Concept Article

The SEURAT-1 Approach towards Animal Free Human Safety Assessment

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Summary

SEURAT-1 is a European public-private research consortium that is working towards animal-free testing of chemical compounds and the highest level of consumer protection. A research strategy was formulated based on the guiding principle to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health. The proof of the initiative will be in demonstrating the applicability of the concepts on which SEURAT-1 is built on three levels: (i) Theoretical prototypes for adverse outcome pathways are formulated based on knowledge already available in the scientific literature on investigating the toxicological modes-of-action leading to adverse outcomes (addressing mainly liver toxicity); (ii) adverse outcome pathway descriptions are used as a guide for the formulation of case studies to further elucidate the theoretical model and to develop integrated testing strategies for the prediction of certain toxicological effects (i.e., those related to the adverse outcome pathway descriptions); (iii) further case studies target the application of knowledge gained within SEURAT-1 in the context of safety assessment. The ultimate goal would be to perform *ab initio* predictions based on a complete understanding of toxicological mechanisms. In the near-term, it is more realistic that data from innovative testing methods will support read-across arguments. Both scenarios are addressed with case studies for improved safety assessment. A conceptual framework for a rational integrated assessment strategy emerged from designing the case studies and is discussed in the context of international developments focusing on alternative approaches for evaluating chemicals using the new 21st century tools for toxicity testing.

Keywords: alternatives to animal testing, integrated testing strategy, mode-of-action theory, proof-of-concept case studies, safety assessment

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1 Introduction

The full ban on animal testing for cosmetic products within the European Union came into force on 11 March 2013, despite the lack of validated alternative methods for reproductive toxicity (including teratogenicity), carcinogenicity, toxicokinetics and repeated dose systemic toxicity. Independent expert panels of scientists concluded that they could not even estimate the time required to establish alternative methods for the full replacement of animal testing in the fields of repeated dose systemic toxicity, carcinogenicity and reproductive toxicity (Adler et al., 2011; Hartung et al., 2011). Consequently, roadmaps for future research aiming at the development of alternative non-animal testing methods were formulated; these roadmaps highlighted the current state of research as well as knowledge gaps to be addressed in ongoing and upcoming research programs (Basketter et al., 2012). These roadmaps, and the call for proposals under the Health Theme of the 7th European Framework Programme "Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity Testing", led to the creation of SEURAT-1 in 2011. This major European research consortium was established to develop the science needed to evaluate the safety of chemicals for repeated exposure in humans without using animals. The research initiative is co-financed through a new model of public-private partnership by the European Commission's FP7 Health Programme and Cosmetics Europe.

SEURAT-1 is inspired by the fundamental considerations published in the report of the U.S. National Research Council (NRC) entitled "Toxicity Testing in the 21st century: A Vision and a Strategy" (NRC, 2007a) and the European Partnership for Alternatives to Animal Testing (EPAA) report "New Perspectives on Safety" (EPAA, 2008), as well as numerous publications highlighting the consequences for future research (e.g., Andersen et al., 2011), the demands for the development of new testing approaches (Hartung et al., 2013a) and their implementation into safety assessment procedures (Krewski et al., 2011). No matter whether the new field of research is named predictive toxicology (NRC, 2007b) or systems toxicology (Hartung et al., 2013b; Sturla et al., 2014), all these activities aim to use the mechanistic understanding of toxicological effects for the development of innovative testing methods and, ultimately, improved safety assessment. SEURAT-1 shares these perspectives.

The acronym "SEURAT" indicates the long-term goal of the initiative – Safety Evaluation Ultimately Replacing Animal Testing. The first execution phase, named SEURAT-1, was launched in January 2011 and focuses on the replacement of repeated dose systemic toxicity testing on animals. SEURAT-1 is intended to be the first step towards the specific goal of addressing the global, long-term strategic target of replacing animal testing in safety assessments. It comprises a cluster of five complementary research projects combining expertise in: (i) stem cell differentiation and cell culturing for providing human-based, organ-specific target cells for toxicity testing (SCR&Tox); (ii) the identification of new biomarkers for repeated dose toxicity (DETECTIVE); (iii) the development of organ-simulating devices mimicking the complex structure and

