png files in the Supplementary Material for improved readability. In a few instances, the same leaf term was present in more than one ontology hierarchy. For better readability, only one path for such leaf terms is depicted. The interested reader can visualize the full ontological structure in the owl of PaCCO (https://github.com/innovamol/PaCCO) while the path in a human-readable format is provided in the supplementary Excel file (column: ontology term hierarchy). Supplementary Material also provide high-resolution images.

potential carcinogenic concerns. Moreover, these studies contribute to a weight of evidence approach, supporting conclusions about tumorigenic potential.

For example, our database lists 679 'hyperplasia' events in different organs and tissues (for example, adrenal glands, bone marrow, gastric glands, mammary glands, liver, and thymus) among 65,403 reported effects across the 2,270 oral repeat-dose toxicity studies (Evangelisti et al., 2023). Another valuable example can be represented by liver and thyroid tumorigenesis data. The database reports 581 liver effects (for example, increased liver weight, enlarged liver, liver inflammation, abnormal liver morphology) and 184 thyroid effects (for example, thyroid gland hyperplasia, thyroid carcinoma, thyroid follicular adenoma). All carcinogenicity studies on more than 50 compounds found a total of 3,365 effects in the ATC class connected to the nervous system, which is one of the most represented categories. Out of these, 93 individual effects are classified as histopathology neoplastic (duplicates excluded). The most common type of tumour found is hepatocellular adenoma.

Author's note

The carcinogenicity study information is available in Excel form as Supplementary Material. The dataset has been created by extracting the pre-clinical carcinogenicity study information from the IUCLID dossiers (Evangelisti et al., 2023; Bartsch et al., 2018) that were compiled from the original pharmacological reviews provided by the US Food and Drug Administration (USFDA) (U.S. Food and Drug Administration). The dataset contains the following information in the corresponding color-coded column groups:

- substance name, CAS number, IUPAC name, application number (NDA) and ATC anatomical class
- administrative information for the carcinogenicity study, such as the dossier and endpoint study record UUID in the IUCLID database; the column esr_data contains all available information for the study as a json string, to facilitate the extraction of additional information other than what has already been included in columns; columns that contains "(code)" in the name contain the integer code of a IUCLID pick list entry and are accompanied by a column that has "(text)" in the name in which the integer code has been mapped to the corresponding IUCLID phrase
- species, strain, route of administration, duration and frequency of treatment, and doses
- description of the incidence and severity of effects that includes a qualitative description of the observed effects, and if the data allows, whether they are adverse, non-adverse, reversible, or irreversible
- details on results, carcinogenic effects and potential, conclusions and the study executive summary
- effect levels, i.e., the exposure level that corresponds to a quantified level of effects, e.g., NOAEL (No Observed Adverse Effect Level) or LOAEL (Lowest Observed Adverse Effect Level); the dataset contains one row per effect level, i.e., there may be more rows than the number of unique studies that can be identified by the column combination UUID (dossier) and UUID (parent)
- ontology information (Evangelisti et al., 2023) with the ontology ID and label assigned to the effect level; we also include the parent ontological identifier (parent ID) and the ancestral path of labels, beginning with the current term and extending upward to the root of the hierarchy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

PGK: Conceptualization, Data curation, Validation, Visualization, Writing–original draft, Writing–review and editing. ME: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. MP: Conceptualization, Data curation, Formal Analysis, Methodology, Software, Validation, Visualization, Writing–original draft, Writing–review and editing. IB: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

Authors ME and AD were employed by Innovamol Srl. Authors PGK, JB, and IB were employed by European Chemicals Agency.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2024.1450612/full#supplementary-material

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A new approach methodology to identify tumorigenic chemicals using short-term exposures and transcript profiling

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Current methods for cancer risk assessment are resource-intensive and not feasible for most of the thousands of untested chemicals. In earlier studies, we developed a new approach methodology (NAM) to identify liver tumorigens using gene expression biomarkers and associated tumorigenic activation levels (TALs) after short-term exposures in rats. The biomarkers are used to predict the six most common rodent liver cancer molecular initiating events. In the present study, we wished to confirm that our approach could be used to identify liver tumorigens at only one time point/dose and if the approach could be applied to (targeted) RNA-Seq analyses. Male rats were exposed for 4 days by daily gavage to 15 chemicals at doses with known chronic outcomes and liver transcript profiles were generated using Affymetrix arrays. Our approach had 75% or 85% predictive accuracy using TALs derived from the TG-GATES or DrugMatrix studies, respectively. In a dataset generated from the livers of male rats exposed to 16 chemicals at up to 10 doses for 5 days, we found that our NAM coupled with targeted RNA-Seq (TempO-Seq) could be used to identify tumorigenic chemicals with predictive accuracies of up to 91%. Overall, these results demonstrate that our NAM can be applied to both microarray and (targeted) RNA-Seq data generated from short-term rat exposures to identify chemicals, their doses, and mode of action that would induce liver tumors, one of the most common endpoints in rodent bioassays.

KEYWORDS

new approach methodologies, biomarkers, liver cancer, transcript profiling, adverse outcome pathway, 2-year cancer bioassay

1 Introduction

In the United States, cancer is the second leading cause of death, imposing a tremendous burden on individuals and their families, as well as the US economy (Ahmad and Anderson, 2021; CDC, 2017). Most chemicals in commerce have not been adequately tested for the ability to cause cancer in humans and animals. The 2-year cancer bioassay conducted in mice and rats remains the "gold standard" for carcinogenicity testing, but due to the

resources needed to assess a chemical ($2–4 M USD; 800 rodents; histopathological analysis of more than 40 tissues; 2+ years to complete the in-life study and years to analyze results), only ~1500 commercial chemicals have been examined to date (Bucher and Portier, 2004; Gold et al., 2005; Waters et al., 2010). In contrast, there are tens of thousands of chemicals in commerce with inadequate information on cancer hazard. These include over 140,000 substances registered by the European Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (REACH, 2008), ~30,000 chemicals being used commercially in the United States and Canada (Muir and Howard, 2006), and ~41,000 chemicals on the US EPA's Toxic Substances Control Act Inventory (https://www.epa.gov/tsca-inventory; accessed 1 August 2022). There are also concerns about the human relevance of rodent cancer outcomes. New resource-efficient methods are needed to move away from reliance on the 2-year cancer bioassay and to identify the carcinogenic potential of a chemical in shorter term *in vivo* assays or through sets of assays carried out in appropriate *in vitro* systems allowing identification of human-relevant risk that can be put into the context of boundaries of exposure.

There are increased efforts across broad sectors of the toxicity testing community to develop new approach methodologies (NAMs) to reduce or entirely replace animal testing. The Organization of Economic and Cooperative Development (OECD) (Jacobs et al., 2020), institutions in the United States (ICCVAM, 2018; Sciences, 2018) (ICCVAM 2018; NIEHS 2018; Hood 2019; U.S. EPA 2020a), and the European Union (Annys et al., 2014; Corvi et al., 2017; Luijten et al., 2020) have efforts to replace the rodent chronic bioassay using more human-relevant testing methods, that if implemented will significantly reduce or replace animal testing (Felter et al., 2021). The NAMs being developed and validated can include relevant *in vitro* assays that do not use animals as well as *in vivo* studies that are for shorter durations of exposure and use fewer animals per treatment group than the 2-year bioassay (Cohen et al., 2019; Madia et al., 2019). Some NAMs can already be used to help predict human carcinogenic risk in a regulatory setting including *in silico* mutagenicity prediction models used to classify an impurity of concern in an active pharmaceutical ingredient and reduce further testing to assess carcinogenic risk (ICH (2017) M7 regulations). Additionally, activities are ongoing to include weight-of-evidence for the carcinogenicity assessments for agrochemicals (Hilton et al., 2022) and pharmaceuticals (Bourcier et al., 2024) which incorporate all available relevant data. The past work highlights the considerable challenges to using NAMs to accurately predict human cancer risk including what endpoints to measure *in vitro* assays and when to measure them. Although NAMs are starting to be used and/or considered by some regulatory agencies (Jacobs et al., 2020; Luijten et al., 2020; Yauk et al., 2020; Heusinkveld et al., 2020), there is currently limited regulatory acceptance for decision-making.

Genomic biomarkers are being increasingly recognized by broad sectors of the scientific community to have the potential to reduce the need for conventional rodent carcinogenicity studies of chemicals through a weight-of-evidence approach. Biomarker-based NAMs could be used in integrated approaches to testing and assessment (IATA) strategies or could be used as standalone NAMs for an intended use (Corton et al., 2022a). Gene expression biomarkers have been developed and applied for hazard identification in a number of contexts. One of the first biomarkers to be developed was the TGx-DDI biomarker, currently under regulatory review by the FDA through the Center for Drug Evaluation and Research Biomarker Qualification Program (Avila et al., 2020). The biomarker was developed to enable differentiating between true positive DNA damage-inducing (DDI) agents and non-DDI irrelevant positive agents using a number of human cell lines (Li et al., 2017; Corton et al., 2018; Cho et al., 2019). Another set of biomarkers were developed to identify molecular initiating events (MIEs) in cancer and liver steatosis adverse outcome pathways (AOPs) by leveraging microarray data from livers of chemically-treated wild-type and transcription factor-null mice, allowing for the identification of well-defined mechanistic gene sets (Oshida et al., 2015a; Oshida et al., 2015b; Oshida et al., 2016; Rooney et al., 2018a; Rooney et al., 2018b; Rooney et al. 2,019). These biomarkers have been applied to sets of chemicals to identify relationships between exposure and hazard (Rosen et al., 2017), as well as to identify the most likely AOP responsible for rodent liver tumors (Peffer et al., 2018; Rooney et al., 2017; Rooney et al., 2018c). Given the growing emphasis on tiered screening of chemicals using high-throughput transcriptomics (HTTr) in human cell lines (Thomas et al., 2019; Harrill et al., 2021), a number of groups have constructed biomarkers that identify important molecular targets underpinning *in vivo* toxicity including estrogen receptor (ER) (Ryan et al., 2016) and androgen receptor (Rooney J. P. et al., 2018) modulation as well as stress factor induction (Jackson et al., 2020; Rooney et al., 2020; Cervantes and Corton, 2021; Korunes et al., 2022) and histone deacetylase inhibition (Cho et al., 2021; Corton et al., 2022b). Future NAMs may one day use gene expression biomarkers used to interpret transcript profiles derived from *in vitro* HTTr human-derived multicellular models (micro-physiological systems, organoids, organ-on-a-chip) that better mimic the physiological and toxicological behaviors of human organs compared to the current screening paradigm carried out in two dimensional human cell cultures. Like NAMs, regulatory acceptance of biomarker use for toxicological assessments is rare; only the GARDskin/GARDpotency used to identify skin sensitizers in a human myeloid dendritic-like cell line have been accepted for regulatory studies (OECD TGP 4.106).

