Food for Thought ...

Making Better Use of Toxicity Studies for Human Health by Extrapolating across Endpoints

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Abstract

To develop and evaluate scientifically robust and innovative approaches for the safety assessment of chemicals across multiple regulatory sectors, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) has started a project to explore how to better use the available information, including that from existing animal studies. The aim is to minimize reliance on *in vivo* testing to avoid redundancy and to facilitate the integration of novel non-animal methods in the regulatory setting with the ultimate goal of designing sustainable testing strategies. In this thought-starter paper, we present a number of examples to illustrate and trigger further discussions within the scientific and regulatory communities on ways to extrapolate useful information for predicting toxicity from one toxicity endpoint to another or across endpoints based on mechanistic information.

1 Introduction

Toxicity data requirements for substance authorization and/or registration, laid down in the relevant piece of EU legislation, vary depending on the product sector (Box 1).

Typically, requirements are fulfilled by data generated using standard test methods, which are testing procedures with a recognized scientific acceptance that are included in current regulatory guidelines. Among the available methods, both *in vitro* and *in vivo*, the OECD Guidelines for the Testing of Chemicals[1] represent a harmonized approach for assessing the potential effects of chemicals on human health and the environment. Currently, the testing of complex systemic toxicity effects, such as target organ toxicities, neurodevelopmental toxicity and toxicity to reproduction, genotoxicity and carcinogenicity, requires the use of experimental animals. In contrast, topical toxicity endpoints, such as eye damage/irritation, skin irritation and skin sensitization, already can be assessed with a number of *in vitro* test methods (Bos et al., 2020; Zuang et al., 2020).

Several pieces of EU legislation encourage or require the use of non-animal approaches, including *in vitro* methods, *in silico* and toxicokinetic models as well as read-across analysis (OECD, 2014b). However, these approaches are not yet used in a systematic way for hazard assessments, especially as stand-alone methods to assess a toxicity endpoint (Mahony et al., 2020). So far, the pieces of legislation with the largest potential impact on the 3Rs (Replacement, Reduction and Refinement of animals used for scientific purposes) have been the EU Cosmetics Regulation (EC, 2009), because of the ban on animal testing, and the REACH chemicals legislation (EC, 2006), where the use of animals should be considered as last resort. Moreover, in light of the EU Directive on the protection of animals used for scientific purposes (EU, 2010), the use of standard and non-standard test methods not requiring experimental animals is encouraged in all sectors of EU Chemicals Policy.

Hence, to further the use of non-animal approaches and, in a broad context, new approach methodologies (NAMs) (ECHA, 2016) (Box 2), it is necessary to make better use of the overall toxicity information in the form of data or mechanistic knowledge. This will also add more human-relevant mechanistic information and avoid repeating redundant studies, particularly *in vivo* studies (e.g., sub-acute, sub-chronic, chronic toxicity studies). In addition,

[1] https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals_72d77764-en

Received May 6, 2020; Accepted July 28, 2020;
Epub July 28, 2020; © The Authors, 2020.

ALTEX 37(4), 519-531. doi:10.14573/altex.2005061

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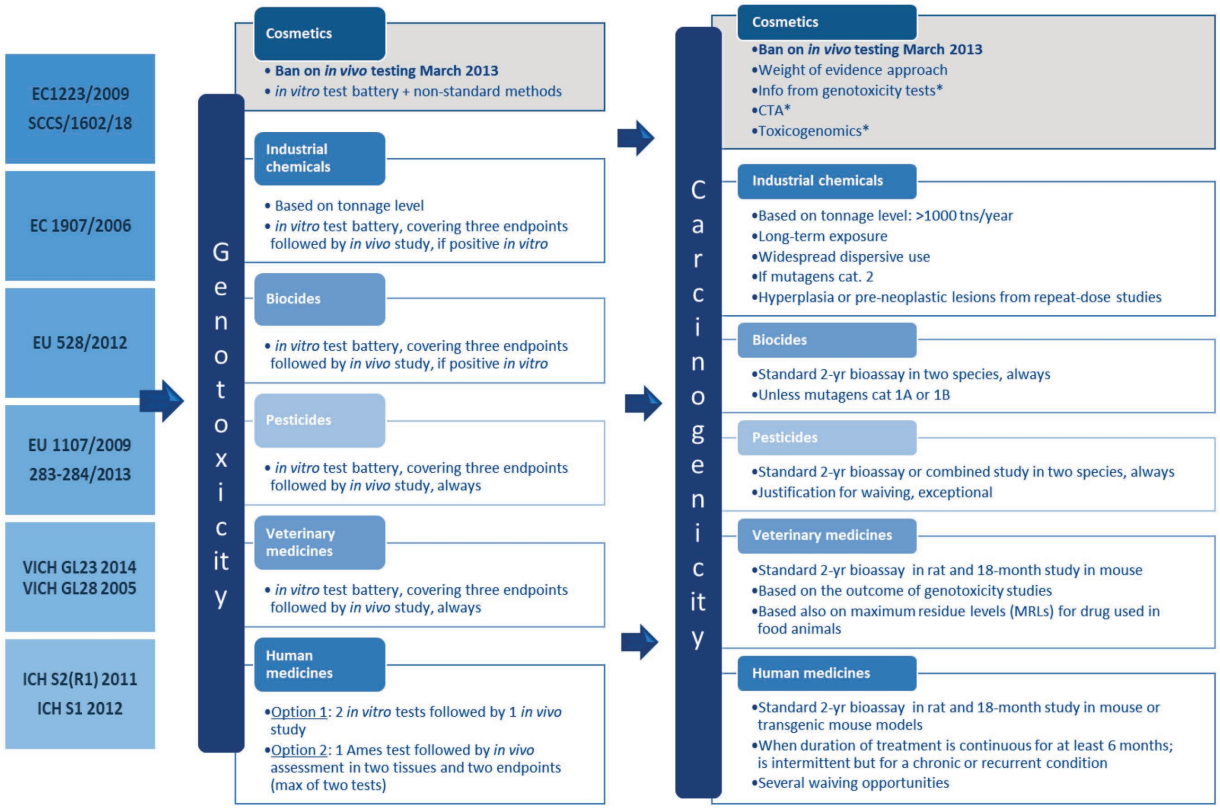
Box 1

Data requirements for the assessment of toxicity endpoints differ across product sectors. Differences may depend on the scope of each piece of legislation, provisions, and/or specific approaches used for the overall safety assessment. EU regulatory needs for the assessment of genotoxicity and carcinogenicity are two examples.



