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Paradigm shift in safety assessment using new approach methods: The EU-ToxRisk strategy

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Abstract

The EU-ToxRisk research project is an interdisciplinary research project that aims to advance the paradigm shift in toxicology towards new approach methodology (NAM)–based approaches for risk assessment. In this European research project, experts in the fields of *in vitro* and *in silico* techniques and risk assessment from academia, industry and regulatory agencies work together. Using a series of custom-designed case studies, the EU-ToxRisk battery of NAMs is being evaluated to learn how to carry out safety assessment using NAMs. This review article provides an overview of the project, its aims and approach and the methodologies that are being used.

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Introduction

EU-ToxRisk is a 38-partner European research project that aims to advance the paradigm shift in toxicology towards mechanism-based testing (www.eu-toxrisk.eu). The core principle of the project is to utilize new approach methodologies (NAMs) such as *in silico* models and *in vitro* techniques to generate human-relevant data. EU-ToxRisk envisages to combine different kinds of NAM data for hazard characterization, which can then be applied in human risk assessment, for example, in read-across approaches. By using *in silico* and *in vitro* techniques, we will gain insights into the mechanisms that underlie typical adverse toxicological effects and will learn how to better predict toxic effects of chemicals. Traditional *in vivo* toxicological studies often do not provide such mechanistic information. Beyond hazard assessment, we also think that NAM data can be applied for prioritization and screening purposes.

The focus of the project is on two endpoints, repeated-dose toxicity (RDT) and developmental and reproductive toxicity (DART). In Europe, these two endpoints are common information requirements under several regulations. While the use of NAMs is encouraged in these legislations, the current standard test requirements are *in vivo* studies to determine adverse toxicological effects of chemicals (with the exception of cosmetics, for which *in vivo* testing has been banned in Europe since 2016). The dose level at which no adverse effects have been observed in the test animal is then extrapolated to a safe exposure level for humans.

Numerous, individual *in vitro* tests have been developed with the aim of predicting a certain endpoint or to replace either an entire or a part of an *in vivo* study [1–3]. However, it is currently not possible to replace an *in vivo* study for a complex endpoint with a single or a battery of *in vitro* tests, unless specific exposure scenarios are considered where human risk can be neglected, that is, the effects are below a threshold of toxicological concern [4,5]. It is currently believed that we need a testing strategy that will integrate data from several NAMs to evaluate relevant aspects in hazard characterization, in particular the interplay of toxicokinetics and toxicodynamics. Several defined approaches are already under

review by authorities for well-understood endpoints such as skin sensitization [6]. Defined approaches or integrated approaches to testing and assessment (IATA) for RDT or DART, however, do not yet exist.

EU-ToxRisk has designed several case studies to explore how and to what extent NAMs can be used for hazard and risk assessment. The actual integration of NAM data into concrete case studies by EU-ToxRisk project partners helps to develop a mutual understanding on the needs for hazard assessment between academia, industry and authorities. EU-ToxRisk follows a tiered approach. First, the applicability of NAMs will be evaluated within a read-across scenario, in which anchoring *in vivo* data is available for at least one compound. In a next step, this approach will be expanded to address *ab initio* assessments, where compounds without available *in vivo* toxicity data will be tested. Lessons from previous research projects have been integrated into the design of the EU-ToxRisk case studies. For example, the Seurat-1 project has demonstrated the importance of the assessment of toxicokinetics, both in the *in vitro* experimental set-up and the extrapolation to safe human doses [7,8]. These two aspects were integrated into the design of EU-ToxRisk case studies through the use of absorption, distribution, metabolism, and excretion (ADME) models and generation of ADME *in vitro* data. As of 2018, the results of the first set of NAM-supported read-across case studies are being compiled and evaluated.

Use of NAMs in EU-ToxRisk case studies: *in silico* modelling and *in vitro* test systems

EU-ToxRisk unites both *in silico* and *in vitro* expertise from different project partners. This is essential for this project because the success of 'mechanistic' hazard assessment depends on integrating complementary NAMs in a testing strategy. Linking these data to established knowledge of pathways of toxicity using adverse outcome pathways (AOPs) helps to tie together the information and make sense of the data that were generated.

How are *in silico* models used in EU-ToxRisk?