In the present study, we describe and test the predictive capability of a NAM using a set of gene expression biomarkers, that was designed to meet 21st century goals of reduced reliance on animals to identify potential carcinogens using short-term exposures in rats and transcript profiling. The NAM was trained to not only identify chemicals and their doses that would cause rat liver tumors but to also identify the underlying chemical mode of action (MOA). Regulatory agencies would then be able to use prior knowledge to determine if the MOA is of human relevance and whether the chemical would need to be examined in a 2-year bioassay. As the NAM was trained and tested on microarray data, we determined if the NAM would be able to accurately identify liver tumorigens using (targeted) RNA-Seq data. We found that the NAM accurately identifies chemicals and their doses that cause liver tumors in rat chronic studies under a wide variety of acute testing conditions. The information derived from the NAM then can be used to determine if the predicted mode of action would be relevant to humans.

TABLE 1 Chemicals used in the rat 4-day study (study A).

Common chemical name (abbreviation used in the study)	CASRN	DTXSID	Dose level used in the study (mg/kg/day)	Dose classification[1]	Lowest tumorigenic dose (mg/kg/day)	Highest non-tumorigenic dose (mg/kg/day)
2,5-Pyridinedicarboxylic acid, dipropyl ester	136-45-8	DTXSID8032544	600	3	1000	500
Acetochlor	34256-82-1	DTXSID8023848	250	1	250	69
Ametryn	834-12-8	DTXSID1023869	176	1	176	26.2
Bisphenol A	80-05-7	DTXSID7020182	450	3		95.4
Carbaryl	63-25-2	DTXSID9020247	100	2	500	100
Cyclanilide	113136-77-9	DTXSID5032600	58.6	3		43.1
Cyfluthrin	68359-37-5	DTXSID5035957	23	2		23
Cyprodinil	121552-61-2	DTXSID1032359	74	2		73.6
Di (2-ethylhexyl) phthalate	117-81-7	DTXSID5020607	600	1	99	19.8
Estragole	140-67-0	DTXSID0020575	600	1	600	
Ethyl methanesulfonate	62-50-0	DTXSID6025309	200	3		
Flusilazole	85509-19-9	DTXSID3024235	13	3		
Flutamide	13311-84-7	DTXSID7032004	10	3		50
Indoxacarb	173584-44-6	DTXSID1032690	10	2		10
Lipopolysaccharide (LPS)	NOCAS_36695	DTXSID4036695	2	3		
N,N-dimethyl-p-toluidine	99-97-8	DTXSID0021832	60	1	60	20
Perfluorooctanoic Acid	335-67-1	DTXSID8031865	15	1	4	1.9
Pirixinic acid (WY-14,643)	50892-23-4	DTXSID4020290	10	1	10	
Simazine	122-34-9	DTXSID4021268	63	2		1000
Tebufenpyrad	119168-77-3	DTXSID0034223	17	1	6.5	
Triclosan	3380-34-5	DTXSID5032498	1000	3		127
Vinclozolin	50471-44-8	DTXSID4022361	225	1	225	83

[1]Tumorigenicity classification of the dose used in the study: 1 = tumorigenic; 2 = not tumorigenic; 3 = not known.

2 Methods

2.1 Overview of datasets used in the analysis

There were three datasets used in our analysis which are described in greater detail below.

- "Study A": To confirm that our approach could be used to identify hepatotumorigens using Affymetrix data at a single dose and time, we utilized a dataset generated in male Sprague-Dawley rats exposed to 22 chemicals at a single dose level each day for 4 days (rat 4-day study). This study has not been previously described. (Quick summary: 22 chemicals; 1 dose; 4 days; Affymetrix)
- "Study B": To compare biomarker activation levels generated using Affymetrix and RNA-Seq, we utilized a published dataset that was generated in male Sprague-Dawley rats exposed to 27 chemicals at one dose level each day for 3, 5 or 7 days (rat Affymetrix-RNA-Seq comparison study) derived from the DrugMatrix study. The livers of the rats were evaluated for gene expression changes using Affymetrix arrays and in later studies by RNA-Seq. The data from this study came from (Bushel et al., 2018; Svoboda et al., 2019; Wang et al., 2014). (Quick summary: 27 chemicals; 1 dose; 3, 5, or 7 days; comparing Affymetrix vs. RNA-Seq)
- "Study C": To determine if the biomarker tumorigenic activation levels (TALs) generated using microarray data could be applied to targeted RNA-Seq data, we used a dataset generated in male Sprague-Dawley rats exposed to 16 chemicals at up to 10 dose levels for 5 days (rat 5-day study). The livers of the rats were evaluated for gene expression changes using TempO-Seq. The livers used in this study came from a previously published study (Gwinn et al., 2020). (Quick summary: 16 chemicals; up to 10 doses; 5 days; targeted RNA-seq). It should be noted that the 5-day study was available to us to use as a dataset to determine if the Tempo-Seq platform could be used in the NAM, not to optimize the minimal number of doses to be used.

2.2 Rat 4-day study (study A)

2.2.1 Chemicals

The chemicals and doses used in the study are found in Table 1 and include those that are carcinogenic and noncarcinogenic at the doses used. There were also a set of chemicals in which the carcinogenic status of the dose used is not known. The following chemicals were obtained from Bayville Chemical Supply Corporation (Deer Park, NY) at label purities >95%: acetochlor, ametryn, cyclanilide, cyfluthrin, cyprodinil, flusilazole, indoxacarb, simazine, and tebufenpyrad. The following chemicals were obtained from Sigma-Aldrich (St Louis, MO) at label purities >97%: bisphenol A, carbaryl, ethyl methanesulfonate, flutamide, lipopolysaccharide, perfluorooctanoic acid, and triclosan. WY-14,643 was obtained from A.G. Scientific (San Diego, CA) at a label purity of 98.5%, and estragol was obtained from Penta Manufacturing Company (Livingston, NJ) at a label purity of 99.7%. Lipopolysaccharide was obtained from Sigma-Aldrich Company (St. Louis, MO). Syringeability, solubility, and concentration were verified for each chemical using either HPLC or GC methodologies.

2.2.2 Rat exposures

Male Harlan Sprague-Dawley rats (Harlan Laboratories, Dublin, VA) (6–9 weeks old) were maintained on a 12-h light/dark cycle at 20-25°C with a relative humidity of 30%–70%, fed NTP-2000 diet (Zeigler Bros., Gardners, PA) and provided food and water *ad libitum*. Rats were housed individually during acclimation and grouped 2 per cage. Animals were assigned to a dose group using a procedure that stratifies animals across groups by body weight such that mean body weight per group did not differ statistically among groups at the start of the study based on analysis of variance (ANOVA) (Statistical Analysis System version 9.1, SAS Institute, Cary, NC). Each vehicle control and treatment group had 6 animals. Studies were run on blocks of 4–6 chemicals at a time, with a common group of vehicle-treated animals for comparison.

Rats were exposed daily to either a 1% acetone/99% corn oil vehicle or test chemical (Table 1) dissolved in vehicle for four consecutive days by oral gavage at a dosing volume of 5 mL/kg. Ethyl methanesulfonate was administered in saline. Lipopolysaccharide was dissolved in saline and administered only once by intraperitoneal injection, 4 h prior to terminal sacrifice. For these last two chemicals, saline was used as the control. Dose volumes were adjusted for body weight daily. The dose of test chemicals used was in most cases based on the liver tumorigenic doses from cancer bioassays. For chemicals that did not cause rat liver tumors, the highest dose in the bioassay was used. To convert the dietary exposures to daily oral gavage exposures, average daily dietary intake was estimated from individual studies based on food intake and chemical concentrations in the diet. This dose was then converted to an oral gavage dose. The dose of flutamide used was based on pilot studies examining its anti-androgenic effects in rats (data not shown).

At 1 and 4 h post dosing, animals were observed cage side. Four hours (±15 min) after the final dose administration, animals were humanely euthanized by CO_2 asphyxiation and blood was collected via cardiac puncture. Death was confirmed by exsanguination. Rats were euthanized in the same order as they were dosed. Livers were excised and weighed. The left lobe of the liver was cut into cubes, flash frozen in liquid nitrogen, placed in cryotubes on dry ice, and then stored at or below −70°C for transcriptomic analysis. All animal procedures were in compliance with the Animal Welfare Act Regulations, 9 CFR 1–4. All animals were handled and treated according to the Guide for the Care and Use of Laboratory Animals (Clark et al., 1997).