* The use of *in vitro* test methods with no specific test guideline (i.e., OECD) is also recommended.

this will help minimize the reliance on apical toxicity endpoints (Box 2). As a step in this direction, we decided to perform comparative analysis of several toxicological effects measured in different studies across endpoints and different sources. This should eventually become common practice in any safety decision, especially for the assessment of complex systemic toxicities.

Comparative analysis, integration of information, and analytical approaches to describe specific modes of action (MoAs) are not new concepts. In fact, the use of the MoA information framework for chronic or cancer risk assessment, introduced as early as 2001, has been considered fundamental to the identification of the human relevance of experimental data and necessary to optimize the design of long-term rodent studies (Sonich-Mullin et al., 2001; Boobis et al., 2008; Meek et al., 2014; OECD, 2014a). In this context, an integrated approach that considers several data streams (physicochemical properties, genotoxicity, target organ toxicity, metabolism, toxicity in short-term studies, QSARs, etc.) based on weight-of-evidence (WoE) was also proposed (OECD, 2014a, 2019). In the area of environmental health, the description of MoAs from acute toxicity data to predict chronic toxicity also has been a matter of investigation, e.g., in aquatic toxicology (Kienzler et al., 2017; May et al., 2016).

The extrapolation of information is mainly based on the analysis of causal events possibly leading to a toxicity effect (adverse outcome) that can be described by means of relevant mechanistic knowledge, data-driven evidence (e.g., lethal dose,

Abbreviations
AOP, adverse outcome pathway; BMD, benchmark dose; CLP, Classification, Labelling & Packaging; LD_{50}, median lethal dose; IC_{50}, half maximal inhibitory concentration; MoA, mode of action; NOAEL, no-observed-adverse-effect level; NAMs, new approach methodologies; LOAEL, lowest-observed-adverse-effect; STOT RE, specific target organ toxicity (repeated exposure); STOT SE, specific target organ toxicity (single exposure); TG, test guideline (OECD); WoE, weight of evidence

Box 2: Terminology

New approach methodologies (NAMs): NAMs, in a broad context, include *in silico* approaches, *in chemico* and *in vitro* assays, as well as the inclusion of information from the exposure of chemicals in the context of hazard assessment. They also include a variety of new testing tools, such as "high-throughput screening" and "high-content methods", e.g., genomics, proteomics, metabolomics, as well as some "conventional" methods that aim to improve understanding of toxic effects, either through improving toxicokinetic or toxicodynamic knowledge for substances (ECHA, 2016).

Mode of Action (MoA): A biologically plausible sequence of key events at different levels of biological organization, starting with the exposure to a chemical and leading to an observed (adverse) effect (WHO definition).

Apical toxicity endpoint: An observable outcome in a whole organism, such as a clinical sign or pathological state, which is indicative of a disease state (e.g., evidence of tumor in rodents) that can result from exposure to a toxicant.

Weight of Evidence (WoE): Generally described as a stepwise process/approach of collecting and weighing multiple lines of evidence to reach a conclusion on a particular problem formulation with a (pre)defined degree of confidence (OECD, 2019).

50% (LD_{50}), half maximal inhibitory concentration (IC_{50}), non-observed-adverse-effect level (NOAEL), lowest-observed-adverse-effect (LOAEL), benchmark dose (BMD)) or by a combination of both.

Indeed, by using these different approaches, a number of attempts have been made to extrapolate from one systemic toxicity endpoint to another and are now included within regulatory documents. For example, a WoE approach introduced as adaptation to the standard information requirement for acute oral toxicity is described in a recent update of the ECHA Guidance on Information Requirements and Chemical Safety Assessment (ECHA, 2017). In this case, NOAEL values from a 28-day toxicity test were evaluated for their ability to predict the outcome (LD_{50} values) of an acute oral toxicity study (Bulgheroni et al., 2009; Gissi et al., 2017; Graepel et al., 2016). This is based on the premise that acute and repeat-dose systemic toxicity studies share common mechanisms of action.

Similarly, other groups have compared quantitative endpoints (NOAEL, LOAEL) derived from toxicological studies of different durations (Batke et al., 2011; Bokkers and Slob, 2005; Kalberlah and Schneider, 1998; Kalberlah et al., 2002; Pieters et al., 1998; Pohl et a., 2010; Schneider et al., 2005). This information has been used by regulatory agencies to derive default factors to extrapolate long-term quantitative estimates from studies with short durations (ECHA, 2012; ECETOC, 2010; EFSA, 2012; Schilter et al., 2014).

More recently, Luechtefeld and co-workers (2018) have developed new computational models called read-across structure activity relationship (RASAR), which allow the prediction of chemical hazards as traditionally done by read-across but in an automated fashion by combining chemical similarity with supervised learning. The so-called data fusion RASAR model, by using all available information of the neighboring chemicals, showed accuracies in the range of 80%-95% across nine health hazards (skin sensitization, eye irritation, acute oral toxicity, acute dermal toxicity, acute inhalation toxicity, dermal irritation, acute/chronic aquatic toxicity, and mutagenicity). By integrating multiple data sources, this model achieved more consistent, accurate and useful predictions than single data sets.

Based on the above, we refer here to further examples where the prediction of toxicity is based on existing, mainly mechanistic, information and is extrapolated from different sources as a mechanistically informed read-across approach. The examples belong to projects under investigation by our group. Although not strictly linked to one another, they are a pretext to trigger further discussions within scientific and regulatory communities on the translation of biological and toxicological information into regulatory decisions on chemical safety.

2 Extrapolation approaches

2.1 Using existing information on common mechanisms

Our understanding of the specific mechanisms by which chemicals exert their toxic effects in humans is continuously growing and should be further exploited in a regulatory context. Also, relevant databases and datasets are becoming increasingly available and can be used to extrapolate chemical related information.