The *in silico* methodologies that are used in EU-ToxRisk fall into several broad categories: i) tools to assess structural/chemical/biological similarity; ii) tools to predict compound behaviour or activity *in vivo* and *in vitro*; iii) models to predict kinetic behaviour of test compounds and iv) tools to provide an overall uncertainty assessment. In the case studies, these tools are used to aid formulation of a read-across hypothesis, the selection of analogues in a read-across context, test system selection and the prediction of metabolism or metabolites. Kinetic modelling is used to determine human-relevant test concentrations and later on the human-equivalent oral dose based on the *in vitro* outcome. Furthermore, kinetic modelling is applied to predict the intracellular concentration of test compounds in different *in vitro* systems, a prerequisite for the extrapolation back to a safe human dose.

A brief overview of the *in silico* methods used in the project is provided in the following paragraphs:

Similarity methods

Most of the methods aiming to infer toxicological properties of new compounds are based on the concept of bioisosterism, which infers that structurally similar compounds are likely to have similar biological properties. We have applied structural similarity metrics based on well-known structural fingerprints (e.g. extended-connectivity fingerprints (ECFP) [9] and the Tanimoto index [10]). However, not all structural features of compounds contribute similarly to the toxicological effects of concern and their relative importance is unknown *a priori*. This uncertainty might lead to so-called 'activity cliffs' [11], compounds with a similar structure showing different biological/toxicological properties. Two approaches are applied to mitigate this problem: enrichment of molecular descriptors with experimental data and the use of supervised metrics obtained by classifiers [12]. These approaches result in enhanced similarity indexes and a better assessment of biological similarity.

Classifiers and Quantitative Structure-Activity Relationship (QSAR) methods

Compounds with known toxicity can be exploited to recognize the structural and physicochemical features associated with their biological properties. This can overcome the aforementioned limitations and build robust mathematical models (classifiers or quantitative structure-activity relationship (QSARs)) describing this relationship. These models can be used to make predictions about the properties of new compounds. In EU-ToxRisk, we have developed more than 50 models covering many diverse endpoints and biological properties. These models use state-of-the-art machine learning algorithms (conformal and nonconformal random forest and partial least square, K nearest neighbour, support vector machines, gradient boosted tree, deep neural nets, etc.) and a wide variety of molecular descriptors (two-dimensional and three-dimensional [3D] fingerprints, physicochemical descriptors, pharmacophoric descriptors, bioactivity spectra, etc.). A key component is the estimation of uncertainties due to limitations of the model or the positioning of the query compound within the model applicability domain. We are applying methods such as conformal regression [13] to obtain highly accurate uncertainty estimations.

Biokinetic and metabolic predictions

In silico methods can be applied to predict the likely metabolism of query compounds [14,15]. To put the findings from *in vitro* assays into context, and allow

hazard assessments to be conducted, two factors need to be considered. First, what is the (unbound) intracellular concentration of target compound within the cells of the *in vitro* test system? Second, what is the (unbound) concentration in the target organs of toxicity *in vivo*? A biokinetic model has been developed (Fisher et al. submitted) that can take physicochemical and/or *in vitro* measured data as inputs and simulate the intracellular concentrations in the *in vitro* system with either steady state or dynamic situations considered. Although other biokinetic models have been published [16,17], the EU-ToxRisk project accounts for some additional critical components that can influence the disposition of compounds into the cells of the *in vitro* system.

The compound concentration in the target organ after an *in vivo* exposure is being assessed using physiologically based pharmacokinetic (PBPK) models [18]. Using this approach, the concentration in target organs at doses that exert toxicity can be estimated and compared with the intracellular concentrations causing toxicity in *in vitro* assays, thus determining the human-equivalent *in vivo* doses.