2.2.3 RNA isolation

Frozen liver samples (approximately 20–30 mg) were submerged in ten volumes of pre-chilled RNAlater®-ICE (Life Technologies, Carlsbad CA) and stored at −20°C ± 10°C for a minimum of 16 h. The RNAlater®-ICE supernatant was then removed and each liver tissue sample, weighing between 23.6 and 30.0 mg, was added to lysis buffer and homogenized using plastic disposable pestles (Fisher Scientific, Pittsburgh, PA). Following homogenization, samples were stored at −80°C ± 10°C until RNA was isolated. Samples were thawed and centrifuged. RNA was extracted from the supernatant, subjected to DNase I digestion, and isolated using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Each RNA sample was analyzed for quantity and purity by UV analysis using a NanoDrop ID-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purity was defined as the ratio of A_{260} to A_{280}; an acceptable purity range was defined as a value between 1.80 and 2.20. A minimum concentration of 35 ng/μL was targeted to ensure reliable amplification using Affymetrix GeneChip® reagents and kits. All samples yielded an acceptable purity and concentration appropriate for use with the Affymetrix GeneChip® 3′ IVT Express Kit. All samples were evaluated for RNA integrity using an RNA 6000 Nano Chip kit with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and were based on the RNA integrity number (RIN) calculated by the 2100 Expert software. A RIN value of 8 and above was met for all samples indicating ideal integrity for microarray processing.

2.2.4 Microarray analysis

Total RNA (100 ng), isolated from each of the rat liver samples, was used to synthesize single-stranded DNA, which was subsequently converted into a double-stranded cDNA template for transcription. An *in vitro* transcription (IVT) reaction, which incorporates biotinylated ribonucleotide analogs, was then used to create labeled amplified RNA (aRNA). This RNA target preparation was performed using the Affymetrix GeneChip® 3′ IVT Express Kit (Affymetrix Inc., Santa Clara, CA). All incubation steps during this preparation were completed using an Eppendorf Mastercycler® thermal cycler (Eppendorf Hamburg, Germany).

Labeled aRNA was fragmented and subsequently hybridized to the Affymetrix Rat Genome 230 2.0 Array (31,099 probe sets) using an Affymetrix GeneChip® Hybridization Oven 645. Washing and staining of the arrays was completed using the Affymetrix GeneChip® Hybridization Wash and Stain kit and performed using the Fluidics Station 450 according to the Affymetrix recommended protocol. After washing and staining, the arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G and the raw microarray data (.cel files) were acquired using Affymetrix GeneChip® Command Console® Software (AGCC). The following QC parameters were evaluated for each array:

TABLE 2 Chemicals used in (study B).

Chemical name	CASRN	DTXSID	Dose (mg/kg/day)
3-Methylcholanthrene	56-49-5	DTXSID0020862	300
Aflatoxin B1	1162-65-8	DTXSID9020035	0.3
17beta-Estradiol	50-28-2	DTXSID0020573	150
5,6-Benzoflavone	6051-87-2	DTXSID8030423	1500
Bezafibrate	41859-67-0	DTXSID3029869	617
Carbon tetrachloride	56-23-5	DTXSID8020250	1175
Cerivastatin	145599-86-6	DTXSID9022786	7
Chloroform	67-66-3	DTXSID1020306	600
Clofibric acid	882-09-7	DTXSID1040661	448
Clotrimazole	23593-75-1	DTXSID7029871	89
Econazole	27220-47-9	DTXSID2029872	334
17alpha-Ethinylestradiol	57-63-6	DTXSID5020576	10
Fluconazole	86386-73-4	DTXSID3020627	394
Gemfibrozil	25812-30-0	DTXSID0020652	700
Ifosfamide	3778-73-2	DTXSID7020760	143
Leflunomide	75706-12-6	DTXSID9023201	60
Lovastatin	75330-75-5	DTXSID5020784	450
Methimazole	60-56-0	DTXSID4020820	100
Miconazole	22916-47-8	DTXSID6023319	920
Nafenopin	3771-19-5	DTXSID8020911	338
N-Nitrosodimethylamine	62-75-9	DTXSID7021029	10
Norethindrone	68-22-4	DTXSID9023380	375
Phenobarbital	50-06-6	DTXSID5021122	54
WY-14,643	50892-23-4	DTXSID4020290	364
Rosiglitazone	122320-73-4	DTXSID7037131	1800
Simvastatin	79902-63-9	DTXSID0023581	1200
Thioacetamide	62-55-5	DTXSID9021340	200

average background, scale factor, percent of genes scored as present, 3′ to 5′ ratios for the internal control genes beta-actin and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), values for hybridization control transcripts, and values for poly (A) controls. Microarrays were normalized in GeneSpring 12.0 using RMA and features were then filtered in which a feature needed to be present at >20% percentile rank in the normalized intensity data in all samples from at least one treatment group. Filtered gene lists were then subject to a Welch test (unpaired, unequal variance *t*-test; treated vs. paired vehicle control). Genes with statistically significant differential expression were those exhibiting a p-value <0.05. The p-values were not subjected to a multiple testing correction, because this is not a standard applied for creating lists of differentially expressed genes in BaseSpace Correlation Engine (BSCE) (Kupershmidt et al., 2010). The genes exhibiting significant differential expression were further filtered by removing genes that exhibited less than an absolute 1.2-fold change. Lists of differentially expressed genes and their fold change values for each chemical treatment were uploaded into BSCE.

2.3 Rat affymetrix-RNA-Seq comparison study (study B)

The analysis of the profiles generated from this study have been described previously (Bushel et al., 2018; Svoboda et al., 2019; Wang et al., 2014). Briefly, male Sprague-Dawley rats were exposed by oral gavage to one of 27 chemicals at one dose level for 3, 5 or 7 days (three rats per chemical with matched controls). Liver RNA was isolated and analyzed using Affymetrix microarrays (Gene Expression Omnibus (GEO) accession number: GSE47875) and Illumina RNA-Seq (GSE55347). The chemicals and their doses

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TABLE 3 Chemicals used in (Study C).

Chemical	Abbreviation	CASRN	DTXSID#	Dose levels (mg/kg/day)	Highest nontumorigenic dose	Lowest tumorigenic dose	Dose classification (in order of dosing order)[1]
Acrylamide	ACR	79-06-1	DTXSID5020027	0.075, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10	2.7		2,2,2,2,2,3,3,3
Bromodichloroacetic acid	BDCA	71133-14-7	DTXSID4024644	1.25, 2.5, 5, 10, 20, 40, 80, 160	43		2,2,2,2,2,3,3,3
Coumarin	COU	91-64-5	DTXSID7020348	3.125, 6.25, 12.5, 25, 50, 100, 200, 400	71.4	200	2,2,2,2,2,3,1,1
Di (2-ethylhexyl) phthalate	DEHP	117-81-7	DTXSID5020607	8, 16, 31.25, 62.5, 125, 250, 500, 1000	19.8	99	2,2,3,3,1,1,1,1
Pentabromodiphenyl ether mixture	DE71	32534-81-9	DTXSID2024246	0.38, 0.75, 1.5, 3, 15, 50, 100, 200, 500	15	50	2,2,2,2,1,1,1,1
Ethinyl estradiol	EE2	57-63-6	DTXSID5020576	0.02, 0.067, 0.2, 0.6, 1.8, 5.4, 16.2, 48.6		0.429	3,3,3,1,1,1,1,1
Fenofibrate	FEN	49562-28-9	DTXSID2029874	8, 16, 31.25, 62.5, 125, 250, 500, 1000	10	45	2,3,3,1,1,1,1,1
Furan	FUR	110-00-9	DTXSID6020646	0.125, 0.25, 0.5, 1, 2, 4, 8, 16		1.4	2,2,2,2,2,3,1,1
Hexachlorobenzene	HCB	118-74-1	DTXSID2020682	0.004, 0.015, 0.0625, 0.25, 1, 4, 16, 64	1.6	5	2,2,2,2,2,3,1,1
Methyl eugenol	MET	93-15-2	DTXSID5025607	4.625, 9.25, 18.5, 37, 75, 150, 300, 600		26.4	3,3,3,1,1,1,1,1
Perfluorooctanoic acid	PFOA	335-67-1	DTXSID8031865	0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20		2.2	3,3,3,3,1,1,1,1
Pulegone	PUL	89-82-7	DTXSID2025975	2.4, 4.7, 9.4, 18.75, 37.5, 75, 150, 300	37.5		2,2,2,2,2,3,3,3
Tetrabromobisphenol A	TBBPA	79-94-7	DTXSID1026081	4, 8, 16, 31.25, 62.5, 125, 250, 500, 1000, 2000	1000		2,2,2,2,2,2,2,2,2,3
3,3′,4,4′-Tetrachloroazobenzene	TCAB	14047-09-7	DTXSID6026086	0.1, 0.3, 1, 3, 10, 30, 100, 200, 400	10	100	2,2,2,2,2,3,1,1,1
Tris (chloropropyl) phosphate	TCPP	13674-84-5	DTXSID5026259	18.75, 37.5, 75, 150, 300, 600, 1000, 2000	395	789	2,2,2,2,2,3,1,1
α,β-Thujone	THU	76231-76-0	DTXSID3040774	1.5, 3, 6.25, 12.5, 25, 50, 100, 200	50		2,2,2,2,2,2,3,3

[1]Tumorigenicity classification of the dose used in the study: 1 = tumorigenic; 2 = not tumorigenic; 3 = not known.

used in the study are found in Table 2. Starting with the raw expression data available in GEO, all statistically filtered gene sets from the study were generated using the BSCE analysis pipeline for Affymetrix or RNA-Seq data that has been described previously (Kupershmidt et al., 2010).