AOP network

The Adverse Outcome Pathways (AOP) Wiki database[2] represents a source of information for identifying common signaling pathways/processes, key events, and their relationships to toxicity outcomes. Within a network of AOPs, the same key/intermediate event may lead to one or more adverse outcomes (Villeneuve et al., 2018a). In fact, by providing a descriptive framework for the overall potential adverse outcomes resulting from particular biological perturbations, AOP network analysis can enable the identification of pathways that have the greatest biological likelihood and/or relevance for risk assessment. Moreover, by describing these pathways, the AOP network can help to identify specif-

[2] AOP Wiki database: https://aopwiki.org/ (accessed December 2019)

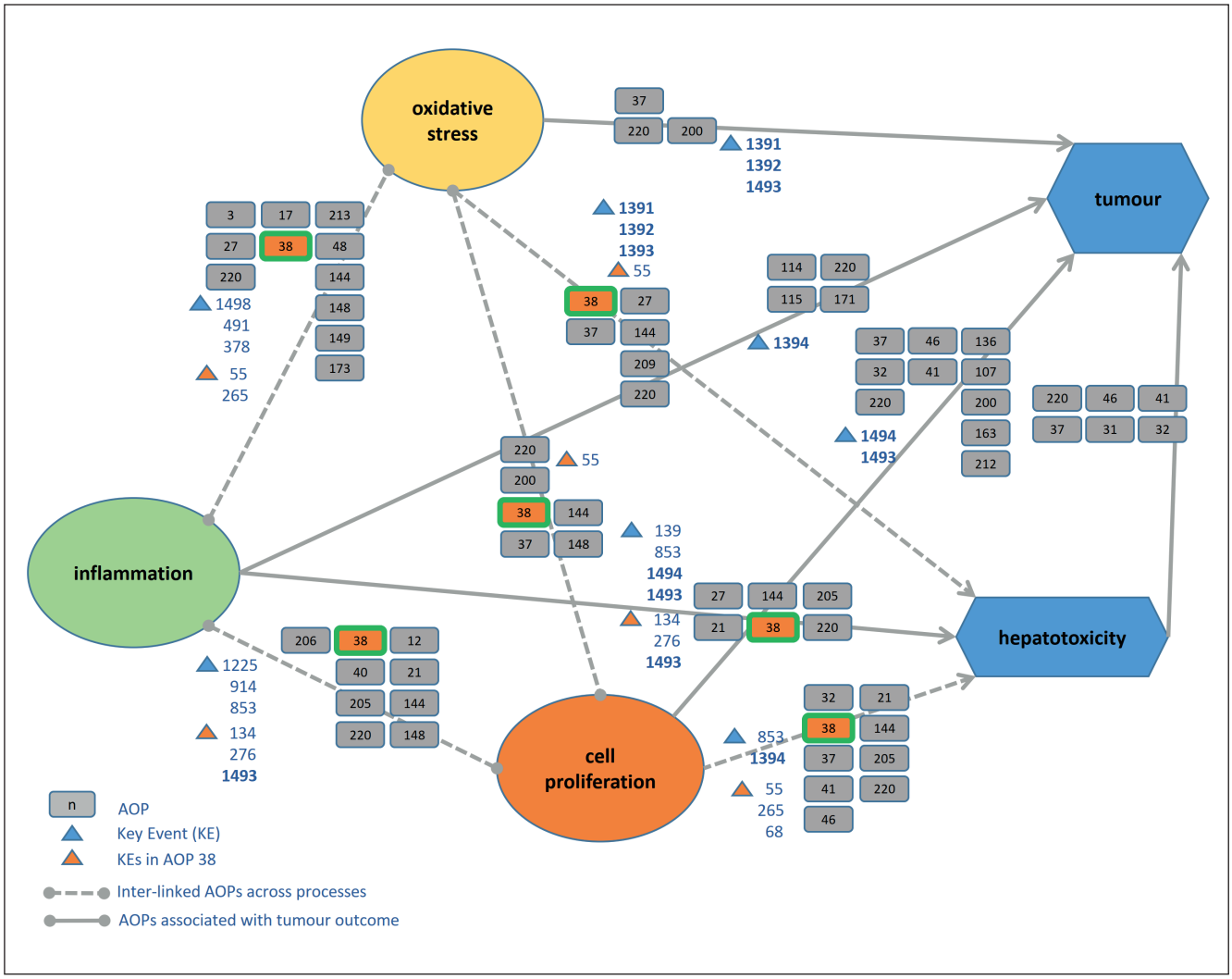


Fig. 1: Inflammation in the AOP network

The AOP-Wiki was consulted to identify AOPs and specific key events (KEs) commonly associated with the three different toxicity processes inflammation, oxidative stress and cell proliferation. Keywords simple search was performed on the database using the AOP or the KE search box. The keyword *inflammation* is found to be associated with 23 different AOPs (grey boxes). Many of the identified AOPs as well as associated KEs (listed numbers) are also shared (bold numbers) across *oxidative stress* and *cell proliferation*, thus describing a network of events that potentially can lead to toxicity outcomes, i.e., hepatotoxicity, tumor in the liver (https://aopwiki.org, December 2019). Representative OECD-endorsed AOP no. 38 on "Protein Alkylation leading to Liver Fibrosis" and its respective shared key events are highlighted (orange boxes).

ic toxicity effects with a good predictive value that can serve as useful alternatives to the direct measurement of apical adverse outcomes (OECD, 2017a). An example of the application of network analytics to an AOP network (for human neurotoxicity) is given in Spinu et al. (2019). Nine AOPs sharing common key events were mapped, and the analysis allowed the identification of points of convergence (common key events) and divergence; the overall connectivity of the key events across the AOP network; the assessment of upstream-downstream gradient of key events across the AOP network and, finally, the identification of the most common/highly connected key events (e.g., cell injury/death is the most hyperlinked key event across the network). The latter can serve as a basis for developing/selecting *in vitro* assays for the assessment of neurotoxicity without animal testing.

This type of analysis is still limited by the information currently captured in the AOP knowledge base (Villeneuve et al., 2018b); however, whilst being populated with new information, the AOP-Wiki database can already be interrogated for specific mechanisms or processes underlying toxicity effects on the most advanced pathways. For example, the keyword "inflammation" in the AOP-Wiki database is found to be associated with 23 different adverse outcome pathways, as reported in Figure 1. We fo-

cused on inflammation since it is a relevant process common to a number of apical toxicity endpoints. In fact, inflammation is a critical step in the etiological process of the majority of chronic diseases, including cancer (Furman et al., 2019; Miklossy and McGeer, 2016; Todoric et al., 2016; Mantovani et al., 2008; Suzuki and Yamamoto, 2015; Hunter, 2012; Bennett et al., 2018). The inflammatory process, as the host response to microbial infections, hypersensitivity, physical agents or chemicals, shares common key events at cellular or organ/tissue level, contributing to different adverse outcomes as in the case of hepatotoxicity or tumor formation (Fig. 1).