Combination of evidence

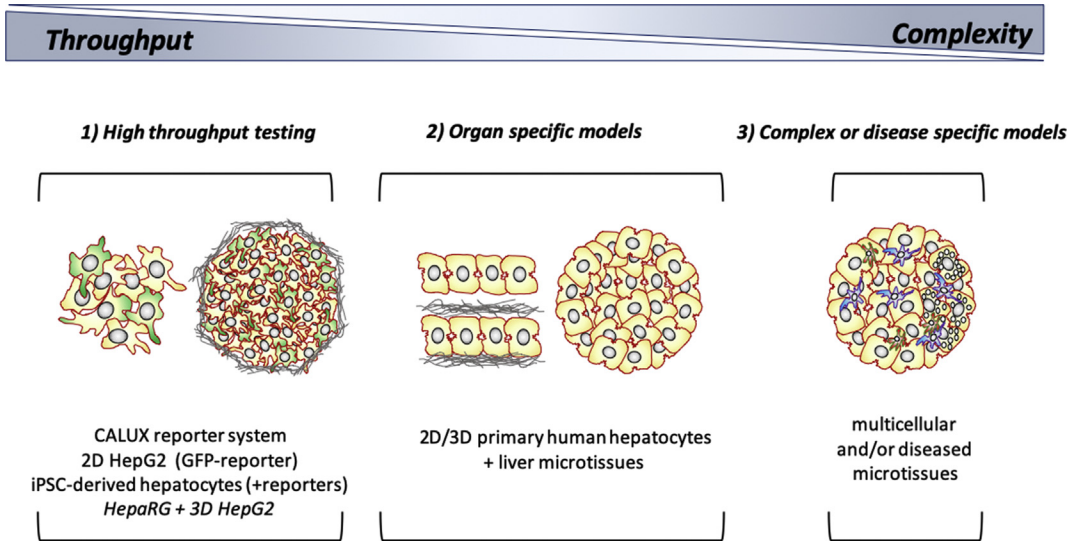
Every piece of evidence obtained in the context of a hazard assessment is associated with uncertainties. Classically, the overall assessment was generated *in cerebro*, by experts who apply their experience and judgements to combine all these elements. We aim to facilitate the work of experts by applying decision theories such as Dempster-Shafer theory [19] to combine what is known about the uncertainty of the results and provide integrated, objective, probabilistic assessments.

How are *in vitro* models used in EU-ToxRisk?

The *in vitro* test systems in EU-ToxRisk were chosen to cover the most frequent and sensitive RDT endpoints (liver, kidneys, neuronal system, lung toxicity [20] as well as the DART endpoint [21]. They cover three levels of complexity: 1) high-throughput testing; 2) organ-specific models and 3) complex or disease-specific models, see Figure 1 as an example for the liver. For RDT, all test systems use human cells because it is thought that these will generate human-relevant data. For the DART endpoint, the situation is different. While human stem cell–derived test systems were chosen to give information relevant to neural crest formation, neurite outgrowth and development of embryonic stem cells, more complex test systems such as zebrafish embryos and mouse embryonic stem cells were also selected to assess the complex endpoint of DART. For DART, no disease-specific NAM models are available.

The test methods in EU-ToxRisk are well established and documented in EURL ECVAM DataBase service on ALternative Methods to animal experimentation (DB-ALM) (https://ecvam-dbalm.jrc.ec.europa.eu/). The generation of new tests addresses key events of AOPs, related to EU-ToxRisk case studies, with a focus on human induced pluripotent stem cells iPSC-derived organ-specific reporter cell lines. Although test systems are mostly well established, they have not all undergone a formal validation procedure. Therefore, the project has internally tested the applicability and sensitivity of the tests by testing 19 well-described toxicants in all EU-ToxRisk assays. In addition, transcriptomics and biokinetic data from all test systems that have been exposed to the 19 toxicants will be generated. All data have been generated

Figure 1



An overview of EU-ToxRisk test systems that are used to assess liver effects for the RDT endpoint. This diagram shows the three levels of complexity from high-throughput testing to organ-specific models and finally complex or disease-specific models. iPSC, induced pluripotent stem cell, GFP, green fluorescent protein. RDT, repeated-dose toxicity.

in a broad concentration range, allowing identification of point of departure for various measurements. These data provide insights into the applicability, behaviour and predictivity of the different test systems used in EU-ToxRisk. Another step towards better understanding the *in vitro* tests was to use RNA sequencing of all test systems without any chemical stressors. These data are expected to give insights into the make-up of the test systems, for example, phase I and II drug metabolism enzymes, transporters etc. This work may support the selection of optimal test systems in the future.

A subset of tests in EU-ToxRisk provides direct mechanistic data for the toxicological effects or endpoints of interest. For example, high-throughput tests such as CALUX (Luciferase reporter assay) and HepG2-BAC-GFP (imaging-based GFP reporter assay) provide information on molecular signalling events such as agonism or antagonism of hormone receptor signalling events and activation of cellular stress response pathways in addition to providing information on cytotoxicity. Organ-specific models can provide information on specific endpoints such as neurite outgrowth as well as cell viability in human-relevant, target organ-relevant test systems. Finally, complex and disease-specific models allow the assessment of the effects of chemicals in, for example, diseased 3D liver spheroids (over-accumulation of lipids) as well as the effect of chemicals on developmental processes as reflected in the differentiation of mouse embryonic stem cells (the embryonic stem cell test) or complex systems such as the zebrafish embryo test. The applicability of tests and models has been supported by a series of exploratory studies within EU-ToxRisk and together with other partners [22–26].