2.4 Rat 5-day study (study C)

This study has been described previously (Gwinn et al., 2020). Briefly, male Sprague Dawley (Hsd: Sprague Dawley SD) rats were exposed by oral gavage to 16 chemicals at up to 10 doses once per day for 5 consecutive days (Days 0–4) with $n = 4$ rats per exposure concentration and vehicle control. The rats were sacrificed on the 5th day. The chemicals and their doses used in the study are found in Table 3. In the original study, the liver RNAs were evaluated using the rat S1500$^+$ TempO-Seq platform. To comprehensively evaluate transcriptional benchmark dose (BMD) approaches, the RNAs used in the original study were re-isolated and evaluated using the rat full genome TempO-Seq platform. For RNA isolation, frozen RNA stabilized tissues were obtained from the National Toxicology Program, thawed on ice and ~10 mg liver were distributed at one sample per well in nuclease-free 96-well plates (Cat. 89218-298, VWR, Radnor, PA, United States) preloaded with 50 µL/well RNAlater™ Stabilization Solution (Cat. AM7021, Invitrogen by ThermoFisher Scientific, Vilnius, Lithuania). Plates were sealed with nuclease-free aluminum seal (Cat. 75805-268, VWR® Aluminium Foil Seals, Radnor, PA, United States) suitable for ultracold storage and stored at < −70°C until RNA isolation and purification was performed (BioSpyder Technologies, Carlsbad, CA, United States). For RNA isolation and purification, samples were processed using the RNAdvance purification kit (Beckman Coulter, Indianapolis, IN, United States) according to the manufacturer protocol. First, tissues were removed from RNAlater™ and transferred to deep-well homogenization plates loaded with RNAdvance lysis buffer and two stainless steel balls. Following homogenization, sample supernatants were digested in lysis buffer and RNA bound to kit provided SPRI beads. Bound RNAs underwent several rounds of incubation and washing followed by DNAse treatment according to RNAdvance protocol with purified RNA eluted in 40 µL nuclease-free water. Purified RNA was stored at < −70°C until sequenced. Raw TempO-Seq reads were aligned to all known probe sequences for the Rat Whole Transcriptome v1.0 probe set, as described previously (Harrill et al., 2021) (Everett et al., 2024 in preparation). Individual samples with <50% of reads uniquely aligned to known probe sequences, or <1 million uniquely aligned reads were removed from further analysis. Outlier samples were identified using PCA plots for each chemical and removed from further analysis as previously described (Everett et al., 2024 in preparation). For each chemical, differential expression analysis was performed using DESeq2 as previously described (Harrill et al., 2021). Briefly, probe counts were tabulated for all samples passing the quality checks described above, corresponding to each dose group and study-matched vehicle controls. Only those probes with mean count ≥5 were used for DESeq2 analysis. A single DESeq2 model was fit per chemical, with each dose group considered as an additional treatment factor. *p*-values and fold-changes were then computed for each dose group *versus* the vehicle control group. To derive a gene list for each dose group, genes were filtered to those with unadjusted *p*-value ≤0.05 (Wald test), and normal shrinkage was used to derive moderated log2 fold-change values. The gene lists were imported into BSCE and compared to the 6 biomarkers as described below. Outlier samples were removed as described (https://www.epa.gov/etap). There was one outlier removed in the following groups: DE71, 15 mg/kg; TBBPA, 31.25 mg/kg.

2.5 Determination of hepatocarcinogenicity of chemicals

We utilized a number of databases that had annotations for tumor induction after chronic exposure in rats. Most of the data came from the Lhasa Carcinogenicity Potency Database (CPD) (https://carcdb.lhasalimited.org/). Data for pesticides not in the CPD came from annotations in the ToxRef database (Watford et al., 2019) or National Toxicology Program studies. Carcinogenicity information for fenofibrate was kindly provided by Drs. Frank Sistare and Rachel Hao using the Pharmapendium database (https://www.elsevier.com/solutions/pharmapendium-clinical-data; accessed 13 August 2022). For all chemicals, we annotated effects described in these studies after chronic exposure on incidence of the following liver effects: hepatocellular carcinomas and adenomas, multiple liver tumor types, neoplastic nodules, trabecular hepatocellular carcinomas, and hepatocellular cholangiocarcinomas. The dose ranges and associated incidences were used to determine the highest non-tumorigenic dose and the lowest tumorigenic dose (if relevant). Any incidences greater than 5% over the control were considered tumorigenic, especially if higher doses resulted in greater incidences. Chemicals evaluated using the 2-year bioassay in which there were no increases in liver tumor incidences were assigned a highest non-tumorigenic dose representing the highest dose used in the study. For the most part, data was collected from 2-year bioassays. For one chemical (WY-14,643), only 1-year studies were available but allowed the derivation of lowest tumorigenic dose levels. Annotations were only made for chemicals with clear positive or negative responses, in female or male rats from any strain. All tumor data used in the analysis is found in Supplementary Files S1, S2.

2.6 Comparison of established biomarkers to gene lists

The six biomarkers for AhR, CAR, PPARα, ER, cytotoxicity and genotoxicity have been previously described (Rooney et al., 2018a; Hill et al., 2020). The biomarker genes and associated fold-changes along with the gene lists generated from the 4-day and 5-day rat studies described above were uploaded into BaseSpace Correlation Engine (BSCE), in which internal protocols rank the genes by absolute fold-change (Kupershmidt et al., 2010). The Running Fisher test is then used to compare the ranked biomarker genes to each ranked gene list from the three studies, calculating a pair-wise correlation *p*-value for the genes that overlap between lists. The *p*-values were converted to -Log (*p*-values) and negative correlations were converted to negative numbers. These procedures allowed the

evaluation of the correlation of the overlaps between gene lists. Thus, the higher the -Log (*p*-value), the greater the correlation.

2.7 Application of tumorigenic biomarker activation levels

The activation levels of each of the biomarkers associated with tumorigenicity were derived as described earlier (Corton J. et al., 2020). Briefly, biomarker activation levels associated with liver tumor induction were derived from two large datasets: the TG-GATES study and the DrugMatrix study. Because we are at an early stage in potential use of the NAM, we wished to determine if one set of tumorigenic activation levels (TALs) are more predictive than another. Using chemical-dose pairs annotated for liver tumorigenicity, biomarker activation levels associated with the maximum -Log (*p*-value)s that did not generate a liver tumorigenic response were used as the TALs. The levels were derived from CodeLink microarray data from the DrugMatrix study or Affymetrix data from the TG-GATES study. The biomarker TALs are found in Supplementary File S3. Each biomarker -Log (*p*-value) derived from the three studies described above was evaluated relative to the biomarker TG-GATES and DrugMatrix TALs resulting in 12 tumorigenic biomarker activation levels to determine if exposure to a dose of a chemical exceeded or not the biomarker activation level. The datasets used to determine the TALs were not used in the present study. If any biomarker in each set of six exceeded the TAL, then the dose was predicted to lead to liver tumors in chronic studies, otherwise the dose was not predicted to be tumorigenic.

2.8 Determination of accuracy of the approach

For Study A and Study C, the predictive accuracy was determined at the level of the individual chemical-doses. For Study C, predictions were also made by chemical at any dose level. Predictions in this scenario would be similar to those used in preliminary testing of a new chemical entity to assist in avoiding any potential liabilities. The biomarker TALs derived from the TG-GATES or DrugMatrix studies (described above) were used to determine if the test chemical-dose exceeded or not the activation levels. The predictions for tumorigenicity were assigned a score of false positive (FP), false negative (FN), true positive (TP), or true negative (TN). Regarding the predictions based on any dose of a chemical (Study C), FN was assigned if all of the doses for a tumorigenic chemical were beneath all biomarker TALs. FP was assigned if any dose for a nontumorigenic chemical exceeded one or more of the biomarker TALs. These were the equations used in determining scores: balanced or predictive accuracy = (sensitivity + specificity)/2; sensitivity (TP rate) = TP/(TP + FN); specificity (TN rate) = TN/(FP + TN); positive predictive value (PPV) = TP/(TP + FP); negative predictive value (NPV) = TN/(TN + FN).

3 Results

3.1 Use of a NAM computational model to identify liver tumorigens after short-term exposures

Our study was designed to achieve three objectives. First, we wished to confirm that our NAM approach could be used to identify liver tumorigens when examining only one dose level. In this case, the study was conducted using Affymetrix arrays. The second objective was to compare the transcriptional responses between Affymetrix and RNA-Seq to determine if the derived biomarker -Log (*p*-value)s would be different between the two methods that may preclude accurate predictions using (targeted) RNA-Seq. Lastly, we wished to determine if the NAM could be applied to transcript profiles derived from (targeted) RNA-Seq (TempO-Seq) analyses without having to rederive the TALs.

To accomplish these objectives, we utilized a previously described NAM that can predict liver cancer outcomes using transcript profiles derived from the livers of rats treated with chemicals with unknown potential to cause liver cancer (Figure 1). The computational model consists of three major components necessary for prediction. First, there are six well-characterized gene expression biomarkers predictive of the modulation of the major MIEs of rodent liver cancer. Each biomarker consists of 7–113 genes and associated fold-change values that are used to determine whether a chemical is an activator of one or more MIEs (Rooney et al., 2018b; Corton J. C. et al., 2020; Corton J. et al., 2020; Hill et al., 2020; Lewis et al., 2020). As it is well known that activation by itself is not sufficient to generate the signals that lead to the adverse outcome, we had previously identified activation levels for each biomarker associated with tumor induction (called tumorigenic activation levels or TALs). We derived the TALs to predict induction of hepatocellular adenomas and/or carcinomas. The TALs have not been tested for other types of liver tumors, e.g., cholangiocarcinomas in part due to their rarity as outcomes. The last component is the Running Fisher statistical test within the BaseSpace Correlation Engine environment used to compare each of the biomarkers to the chemical-induced transcript profiles. We had previously determined that gene lists derived from the livers of rats exposed to a chemical up to 29d could be used by the NAM for accurate prediction or tumorigenicity (Rooney et al., 2018c; Corton J. C. et al., 2020; Corton J. et al., 2020; Hill et al., 2020; Lewis et al., 2020). Our previous studies showed that the predictions coming from the NAM computational model can not only be used to identify which MIEs are activated but whether the level of activation exceeds a tumorigenic level. Here, we apply this NAM to rat studies that vary by chemical, dose level, time of exposure, and profiling platform.