Villeneuve and colleagues have identified aspects of the inflammatory process that are common across multiple tissues/organs and those that are context-specific. They described three common key events, which are independent of the organ/tissue involved and the final adverse outcome (i.e., tissue resident cell activation, increased pro-inflammatory mediators, and leukocyte recruitment/activation). Within an AOP network, these key events represent points of convergence and divergence between a wide range of potential stressors (upstream signals) and a wide range of tissue and context-dependent adverse outcomes (downstream), respectively (Villeneuve et al., 2018b). Moreover, the majority of AOPs that include inflammation share other key events. Oxidative stress, for example, contributes to other processes, including inflammation and/or cell proliferation, and is common across different adverse outcomes. In Figure 1, the inflammation process is interlinked with oxidative stress and cell proliferation and can lead to hepatotoxicity, as in the case of liver fibrosis described in AOP no. 38, or through hepatotoxicity it can also lead to liver tumor (e.g., AOP no. 220 "*Cyp2E1 Activation Leading to Liver Cancer*").

This shows how a keyword search on the AOP-Wiki database allows the identification of associations across processes and related adverse outcomes. It also allows the identification of shared key events, informs on their relationships and possibly on the strength of such relationships. Finally, it may report on the supporting qualitative and quantitative evidence, as described in the AOP-Wiki database. In addition, the organized inclusion of AOP information in an integrated approach to testing and assessment (IATA) can assist the prediction of a specific toxicity, as is well-described in the IATAs of skin sensitization, developmental neurotoxicity and, more recently, non-genotoxic carcinogens (OECD, 2017b; Bal-Price et al., 2018; Jacobs et al., 2020).

Cell type-specific mechanisms dataset

Available datasets can be explored to retrieve relevant chemical-related information. In this context, mechanisms described as the basis of one toxicity endpoint can infer on another toxicity endpoint. For example, the mechanistic knowledge derived by literature search for acute oral systemic toxicity (Prieto et al., 2019) is a valuable starting-point to inform other adverse outcomes. It is possible to evaluate the extent to which such mechanisms could play a role after repeated dose exposure scenarios. This is the case, for example, for cell type-specific mechanisms involved in acute and/or chronic adverse outcomes underlying toxicity effects in the blood, such as depletion of the different cell types (Fig. 2) or neurotoxicity mediated by dysfunction of, e.g., N-methyl-D-aspartate (NMDA) or γ-aminobutyric acid (GABA) receptors (Prieto et al., 2019; Carvajal et al., 2016).

It is also possible to interrogate the inventory of classified substances (Classification, Labelling and Packaging (CLP) Inventory))[3] with the aim to verify whether substances classified for acute oral toxicity (Acute Tox) are also classified for specific target organ toxicity after repeated (STOT RE) or single (STOT SE) exposure. For example, in the dataset of 178 chemicals described in the Prieto et al. (2019) study, it is shown that in 8 chemicals out of 22 bearing both Acute Tox (oral) and STOT RE harmonized classifications (i.e., assigned by a regulatory authority), the target organ derived from mechanistic information for acute oral toxicity is also reported in the CLP notifications assigned by the registrants for STOT RE toxicity classification (kidney for 4-ammonio-m-tolylethyl(2-hydroxyethyl)ammonium sulphate; peripheral nervous system for acrylamide; lung for cadmium chloride; liver for carbon tetrachloride; central nervous system for chloroform; lung for paraquat dichloride; central nervous system for phenol; blood for warfarin) (Tab. S1[4]).

Furthermore, 13 out of 35 acutely toxic chemicals with mechanistic information (Prieto et al., 2019) but lacking harmonized classification affect the same target organ as reported in the CLP notifications for STOT RE and/or STOT SE (5,5-diphenylhydantoin, central nervous system; acetylsalicylic acid, central nervous system; cis-diammineplatinum (II) dichloride, kidney; cyclosporine A, kidney; digoxin, heart; epinephrine bitartrate, heart, haloperidol, central nervous system; isoniazid, central nervous system and kidney; lithium carbonate, kidney; lithium sulfate, kidney; methadone hydrochloride, central nervous system; ochratoxin A, kidney; potassium cyanide, central nervous system) (Tab. S1[4]). As such, the use of mechanistic knowledge and the identification of specific target organs may serve as anchors to design and/or to select appropriate assays and, finally, relevant alternatives, which can be integrated to predict acute and/or repeated-dose adverse effects.

2.2 Using information on chemical properties to infer toxicity mechanisms: the examples of skin sensitization and mutagenicity

Useful information from different sources can be integrated to help prediction of toxicity. *In silico* and *in chemico* mechanistic information, for example, when coupled with other data, can be exploited further to explore common mechanisms across different endpoints. This is the case for skin sensitization prediction. Patlewicz and colleagues (2014) have proposed the use of genotoxicity data from both the bacterial reverse mutation test (Ames) and the chromosomal aberration *in vitro* test to improve the prediction of non-standard skin sensitization methods (i.e., QSARs and read-across). Such information can be used in an IATA. In

[3] C&L Inventory: https://echa.europa.eu/information-on-chemicals/cl-inventory-database (accessed December 2019)