Novel advances in EU-ToxRisk *in vitro* test systems toolbox

To improve the predictive scope of NAMs, EU-ToxRisk is working on establishing fluorescent protein reporter cell lines in iPSC. These cell lines are highly desirable because they would offer the advantage of a genetically stable cell line that can proliferate indefinitely and be differentiated into numerous target organ-specific cell lineages. To date, all reporter cell lines in EU-ToxRisk have been established in immortalized or cancer cell lines that are genetically unstable [27,28]. Primary human cells on the other hand have limited proliferative potential, making it virtually impossible to generate reporter lines from them. These problems could be overcome with stem cell–based reporter technology. Here, the CRISPR/Cas9 technology is used to generate iPSC-based fluorescent reporter cell lines. At this point, EU-ToxRisk has generated a functional induced pluripotent stem cell line iPSC heme oxygenase 1 (HMOX1) green fluorescent protein (GFP) reporter for monitoring oxidative stress responses, and the functionality of this reporter is evaluated in different cell lineages. In combination with live cell imaging, this will allow a temporal and quantitative assessment at the single cell level of modulation of stress response pathway activation in different target tissues.

Also, we have established dual reporter cell lines in HepG2 using bacterial artificial chromosome (BAC) reporter technology. By using live cell imaging, these dual fluorescent reporters do allow the simultaneous monitoring of two different cellular stress response pathways in individual cells or the monitoring of two different components within one pathway. This allows us to directly observe the interplay between different stress pathways and the sequential activation of proteins within one stress pathway, respectively.

Another dimension of new approaches developed and applied in the project is the application of microphysiological systems (MPSs). This comprises the use of (i) 3D organoids and (ii) coculture of relevant cell types, such as neuronal and glial cells [29]. Moreover, it is directed to a multiorgan-on-a-chip technology (e.g. developed by the partner TissUse) [30,31] which involves four different organs on one chip that are integrated with microfluidics. The four-organ-on-a-chip allows prolonged exposure scenarios and will be evaluated in future case studies. This important microphysiological system (MPS) development as a future NAM toolbox component will be the subject of a high-level workshop coorganized by EU-ToxRisk in June 2019.

Read-across approach

The first set of case studies in EU-ToxRisk uses a read-across approach. The concept, its building blocks and assessment steps will be published in full detail elsewhere. Briefly, groups of structurally similar compounds were selected, for which existing *in vivo* animal data (e.g. from ToxRef DB or RepDose) indicated critical shared toxicological effects. In some cases, closely related compounds without *in vivo* data were added to test in how far the 'mechanistic' data from NAMs can be used to draw a conclusion on their hazard compared with other group members. Whenever possible, we included structurally closely related compounds, which did not show the critical toxicological effect *in vivo*. The absence of the effects might either be a consequence of differences in toxicodynamics and/or in toxicokinetics. With the help of NAMs, we will learn how to discriminate active and inactive analogues and predict the hazard of those being active correctly.

The reference *in vivo* data are used to guide the choice of *in vitro* test systems, while physiologically based pharmacokinetic PBPK modelling is used to determine relevant *in vitro* testing concentrations to provide information on *in vitro* kinetics. *In vitro* test systems provide data on endpoint and target organ-specific effects,

as well as insights into activation of cellular receptors, stress response pathway activation and transcriptomic changes. Finally, databasing expertise provides a platform to assemble, analyze and integrate data that are generated in the project. The EU-ToxRisk *in vitro* test systems are used to provide mechanistic data to demonstrate the similarity (or dissimilarity) of the dynamic and kinetic behaviour of test substances. This testing is based on a read-across hypothesis mainly derived from the available *in vivo* and to some extent *in vitro* data of the source compounds.