3.2 Prediction of tumorigenicity of chemicals examined at a single dose

To accomplish our first objective, we evaluated the transcript profiles derived from the livers of rats treated with 22 chemicals at one dose level for 4 days (Study A). These chemicals included pesticides, industrial chemicals and reference chemical activators of one or more

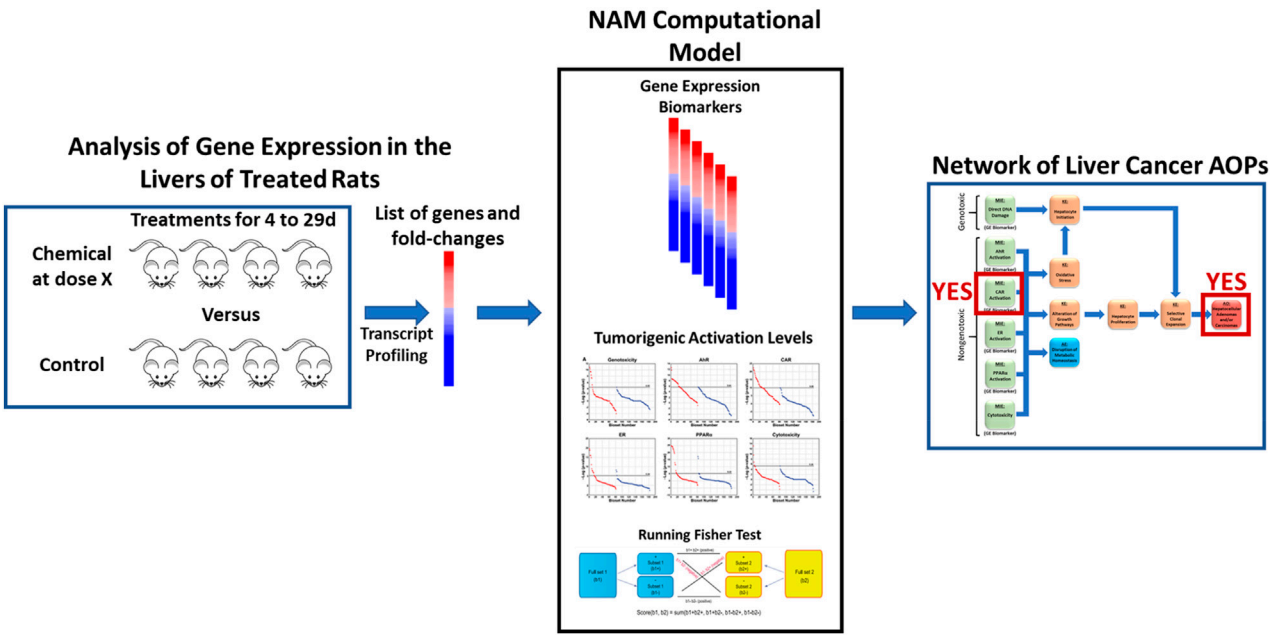

FIGURE 1
Use of a new approach methodology computational model to identify liver tumorigens. The computational model consists of three major components. There are six well-characterized gene expression biomarkers predictive of the modulation of the major molecular initiating events of rodent liver cancer. Each biomarker consists of 7–113 genes and associated fold-change values. The model includes biomarker activation levels associated with liver tumor induction after chronic exposure. The Running Fisher test within the BaseSpace Correlation Engine environment is used to compare each of the biomarkers to a transcript profile derived from the liver of a rat exposed to a new chemical entity for up to 29d. The model is able to identify MIEs activated and whether the level of activation exceeds a tumorigenic level.

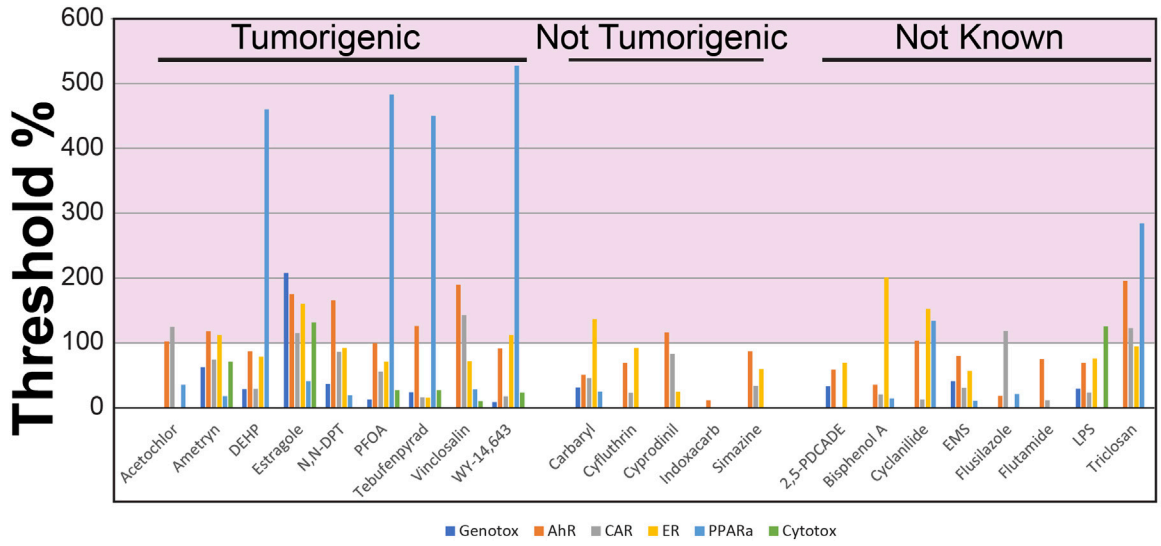

FIGURE 2
Identification of chemical-dose pairs that are tumorigenic. The 6 biomarkers were compared to the transcript profiles derived from the livers of rats exposed to the indicated chemicals (dose levels described in Table 1). The 6 -Log (*p*-value)s representing the correlation of each chemical to the 6 biomarkers was compared to the tumorigenic thresholds derived from the TG-GATES study. Values on the *y*-axis represent the (biomarker -Log (*p*-value)/the tumorigenic threshold) × 100. Any treatment that exceeds 100% for any of the biomarkers (pink shaded area) would be predicted to cause increases in liver tumors under chronic conditions.

MIEs. Each dose level was classified as tumorigenic, not tumorigenic or not known. There were 14 chemical-dose pairs that could be annotated for cancer outcome. Each profile was compared to the set of 6 biomarkers using the Running Fisher test. The level of activation of each biomarker was compared to the TAL derived from the TG-GATES study (TG-TAL) (Figure 2) or from the DrugMatrix study

TABLE 4 Predictive accuracies derived using the NAM.

Study	Unit of prediction	Tumorigenic activation level	Total number of biosets or chemicals examined	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV	Balanced accuracy
Study A	Chemical-Dose	TG-GATES	14	9	3	2	0	1	0.6	0.82	1	0.8
Study A	Chemical-Dose	DrugMatrix	14	7	5	0	2	0.78	1	1	0.71	0.89
Study C	Chemical-Dose	TG-GATES	100	31	51	7	11	0.74	0.88	0.82	0.82	0.81
Study C	Chemical-Dose	DrugMatrix	100	22	56	2	20	0.52	0.97	0.92	0.74	0.75
Study C	Chemical	TG-GATES	16	11	3	2	0	1.00	0.60	0.85	1.00	0.80
Study C	Chemical	DrugMatrix	16	9	5	0	2	0.82	1.00	1.00	0.71	0.91

(DM-TAL) (Supplementary File S4). Figure 2 shows the TG-TALs relative to the tumorigenic levels for each of the biomarkers for the chemicals. There were 9 chemicals that were examined at tumorigenic dose levels (acetochlor, ametryn, di (2-ethylhexyl) phthalate (DEHP), estragole, N,N-dimethyl-p-toluidine, perfluorooctanoic acid (PFOA), tebufenpyrad, vinclozalin, WY-14,643 (WY)). DEHP and PFOA activated only one MIE (PPARα) at tumorigenic levels. The other chemicals activated a mixture of two or more MIEs but most commonly, AhR and CAR. Using the DM-TALs, the analysis was repeated and is shown in Supplementary File S4. The MIEs that were activated to tumorigenic levels were similar to the analysis with the TG-TALs. However, two chemicals were called false negatives as they were not correctly identified as tumorigenic (acetochlor, ametryn).

There were 5 chemicals examined at doses that did not induce liver tumors at the highest dose tested (carbaryl, cyfluthrin, cyprodinil, indoxacarb, simazine). Using the TG-TAL, cyprodinil was predicted to cause liver tumors through an AhR mechanism and carbaryl through an ER mechanism (false positives) (Figure 2). Using the DM-TAL, no chemicals were predicted to be tumorigenic (Supplementary File S4).

In addition to the tumorigenic and nontumorigenic chemicals, there were 8 chemicals (2,5-pyridinedicarboxylic acid, dipropyl ester (2,5-PDCADE), bisphenol A, cyclanilide, ethyl methanesulfonate (EMS), flusilazole, flutamide, lipopolysaccharide (LPS), triclosan) that could not be classified for tumorigenicity, either because the dose examined in the study was higher than the highest nontumorigenic dose or that the chemical had not been examined in a chronic study. For all but EMS, flutamide and 2,5-PDCADE, the chemicals were predicted to increase liver tumor incidence after 2 years using the TG-TALs (Figure 2), while only cyclanilide and triclosan would be predicted to cause liver tumors in chronic studies using the DM-TALs (Supplementary File S4).