[4] doi:10.14573/altex.2005061s

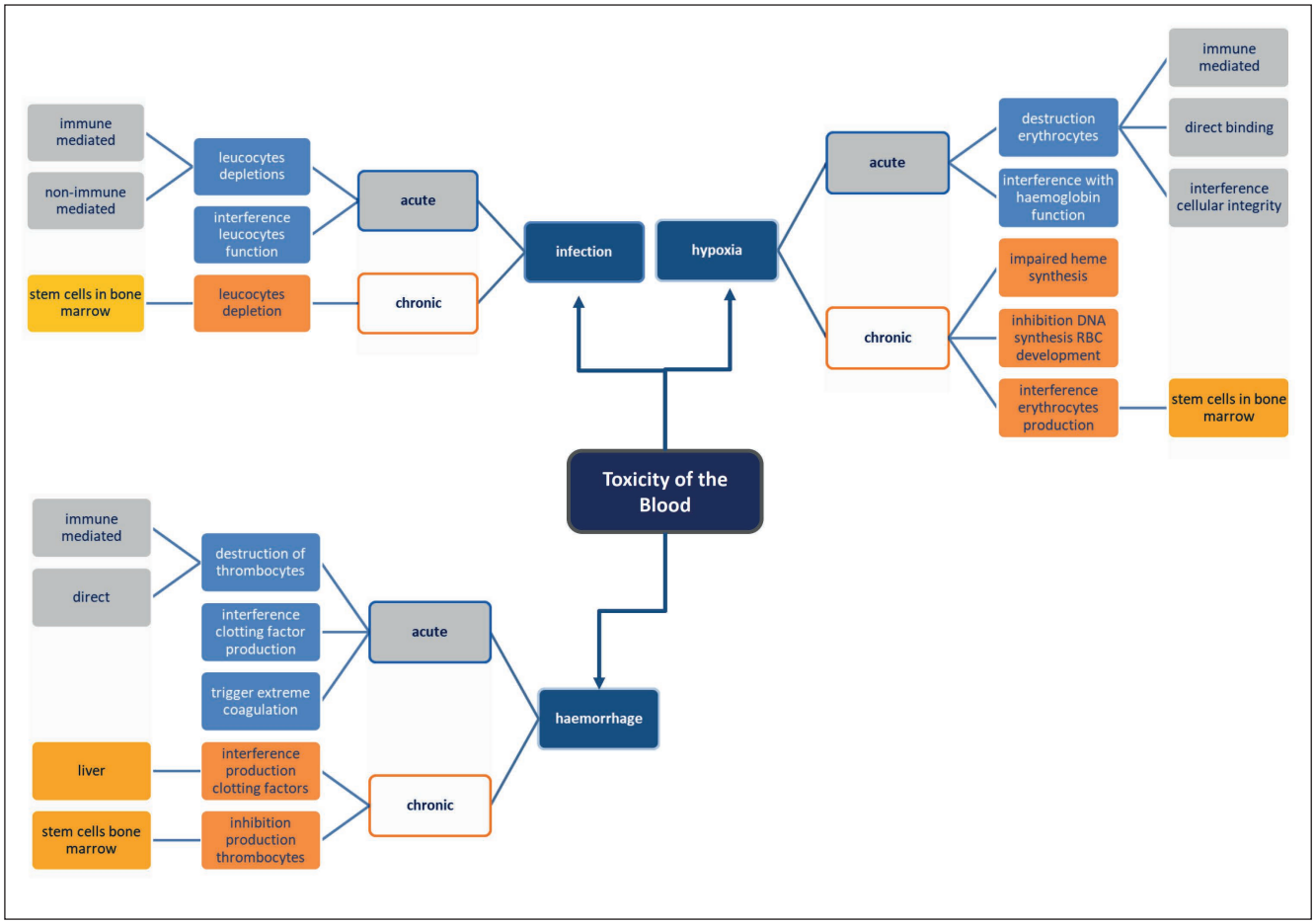


Fig. 2: Mechanisms underlying toxicity of the blood

Mechanistic knowledge derived by literature search, as for acute oral systemic toxicity, is relevant to inform other endpoints, e.g., chronic toxicity. For example, toxicity effects described in blood such as hemorrhage, hypoxia and infection (Bloom, 1993; Bloom and Brandt, 2001; Budinsky, 2003) can be either acute or chronic, depending on which underlying pathway or function is disturbed by the toxicant as well as on the dose of the toxicant and the duration of the exposure.

this case, one common key chemical property ("key characteristic") explaining the mechanism and the relationship between mutagens and skin sensitizers is electrophilicity, as both types of toxicants can act as electrophiles.

In support of this, we evaluated the Ames test outcomes together with different *in silico* methods as predictors for skin sensitization (i.e., local lymph node assay, LLNA) with the aim of analyzing structural and mechanistic patterns of 127 chemicals selected according to their mutagenic and sensitizing properties in depth. The data sources were the EURL ECVAM skin sensitization database[5] (Asturiol et al., 2016; Basketter et al., 2014); the EURL ECVAM Ames positives consolidated genotoxicity and carcinogenicity database[6]; the ISSTOX Chemical Toxicity Database[7], and a preliminary version of the EURL ECVAM Ames negative database (Madia et al., 2020). Hence, chemical properties (i.e., organic functional groups and DNA or protein binding alerts) have been analyzed in order to define common characteristics of correlating compounds and outliers (Tab. S2[4]).

Chemicals characterized by the presence of ether, phenol, carboxylic acid, carboxylic acid ester, alcohol or aniline groups are in the majority of cases negative in the Ames test. In addition, both carboxylic acid and carboxylic acid ester but also aldehyde groups may be indicators of non-sensitizing properties of chemicals (LLNA negative). On the other hand, the acrylate group characterizes chemicals generally giving a positive result in the LLNA and a negative Ames result. Chemicals having a nitroben-

[5] EURL ECVAM Skin sensitization database. https://ec.europa.eu/jrc/en/scientific-tool/chemical-lists-information-system

[6] EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames Positive Chemicals. http://data.europa.eu/89h/jrc-eurl-ecvam-genotoxicity-carcinogenicity-ames

[7] ISSTOX Chemical Toxicity Databases. http://old.iss.it/meca/index.php?lang=1&anno=2013&tipo=25 (accessed September 2018)

zene group or being a precursor of quinoid compounds are usually positive in both tests. These compounds are typically strong (1A) sensitizers. Chemicals having allyl groups in many cases are non-mutagenic but have skin sensitizing properties and are classified as low/moderate sensitizers (1B).

The analysis of the data has shown that chemicals yielding a positive response in the Ames test are also positive in the LLNA, suggesting that mutagenicity data can support, in a WoE approach, skin sensitization assessment. This trend is especially evident for strong skin sensitizers, which are described by structural alerts for nucleophilic substitution (SN1 or SN2) and radical formation (Tab. S2[4]).

2.3 Using common adverse outcomes in the time-response relationship: the example of repeated dose toxicities

Existing information also can be exploited to combine empirical evidence (knowledge-driven) across endpoints with relevant mechanistic information. The collection of toxicological data developed in the context of an endocrine disruptors impact assessment (JRC, 2016) represents an example of such a source of information.