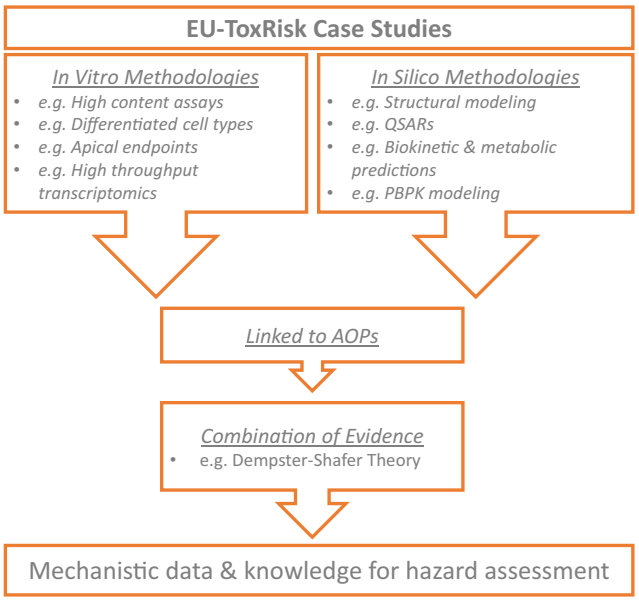
One RDT read-across case study in EU-ToxRisk, for example, uses valproic acid as a source compound. It is well established that exposure to valproic acid can lead to the formation of microvesicular liver steatosis in humans and animals. AOPs for this effect have been described. *In vitro* tests were chosen that allow testing of key events and a number of molecular initiating events (MIEs) to demonstrate similar dynamics within the grouped valproic acid analogues. Kinetic modelling and testing are used to further ensure that absence of effects is not caused by differences in bioavailability. Similar strategies are used in other case studies, for example, for compounds such as rotenone that targets complex I of the mitochondrial respiratory chain. This effect of rotenone is related to an AOP that has recently been accepted by the Organisation for Economic o-operation and Development (COECD) and describes the key events related to Parkinson's-like neuronal effects caused by inhibition of complex I of the mitochondrial respiratory chain [32]. *In vitro* test systems were chosen that allow for quantitative assessment of almost all key events of this AOP. Within all the case studies, the integration of AOP-related test and toxicokinetic data are used to support and strengthen the read-across hypothesis. The final read-across assessment supported by NAMs has been documented in well-structured dossiers, termed mock submissions, largely based on Organisation for Economic o-operation and Development (COECD) templates for case study reporting. These mock submissions will be presented to various European regulators who are members of the EU-ToxRisk regulatory advisory board for feedback. These case studies will be part of a workshop on read-across approaches that EU-ToxRisk organizes in May 2019. The experience gained from this first set of read-across case studies carried out using NAMs and the outcome of the workshop will be used to establish a manuscript detailing read-across guiding principles.

Conclusions and future steps

The mechanistic knowledge generated in this first set of read-across case studies is linked to well-described AOPs, therefore providing a strong scientific support for read-across approaches, see Figure 2. We believe that this thorough scientific underpinning will be key for the regulatory acceptance of integrative testing approaches developed in EU-ToxRisk. In addition, this first set of case studies has helped to shape the next set of case studies which will address new regulatory and scientific questions. Some case studies will address the issue of low or no toxicity. In these case studies, we will address chemicals with little or no observed adverse effects — will it be possible to predict this using NAMs? In addition, we will address the topic of multitarget organ toxicity. Here, we aim to determine whether an integrated testing strategy can define the liability of chemicals to cause toxicity in multiple different target organs and learn in how far the EU-ToxRisk battery of NAMs will be able to correctly predict these toxicities based on qualitative and quantitative mechanistic information whenever possible related AOPs.

In the next phase of the project, we will also aim to further advance the field of NAM-based hazard assessment. The assessment of a chemical without any knowledge of its *in vivo* effects (*ab initio*), that is, in the absence of any chemicals with structural similarities and available in *vivo* data, using only NAM approaches is an ultimate, very-high-reaching goal of EU-ToxRisk. To learn how this may be achieved in the future, dedicated *ab initio* case studies will be carried out in EU-ToxRisk. We will work closely with the Joint Research Centre (JRC) on the *ab initio* case study. Finally, we will further seek interactions with stakeholders from different

Figure 2



An overview of the general approach taken to the first set of EU-ToxRisk case studies. Data, linked to adverse outcome pathways, were generated using *in silico* and *in vitro* methodologies from the EU-ToxRisk toolbox. The data that were generated in a case study are then combined, using uncertainty analysis and expert judgement to be used in a hazard assessment. AOPs, adverse outcome pathways.

chemical industry sectors to evaluate the application of the NAMs established and/or evaluated within the EU-ToxRisk project in industry-driven case studies. We anticipate that the refinement of existing NAMs, the development of novel NAMs and the application of NAMs in case studies will pave the way for an evolution towards well-established integrated testing strategies for the assessment of chemical safety without the use of animals.

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Conflict of interest statement

Nothing declared.

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- • of special interest

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