We determined how accurate the NAM was at identifying chemical doses that were tumorigenic. The predictive accuracy using the TG-TALs was 80% (100% sensitivity; 60% specificity) (Table 4). The predictive accuracy using the DM-TALs was 89% (78% sensitivity; 100% specificity). The level of accuracy for this set of chemicals is within the range of accuracies demonstrated in previous studies.

3.3 Relationships between biomarker TALs in affymetrix and RNA-Seq profiles

Our second objective was to determine if the biomarker TALs derived from microarray data could be applied to RNA-Seq data (Study B). A unique dataset was used to make comparisons between the two platforms. Male rats were exposed to 27 chemicals at one dose level each day for 3, 5, or 7 days, and the liver RNAs were evaluated using Affymetrix arrays and RNA-Seq. The two transcript profiles from each chemical-dose pair were compared to the set of 6 biomarkers. Figure 3 shows the biomarker activation levels (using -Log (*p*-value)s of the Running Fisher test as metrics) across all of the chemicals for individual biomarkers. The figures show that for the most part, there is a linear relationship between the activation levels determined by Affymetrix arrays and by RNA-Seq, especially within the range of the biomarker TALs (~2–7). Using the TG-TALs (Figure 3), we found that the levels derived from extrapolation to the RNA-Seq data were similar. For all but genotoxicity, the TALs from the RNA-Seq data were somewhat smaller compared to the TALs derived from the Affymetrix data. Using the DM-TALs (Supplementary Figure S4), we found that the levels derived from extrapolation to the RNA-Seq data were also similar. The findings indicate that the TALs could potentially be used to make predictions using RNA-Seq data.

3.4 Identification of chemical-dose pairs that are tumorigenic using TempO-Seq data

There have been no studies applying the NAM to full-genome TempO-Seq-derived transcript profiles to make predictions. We utilized a dataset from the livers of rats treated with 16 chemicals for 5 days at up to 10 dose levels for a total of 132 chemical-dose comparisons (Study C). There were 100 comparisons that could be annotated for potential to induce tumors. Figure 4 shows the biomarker activation levels relative to the TG-TALs for each chemical. The figures derived from the analysis using the DM-TALs are shown in Supplementary File S4.

Predictive accuracies were determined two ways. In the first method, accuracy was based on the 100 chemical-dose pairs that could be annotated for chronic outcomes. Table 4 shows that the balanced accuracies using the TG-TALs or DM-TALs was 81% or 74%, respectively. Using the TG-TAL, there were 11 false negatives

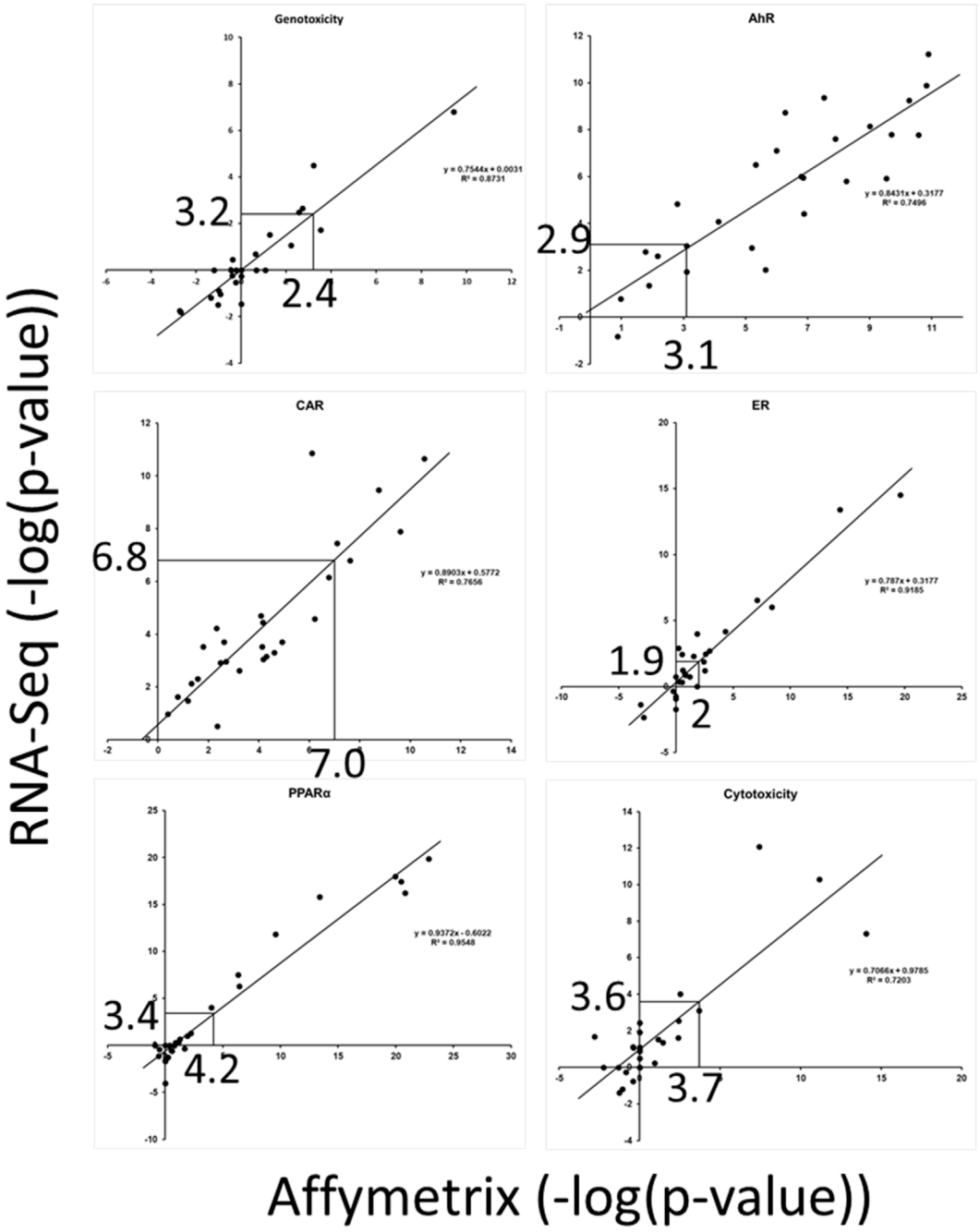

FIGURE 3
Relationships between biomarker activation levels derived using Affymetrix vs. RNA-Seq. Transcript profiles generated using either Affymetrix arrays or RNA-Seq were derived from the same livers of rats exposed to 27 chemicals. The pairs of profiles were compared to each biomarker. The TG-TALs are indicated on the *x*-axes and the derived TALs from the RNA-Seq analysis are shown on the *Y*-axes. The lines indicate linear trendlines. The figures show that the TALs derived from the Affymetrix data are similar to values derived from the RNA-Seq studies.

for 4 chemicals (furan, TCAB, EE, methyleugenol) and 7 false positives for 6 chemicals (coumarin, TCPP, BDCA, BDCA, TBBPA, coumarin, fenofibrate). A number of the false positives were at doses lower than those that were tumorigenic including for coumarin, DE71, fenofibrate, and TCPP, indicating the TG-TALs are sensitive to gene changes that precede overt tumor induction. Using the DM-TALs, there were 20 false negatives for 8 chemicals (coumarin, DE71, EE, furan, HCB, methyleugenol, TCAB, TCPP) and 2 false positives for 2 chemicals (coumarin, fenofibrate). The relatively high level of false negatives compared to the TG-TALs may be due to the higher -Log (*p*-value)s for the DM-TALs for all 6 biomarkers.

In a screening study to identify hazards, all doses would be considered, not just individual chemical-dose pairs. When the accuracy was determined based on evaluation of all dose levels