The database contains approximately 400 chemicals comprising only pesticides and biocides, mainly from regulatory assessment reports (e.g., EFSA, European Food Safety Authority)[8], and it includes information from (sub-)chronic studies (both for human health and wildlife) and toxicological endpoints relevant to an endocrine disrupting mode of action (JRC, 2016). Most of the 400 chemicals are also reported in the Toxicity Reference Database from the US Environmental Protection Agency (EPA)[9]. This database offers the opportunity to identify data-rich chemicals and data-rich studies; compare effects between short- and long-term studies; identify missing data (e.g., clinical or histological observations); and, finally, correlate mechanistic information, if available, to apical endpoints or intermediate measured parameters. Chemicals are prioritized using specific criteria: 1) oral route of exposure, 2) studied in rodents, and 3) data-rich chemicals (e.g., with data provided from at least 10 Test Guideline methods (TGs)) covering mainly TGs related to systemic toxicity endpoints (sub-chronic, chronic, carcinogenicity, general reproductive toxicity, neurotoxicity). *In vivo* studies specific only to the investigation of endocrine disruptor-related effects (e.g., uterotrophic bioassay or Hershberger bioassay) are excluded from the analysis.

Based on the above criteria, 20 chemicals (Tab. S3[4]) were filtered that are characterized by a complete set of information, harmonized classification, and from which it is possible to extract information on affected target organs. As such, the database can be interrogated to explore mechanistic evidence leading to a specific adversity, both at dose-response and temporal level across several toxicity endpoint studies. For example, the data available for the pesticide linuron was used to perform preliminary temporal comparisons across endpoints with regard to the development of tumors. For this particular compound, using the data on testis histopathology, indications of cell hyperplasia are evident in short-term studies (EFSA)[8]:

- Cell hyperplasia and adenomas in short-term studies (7 days oral toxicity in rodents);
- Focal interstitial cell hyperplasia in a two-generation reproduction toxicity study (26 weeks);
- Interstitial cell hyperplasia and an increased incidence of interstitial cell adenoma in a rodent sub-chronic oral toxicity study (6-12 months);
- Unilateral/bilateral Leydig cell benign adenomas, slightly increased incidences of hyperplasia of the Leydig cells in a rat oral chronic toxicity study (24 months);
- Benign testicular interstitial cell tumors, increased incidence of testicular interstitial cell hyperplasia in a rat oral combined chronic toxicity/carcinogenicity study (27 months).

As such, comparisons across different toxicity endpoint studies can be indicative of a potential treatment-related effect. Mechanistic information needs to be added in a WoE approach to conclude on the specific toxic effect. The identification of cell type-specific mechanisms can help to elucidate the temporal dynamics of the adversity and to identify chemicals sharing those underlying mechanisms. More relevant, as also reported in the previous examples of the AOP network for human neurotoxicity and target organ toxicity after acute and repeated dose exposure, there is the possibility to exploit the information on the mechanisms to design *ad hoc* alternative assays. Mechanistic information obtained on specific toxicity effects across studies of different duration also can be combined with, e.g., NOAEL values to evaluate the predictivity of shorter studies for longer studies. This approach has been recently explored by Braakhius and colleagues (2018) with the intent to investigate whether risk assessment of non-genotoxic carcinogens based on NOAELs is protective against cancer.

2.4 Integration of information from multiple endpoints: the example of carcinogenicity

The opportunity to integrate information across systemic health endpoints is particularly relevant to the evaluation of the carcinogenic potential of substances. This is in fact the core component of the recent approach proposed for the carcinogenicity assessment of pharmaceuticals (ICH, 2016). This is based on the hypothesis that knowledge of pharmacological targets and pathways, together with other toxicological data (i.e., genotoxicity, 6-month chronic toxicity study and hormonal perturbation), can provide sufficient information to anticipate, in several cases, the outcome of a 2-year rodent carcinogenicity study (Sistare et al., 2011; van der Laan et al., 2016).

A similar approach is under investigation for agrochemicals by the European Partnership for Alternative Approaches to Animal Testing (EPAA), where data from standard toxicological studies, including the 90-day repeated dose toxicity study, are combined

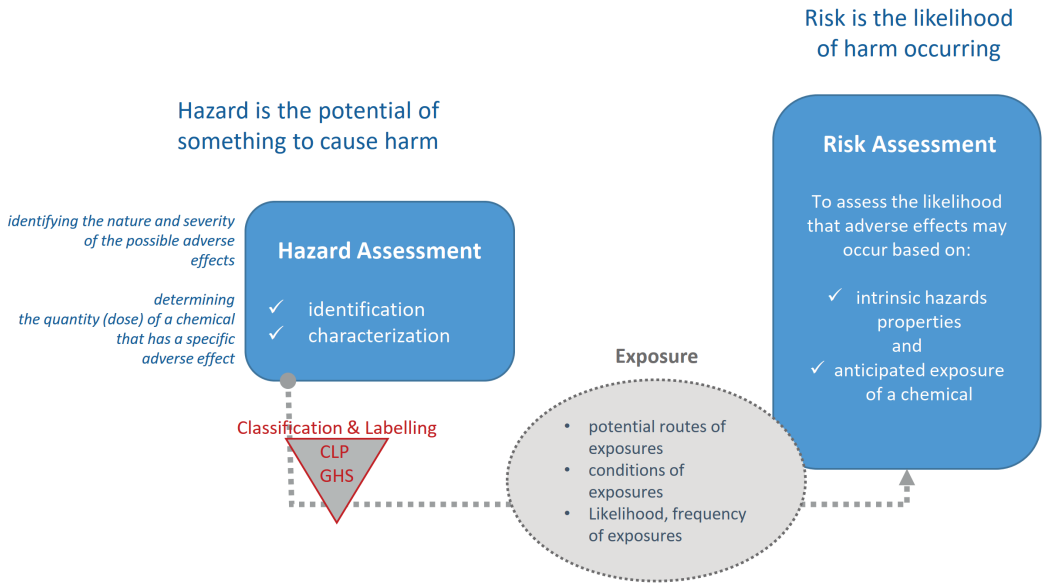
[8] EFSA pesticides dossiers: http://registerofquestions.efsa.europa.eu/roqFrontend/wicket/bookmarkable/eu.europa.efsa.raw.gui.pages.substance.SubstanceSearchPage?12 (accessed October 2019)

[9] ToxRefDB: https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data

Box 3

Concepts of hazard, exposure and risk in the context of risk assessment

Hazard represents the potential of something to cause harm; risk is the likelihood of harm to occur. "Hazard" refers to the intrinsic properties of a chemical such as toxicity, while "exposure" addresses the degree to which a human or the environment will be exposed to the intrinsic hazards of a chemical. Risk assessment consists of hazard identification and characterization, appraisal of exposure, followed by the risk assessment itself. As such, it aims to understand the harm posed by a chemical based on its intrinsic hazards in light of the anticipated exposure (Nordlander et al., 2010).



with mechanistic knowledge across endpoints to reduce or avoid the need for a 2-year carcinogenicity study.[10,11] Likewise, the US EPA, in collaboration with PETA (People for the Ethical Treatment of Animals, PETA International Science Consortium Ltd) and industry, in a project entitled "Rethinking Carcinogenicity Assessment for Agrochemical Projects" (ReCAAP), is examining ways to develop a waiver to the rodent cancer bioassay for agrochemicals by using WoE approaches on a breadth of relevant endpoints that are used in both hazard identification and risk assessment (Box 3) (Cohen et al., 2019; Wolf et al., 2019).