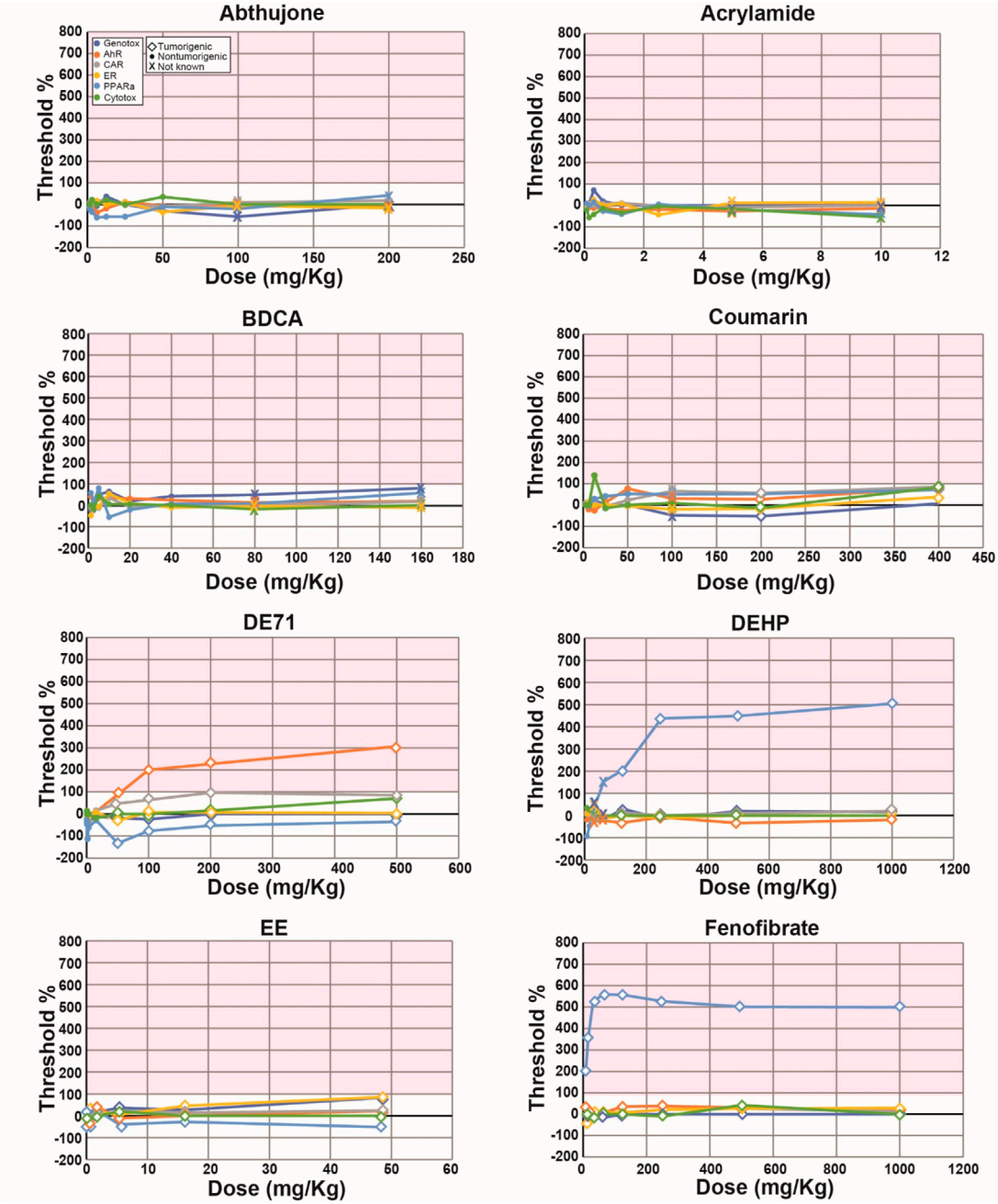

FIGURE 4
(Continued).

for each chemical, the balanced accuracies were 80% and 91% for the TG- and DM-TALs, respectively. There were 2 false positives (BDCA, TBBPA) using the TG-TALs and no false negatives. For BDCA the TALs were not dose-dependent; the activation levels were achieved at 10 mg/kg for ER and at 5 mg/kg for PPARα. This is in contrast to the true positive chemicals in which there was usually more than one dose level that was positive for one of the MIEs and occurred at the higher dose levels. There were 2 false negatives (EE, methyleugenol) using the DM-TALs and no false positives. Thus, the NAM can be accurately applied to TempO-Seq data.

4 Discussion

New approach methodologies (NAMs) have the potential to radically transform carcinogenicity testing. Integrated sets of *in vitro* assays could be used in IATA-type approaches. However, their ability to accurately predict cancer has not been fully tested. Short-term exposures in test species coupled with NAMs have the potential to greatly reduce the number of animals and could act as a bridge between the current requirements for chronic exposure testing and future *in vitro* testing strategies. Here, we describe a novel NAM that can be used with transcript profiling

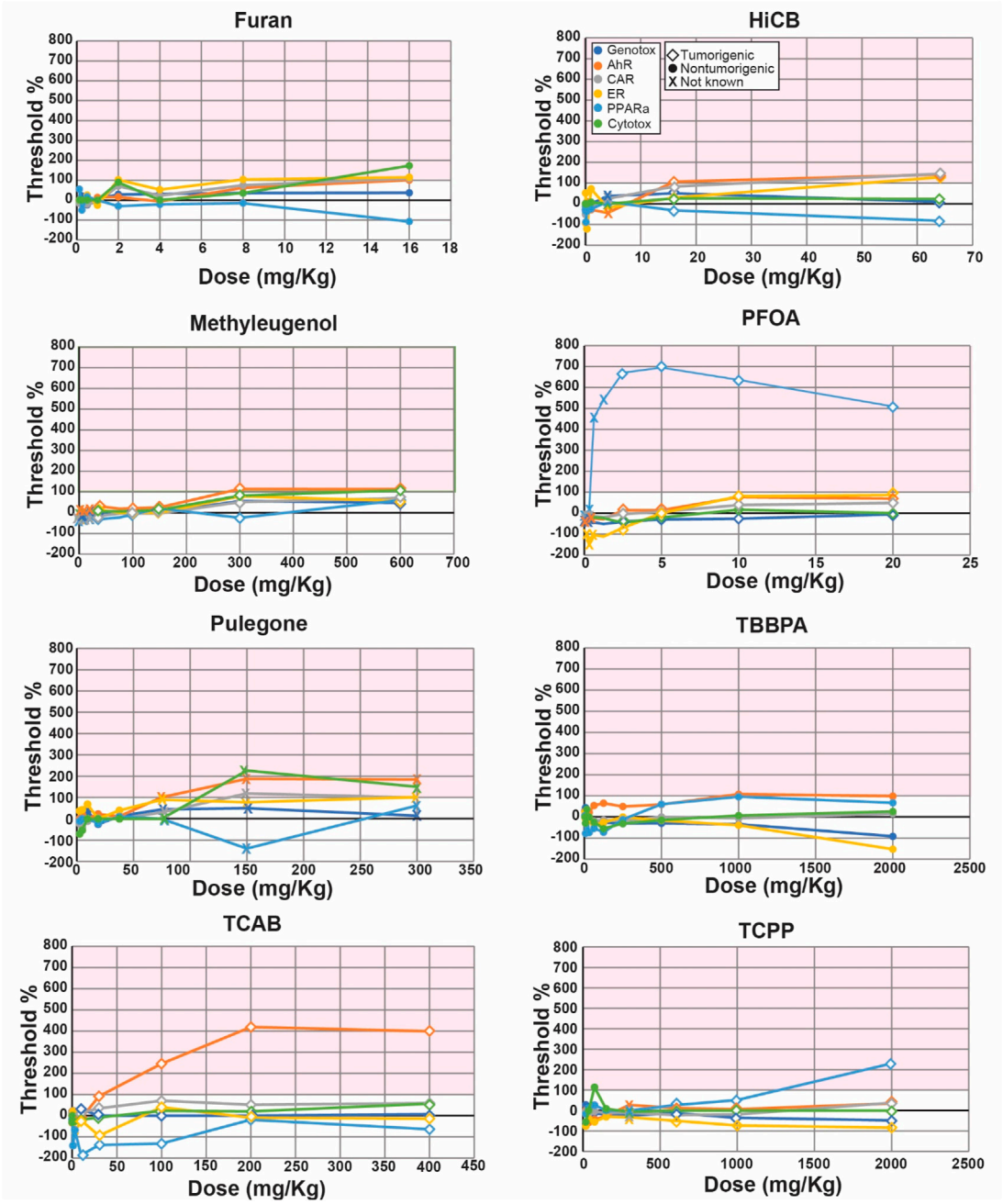

FIGURE 4
(Continued). Biomarker activation levels identify chemical-dose pairs that are tumorigenic in chronic studies. Rats exposed to 16 chemicals at up to 10 dose levels were evaluated for gene expression changes using targeted RNA-Seq (TempO-Seq). Each derived gene list was compared to the 6 biomarkers using the Running Fisher test. Dose-dependent changes in the -Log (*p*-value)s of each biomarker relative to the derived TG-TALs are shown. A similar analysis using the DrugMatrix TALs is found in Supplementary File S4. The TAL for each biomarker was set at 100%. The different color lines track the changes in the TALs for each of the molecular initiating events. Each dose is indicated as a diamond (tumorigenic), a filled circle (not tumorigenic) or x (tumorigenicity at this dose is not known). Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive activated receptor; ER, estrogen receptor; PPARα, peroxisome proliferator-activated receptor α.

measurements to identify in short-term exposures, chemicals and their doses that would cause tumors in the livers of rats (Figure 1). Capitalizing on three studies conducted in rats in which liver gene expression was evaluated after 3–7 days exposures, we demonstrated that 1) using Affymetrix data, the NAM could identify individual chemical-dose pairs that were tumorigenic (80% or 89% accuracy); 2) when comparing the transcript profiles generated from the same liver samples by Affymetrix and RNA-Seq, there were no notable differences in the responses in the -Log (*p*-value) range of biomarker TALs, indicating the TALs derived from microarray data could be applied to RNA-Seq data, and supporting this observation; 3) using TempO-Seq-generated transcript profiles, the NAM was able to

identify chemicals and their dose levels that would be tumorigenic with 75%-91% accuracy. In summary, the NAM can be used for prediction of liver tumor induction under different rat exposure scenarios and using different platforms to interrogate RNA expression.

Due to the diversity and complexity of the biological processes underlying tumor formation, the ability to predict human tumor induction using sets of nonanimal-based NAMs within an IATA framework will be challenging. While rodent tumor formation does not always mimic that in humans, regulatory mandates require rodent carcinogenicity testing, which the current study is meant to support and optimize. While approaches using large sets of *in vitro* assays coupled with *in vitro* to *in vivo* extrapolation to set exposure limits appear to be promising (Paul Friedman et al., 2020), most new chemicals will not be evaluated using even a subset of the assays. Short-term tests in animals that link molecular and cellular changes to subsequent toxicity may provide a way to reduce animal testing, especially if approaches for harmonization of animal tests could be agreed upon. Use of HTTr gives a better understanding of underlying toxicity by indicating the actual toxicological mechanisms, which can be used to infer eventual toxicity and carcinogenesis; thus, this allows for the use of shorter exposures on fewer animals by negating the need to wait for the possible development of cancers over a rodent's lifetime. This approach could be incorporated into new standards to make future animal use more reliable and relevant, whilst reducing animal usage and suffering overall, and falling in step with the 3Rs of toxicology. With this in mind, an approach that has been receiving much attention recently is the *in vivo* application of transcriptomics for establishing a "bioactivity" point of departure (PoD). This approach is based on the hypothesis that any toxicity (including carcinogenicity) is not likely to occur in the absence of changes in gene expression in one or several sentinel tissues (Thomas et al., 2013) (https://www.epa.gov/etap). Promising studies examining adult and fetal tissues (e.g., Johnson et al., 2022) after short-term exposures have shown that the derived PoD could be used to protect human populations from adverse effects. The EPA has proposed to use this approach to determine PoD based on transcriptomics for data-poor chemicals (https://www.epa.gov/etap). Implementing this strategy to large sets of chemicals will be challenging due to the costs of the studies, identification of appropriate exposure conditions, the choice of tissues to examine, and the computational methods for deriving the PoD. Despite these challenges, the approach has the potential to greatly reduce the animal requirements for not only the 2-year bioassay, but other animal tests required by regulatory agencies. Until the toxicity testing community has greater confidence in this approach, NAMs with known predictive accuracies for important endpoints will likely assist in making regulatory decisions.