Several underlying mechanisms involved in the development of cancer have been recently identified, and tools to analyze them are becoming available; as such, they might be captured more fully to ensure a more predictive assessment of cancer risk (Guyton et al., 2009; Corvi et al., 2017; Fielden et al., 2018). This is the basis of an integrated approach to carcinogenicity assessment aimed to better exploit available information derived across different endpoints, which is currently feeding the OECD project on the development of an IATA for non-genotoxic carcinogens (Jacobs et al., 2016, 2020).

This endeavor is in line with a series of recent investigations aimed at organizing and integrating mechanistic information in a systematic and uniform manner. Smith and colleagues have designed a systematic approach to classify potential carcinogens by using key characteristics (i.e., is electrophilic or can be metabolically activated, is genotoxic, alters DNA repair or causes genomic instability, induces epigenetic alterations, induces oxidative stress, induces chronic inflammation, is immunosuppressive, modulates receptor-mediated effects, causes immortalization, and alters cell proliferation, cell death or nutrient supply) that represent established properties by which agents contribute to carcinogenesis (Smith et al., 2016). Schwarzman et al. (2015) have proposed instead to reverse the investigation by first identifying all biological processes that play a role in the etiology of cancer and use them to inform toxicity targets for chemical screening and prioritization.

[10] EPAA Annual Conference Final Report. The European Partnership for Alternative Approaches to Animal Testing (EPAA) 13th Anniversary Conference. Building Synergies to accelerate development & acceptance of alternatives, 22nd November, 2017. https://ec.europa.eu/docsroom/documents/28205

[11] Flashreport – EPAA Expert Workshop 2019 on "Mechanism-based approach to cancer risk assessment of agrochemicals". 12-13 June 2019, Brussels, Belgium. https://ec.europa.eu/docsroom/documents/36296

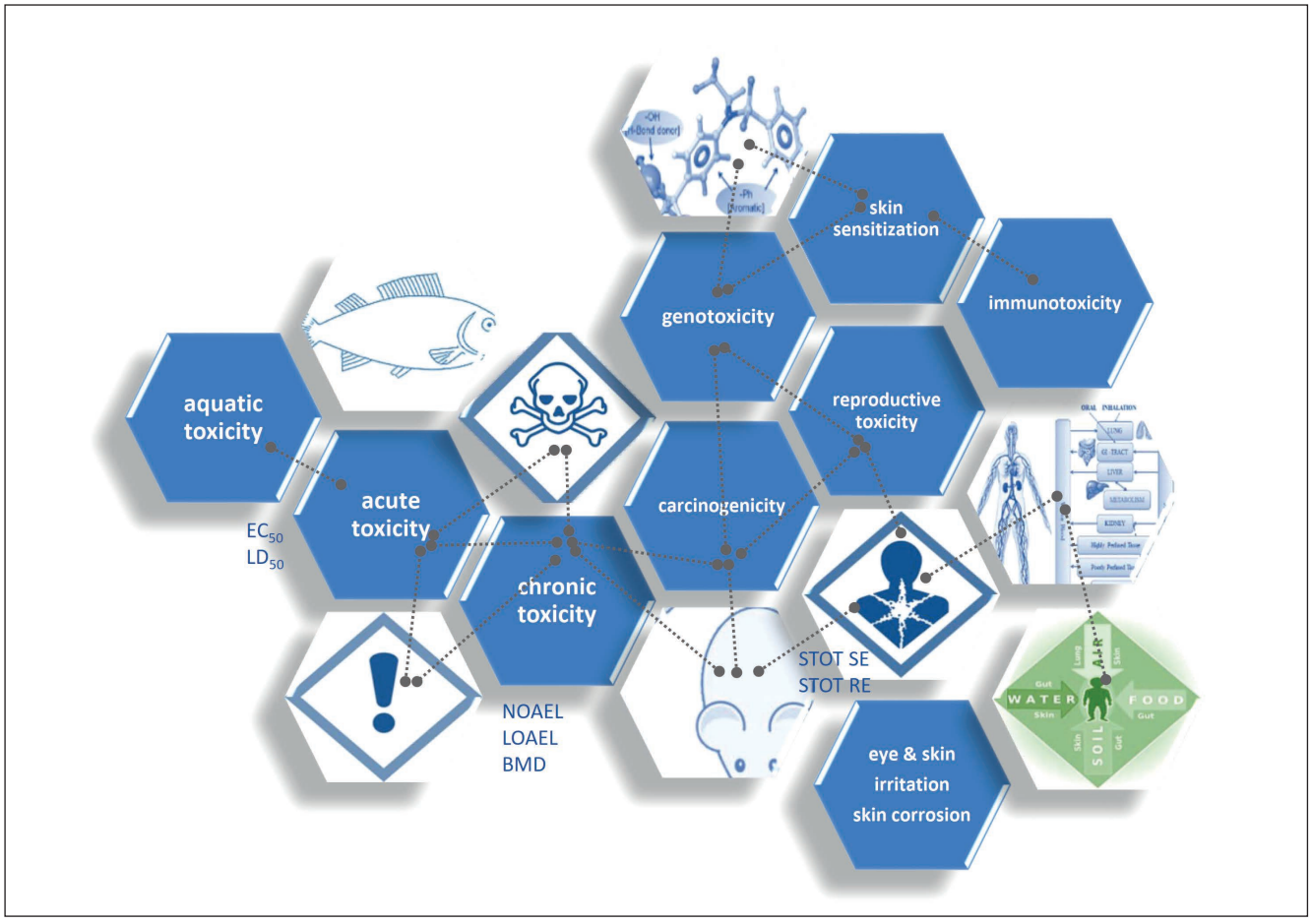


Fig. 3: Making better use of toxicity studies for human health by extrapolating across endpoints

Extrapolation of information based on mechanistic knowledge across multiple sources and endpoints, as reported in the examples described, can help a sustainable and more human-relevant prediction of toxicity. This avoids redundancy of testing and reduces the use of animals. EC_{50}, half maximal effective concentration; IC_{50}, half maximal inhibitory concentration; LD_{50}, median lethal dose; NOAEL, no-observed-adverse-effect level; LOAEL, lowest-observed-adverse-effect; BMD, benchmark dose; STOT SE, specific target organ toxicity (single exposure); STOT RE, specific target organ toxicity (repeated exposure). Dotted lines represent opportunities of cross endpoint extrapolations by means of, e.g., AOP network.