The NAM approach described and tested here was built using the network of liver cancer AOPs as a starting point that can be found in the AOPWiki (https://aopwiki.org/). The importance of using the AOP framework for building and testing NAMs is highlighted by work in which knowledge related to carcinogenicity assessment has been reorganized into AOP networks resulting in the development of the Kaptis model (https://www.lhasalimited.org/products/kaptis.htm) which like the AOPWiki has the potential to facilitate interpretation of the weight of evidence of available information related to carcinogenicity assessment and future integration of existing and emerging *in vitro* and *in vivo* assays used for prediction (Felter et al., 2021). While each of the liver cancer AOPs examined in our study contain key events downstream of the MIEs, many of these KEs cannot be measured using transcript profiling. Thus, we originally focused on methods to predict each of the MIEs of the major liver cancer AOPs using transcript profiling, a now routine method for identifying chemical hazards. The 6 biomarkers were constructed using profiles derived from the livers of rats exposed to reference chemical activators of each of the MIEs. In our past studies, the individual gene expression biomarkers had balanced accuracies of 92%–98% (Rooney J. P. et al., 2018; Hill et al., 2020). We found in these studies that most chemicals have mixed MOAs in that they activate 2 or more MIEs under conditions that would cause cancer. This finding highlights the need for measurement of all MIEs when considering whether exposure to a chemical would be relevant to humans.

Our approach to determining the activation of MIEs is similar to that described by another group. Using a multivariate regression approach applied to liver RNA-Seq data derived from rats exposed to a diverse reference chemical set enabled the identification and refinement of gene sets (biomarkers) predictive of agonists for 5 different canonical xenobiotic receptors (AhR, CAR, Pregnane X Receptor [PXR], PPARα, ER), 3 mediators of reactive metabolite stress responses (NRF2, NRF1, p53), and activation of the innate immune response (Podtelezhnikov et al., 2020). Additionally, a composite transcriptional biomarker of tissue injury and regenerative repair response was described by the same group and could be applied across 8 different tissues (Glaab et al., 2021). These 10 biomarkers along with thresholds for AhR activation (Qin et al., 2019) are used by the group for routine monitoring in initial rat tolerability studies just prior to entering drug development to identify drug candidate potential for activating these MIEs to trigger liver and other organ toxicities with strong (>90%) sensitivity and/or specificity (Monroe et al., 2020; Glaab et al., 2021).

These AOP-based approaches to predicting toxicity and cancer are different from the key characteristics of carcinogens (KCC) approach (Smith et al., 2016; Guyton et al., 2018) originally inspired by the idea of the Hallmarks of Cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) and identified and developed to organize new lines of evidence for assessing carcinogenicity. In the first study using the KCCs, Smith et al. (2016) analyzed the biological effects of chemicals classified as known human carcinogens and defined 10 KCCs. Tice et al. (Tice et al., 2021) reviewed the KCCs as a method to develop an IATA of carcinogenic potential using NAMs. However, their conclusion echoed by others (Goodman et al., 2018) was that the KCCs lack the necessary specificity for carcinogenicity prediction as KCCs are also involved in disease processes that are not related to cancer. Furthermore, no scheme has yet been proposed in which to relate the number of KCCs that are "positive" and carcinogenicity potential, the identification of assays to determine if the chemical exhibits that KCC, and how the KCCs could be used in a quantitative manner. There is general agreement that KCCs could play a role in assembling lines of evidence in assessing carcinogenic potential that would complement other relevant information.

While our MIE biomarkers had demonstrated utility in identifying chemical MOAs for liver tumorigens, it was not possible using the biomarkers alone to identify the doses of a chemical that would cause cancer. Thus, in later studies we capitalized on a central premise of the AOP concept which is

that while MIEs/KEs are required at a qualitative level, they must be activated to a sufficient level and duration to cause an adverse outcome (Conolly et al., 2017). Computationally-derived quantitative effect levels, or "molecular tipping points" can be used as tools for adversity determinations using shorter-term data (Julien et al., 2009; Knudsen et al., 2015). Using biomarker TALs that were derived a number of ways, we found that across 163 chemicals examined at multiple time points, the NAM had predictive accuracies of 96%–97% (Corton J. C. et al., 2020; Lewis et al., 2020). We also found that data requirements for prediction could be reduced to measuring 12 individual genes (2 from each biomarker) (Corton J. et al., 2020), or measuring combinations of liver weight to body weight and clinical chemistry markers (Corton J. C. et al., 2020); these approaches were predictive of liver tumors at up to 97% balanced accuracy. These predictions were based for the most part on legacy microarray and associated data from TG-GATES and DrugMatrix datasets. Remarkably from the current study, we showed that the predictive accuracies using full-genome targeted RNA-Seq (TempO-Seq) transcript profile data was as high as 91%, in the same range as our original studies. Thus, our NAM can be used under a wide number of short-term exposure scenarios (4–29 days) using transcript profiling platforms that are more commonly in use today.

The 6 biomarkers and their TALs discussed here could be applied in a number of ways for toxicological testing of industrial chemicals. After preliminary short-term exposure studies followed by gene expression analysis, the TALs could be used to help bracket the range of doses between the BMD and the calculated dose that would be expected to induce liver tumors. Knowledge of the TALs could be used to allow informed decisions to be made of doses to use in chronic studies to avoid tumor induction. In testing for pharmaceutical candidates, the TALs could be used to support reduced carcinogenicity testing under the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) S1 guidance modification initiatives. Modifications to ICH S1 Carcinogenicity Testing Guidance (ICH, 2015) proposes a more flexible approach to pharmaceutical carcinogenicity testing. This allows for adequate assessment of carcinogenic risk without the need for always conducting a 2-year rat carcinogenicity study. This modification in the guidance may enable drug sponsors to gain 2-year rat carcinogenicity study waivers through a Carcinogenicity Assessment Document (CAD)-based justification process. Our study represents an example of how gene expression thresholds could be leveraged as "new biomarkers" data (ICH, 2015) to strengthen CAD-based predictions. If, for example, after a 6-month study there are histopathological indicators of liver cancer signals, a short-term toxicogenomic study coupled with our biomarker TAL approach would provide information about the underlying AOP and doses that would lead to liver tumors and possibly contributing to the conclusion that a 2-year bioassay is not needed.

Given the convergence of approaches to build and utilize gene expression biomarkers by multiple groups, the HESI Emerging Systems Toxicology for the Assessment of Risk (eSTAR) committee has an ongoing multi-institution effort to identify predictive gene sets. The committee will employ a number of computational approaches to liver transcript profiles of chemicals annotated for liver cancer MIE modulation and cancer outcomes (Corton et al., 2022a). The approach will include data from wild-type *versus* factor-null rats where gene dependence on ligand-activated transcription factors can be confirmed, complemented with a large body of published or to-be-generated data including ChIP-Seq data to further support specific compound MIEs. The hope is that scientific consensus between investigators will result in a validated set of biomarkers and computational techniques that will be accepted by various regulatory agencies for widespread use for internal decision making as well as for regulatory applications.

In summary, the NAM described and tested here to be used to replace carcinogenicity testing exhibits characteristics desired in a method used for prediction. These include accurate prediction of whether the MIE is modulated and most importantly, whether the dose of the chemical would be tumorigenic in chronic studies. The NAM could be used for screening chemicals in short-term exposures to identify potential liabilities or after a chronic study before the appearance of tumors when the liver is found to be a tissue with histopathology findings of concern. The continued use of *in vivo* tests using new animal models or modifications of existing guideline animal studies of shorter duration is increasingly recognized as a necessity for bridging gaps en route to establishing new animal-free regulatory frameworks that are the goal of regulatory Agencies worldwide.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

All animal procedures were in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4 and approved by the NIEHS Institutional Animal Review Board. All animals were handled and treated according to the Guide for the Care and Use of Laboratory Animals (Clark et al., 1997). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VL: Data curation, Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing. SA: Data curation, Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing, Funding acquisition, Resources, Supervision, Visualization. LE: Investigation, Methodology, Writing–original draft, Writing–review and editing, Project administration. BV: Investigation, Writing–original draft, Writing–review and editing, Formal Analysis. AL: Writing–original draft, Writing–review and editing, Conceptualization. GA: Conceptualization, Writing–original draft, Writing–review and editing. WG: Writing–original draft, Writing–review and editing, Data curation, Formal Analysis, Investigation, Methodology. LW: Investigation, Methodology, Writing–original draft, Writing–review and editing. MH: Investigation, Methodology, Writing–original draft,

Writing–review and editing. MD: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. JC: Validation, Visualization, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing, Data curation, Formal Analysis, Investigation, Methodology, Software.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2024.1422325/full#supplementary-material

SUPPLEMENTARY FILE S1
Carcinogenicity information for the chemicals used in the rat 4-day study.

SUPPLEMENTARY FILE S2
Carcinogenicity information for the chemicals used in the rat 5-day study.

SUPPLEMENTARY FILE S3
Tumorigenic activation levels.

SUPPLEMENTARY FILE S4
Supplementary Figures S1–S3.

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