Of note, key characteristics of male and female reproductive toxicants or endocrine disrupting chemicals (Arzuaga et al., 2019; La Merrill et al., 2019; Luderer et al., 2019) have been recently described. Key characteristics of cardio- and neurotoxicants were also recommended (NRC, 2017), as well as a set of characteristics of chemicals inducing repeated-dose systemic toxicity (RDT). Recently, RDT has been the focus of a recent EPAA Partners Forum and was identified as an area that would benefit from data integration across endpoints (Laroche et al., 2019). It has been proposed to gather and organize mechanistic knowledge related to toxicological effects on target organs in animal models after repeated exposure to chemicals, i.e., to map out the mechanisms related to RDT (Laroche et al., 2019). A study aimed to gather, analyze and organize mechanistic knowledge related to the toxicological effects on target organs observed in animal models after repeated exposure to chemicals has been recently awarded (July, 2020) by the European Commission's Joint Research Centre[12].

3 Opportunities for further discussions

The approaches reported above represent a number of opportunities where different kinds of information are extrapolated and integrated for the evaluation of toxicological endpoints of regulatory concern, particularly of complex systemic toxicity endpoints.

Considerable knowledge already exists, is available for standard tests, and more is becoming available for NAMs. However,

[12] https://ec.europa.eu/jrc/en/science-update/call-mechanistic-knowledge-toxicological-effects-caused-chronic-exposure-chemicals

the challenge and priority is the sharing and integration of different data streams (Mahony et al., 2020) and how to exploit available information.

Before embarking on such a challenge, it is considered essential to formulate defined questions addressing specific regulatory needs, as recently highlighted by Bos and co-workers (2020), and to define specific scenarios where new integrated approaches can be applied. The development of an overall strategy will necessitate a strong collaboration between scientists, industry representatives and regulators, e.g., via a multi-stakeholder collaboration program. For example, the EPAA represents an example of a suitable platform for such multi-stakeholder interactions.

We consider that data integration across endpoints, which currently forms the basis of scientific research endeavors, should ultimately inform routine regulatory decisions. It can be envisaged that the assessment of all individual apical toxicity endpoints will not be needed in the future; instead, the protection of human health could be based on the identification of mechanistic intermediate effects as well as the knowledge of kinetics (Fig. 3).

The concept of using key characteristics, for example, as illustrated for the assessment of carcinogenicity, is going in this direction and is relevant to current discussions on new ways of conducting safety assessment. Indeed, by exploiting the key characteristics of carcinogens, we are currently investigating the feasibility of a more holistic approach to inform on the carcinogenic potential of chemicals. Specifically, the approach is intended as a regulatory tool to organize and to guide the provided information in a way that is aligned across toxicity endpoints (manuscript in preparation).

Nevertheless, a number of practical steps need to be made to further evolve such approaches:

- Mechanistically-based approaches can be effective if combined with robust kinetic information. This means better use of tools such as physiologically-based toxicokinetic models that integrate data generated by *in vitro* methods and allow *in vitro* to *in vivo* extrapolation. It is also necessary to standardize methods for human *in vitro* absorption, distribution, metabolism and excretion (ADME) (Bessems et al., 2015).
- Further the integration of quantitative data into AOP networks to allow derivation of quantitative relationships for each key event relationship to improve confidence in the network and to support its regulatory use (e.g., in risk assessment) (Spinu et al., 2020; Foran et al., 2020)
- The integration of human physiology and pathophysiology in the description of toxicity pathways (i.e., the molecular events within a cell which include genes, proteins and metabolites that are altered as a result of a molecular initiating event) to enable the selection of proper assays (Madia et al., 2019). This can also inform chemical grouping based on biological information (mechanistic read-across) and can help to prioritize chemicals. The "key characteristics", as mentioned above, are already going in this direction.
- The importance of understanding exposure (as one of the risk components together with hazard) when dealing with non-animal testing strategies in order to correctly interpret data from these studies and their relevance to toxicity endpoints is crucial and well-recognized. Therefore, inclusion of exposure patterns that reinforce the human biological relevance of the identified toxicity pathways with respect to real chemical exposure scenarios is needed (Krewski et al., 2020).
- In addition, all the information above is essential for demonstrating the scientific validity of novel integrated approaches, including analysis of uncertainties, as well as the utility of novel approaches by means of appropriate case studies. This has been discussed at length in two recent workshops focused on the validation and regulatory acceptance of innovative approaches in regulatory toxicology (Piersma et al., 2018; Burgdorf et al., 2019).

Adding to the above practical considerations, it is anticipated that integrated approaches can be more easily accommodated into the risk assessment process (Box 3) where *in vitro* points of departure can be extrapolated to external doses, whereas difficulties are foreseen for hazard classification, which is largely based on strict, established criteria relating to animal studies (CLP or UN Globally Harmonized System of Classification and Labelling of Chemicals (GHS)). The successful uptake of non-animal methods into hazard classification would require adaptation of regulatory information requirements and of classification criteria, as well as robust tools for toxicity assessment. Progress in this direction would necessitate a strong commitment from policy-makers, regulatory assessors, and research funders. It would also be important to facilitate a sustained dialogue between all stakeholders.

In summary, integration of data can serve three objectives: to allow better use of existing information, hence avoiding redundancy, particularly of *in vivo* studies (short-term goal); to provide new frameworks to introduce data from alternative approaches into the regulatory decision process (medium-term goal); and, finally, to fulfil regulatory requirements with alternative approaches (long-term goal).

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Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

We are thankful to our dear colleague and friend, the late Dr Alfonso Lostia, for his contribution to the evaluation of common adverse outcomes in the time-response relationship: the example of repeated dose toxicities (Section 2.3).