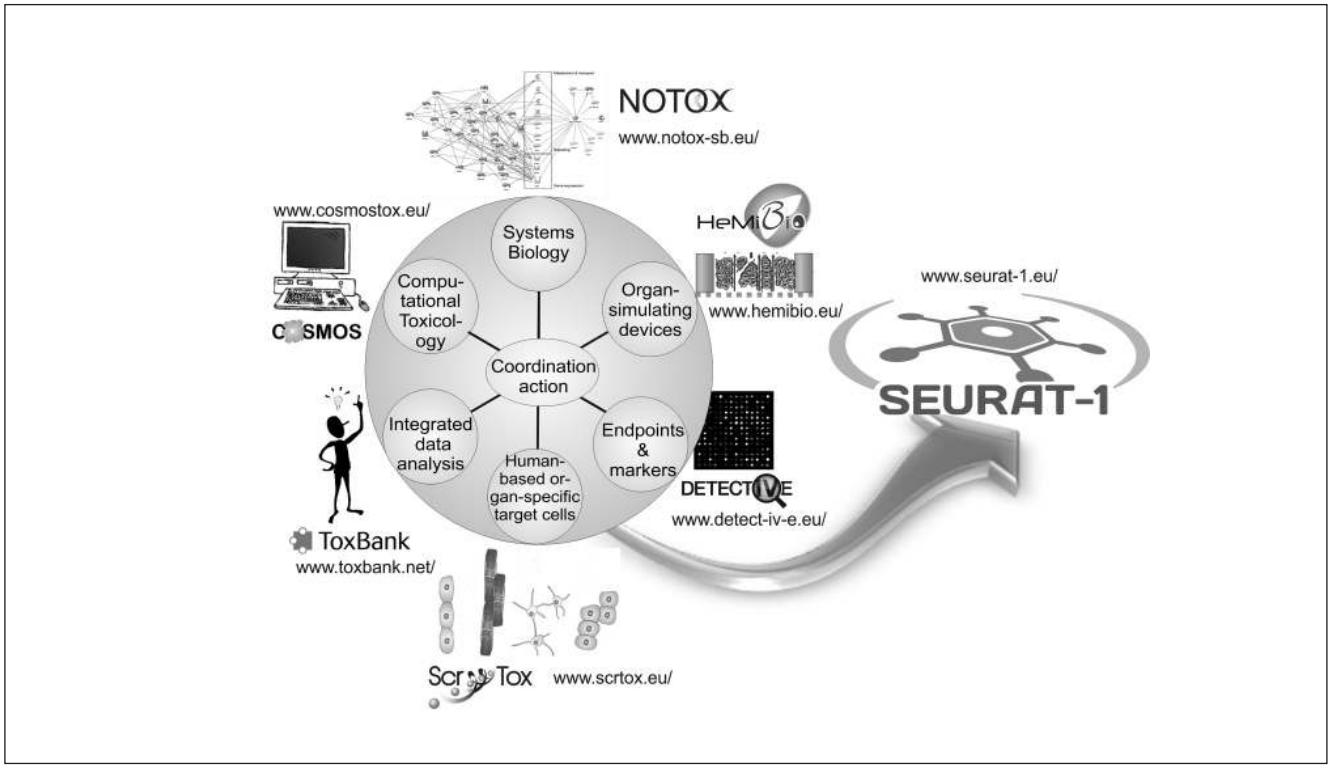

Fig. 1: Building blocks of SEURAT-1 (themes, project logos and respective homepages)
These were established based on the call for proposals under the Health Theme of the 7th European Framework Programme "Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity Testing."

function of the human liver (HeMiBio); (iv) the development of systems biological tools for characterizing long-term toxic effects in organotypic human cell cultures (NOTOX); and (v) the development of integrated computational tools to predict long-term effects of chronic exposure to chemicals in humans (COSMOS). These research projects are accompanied by a data handling and servicing project (ToxBank) and a coordination action project (COACH). Each of the research projects has its own defined research agenda, which can be found on the respective homepages, as indicated in Figure 1.

Overall, the aim of SEURAT-1 is to provide a blueprint for future implementation of mechanism-based, integrated toxicity testing strategies into modern safety assessment approaches. For this, we outline first the key elements of the SEURAT research strategy and then describe how the theory is being brought into practice within SEURAT-1 through the formulation of case studies using mode-of-action descriptions as starting points for the development of *in silico* and *in vitro* test methods. Further case studies target the application level in the context of safety assessment, demonstrating that SEURAT is indeed feasible. This is discussed in the context of a conceptual framework for a modern safety assessment approach, emerging from the work within SEURAT-1 and related international activities. Finally, the SEURAT-1 approach is evaluated against the recommendations from the roadmap for the development of non-animal test methods for the replacement of *in vivo* repeated dose toxicity testing (Basketter et al., 2012).

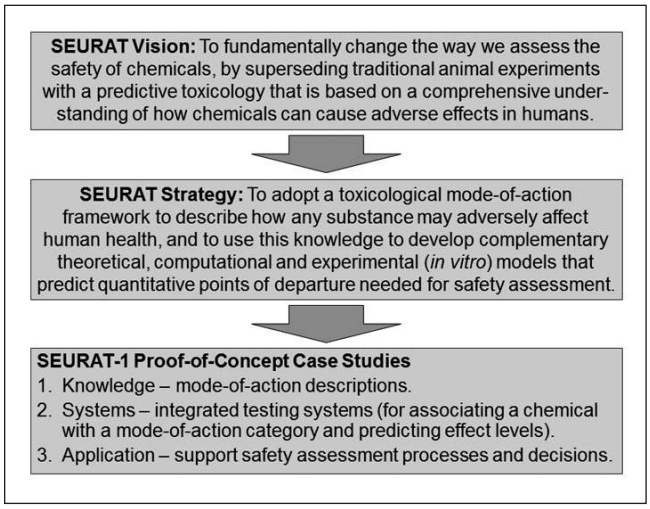

Fig. 2: The SEURAT vision and strategy triggers the formulation of proof-of-concept case studies addressed within SEURAT-1

2 The SEURAT research strategy

2.1 A mode-of-action based framework

Central to new approaches to toxicity testing is a mechanistic redefinition of adverse effects based on *in vitro* toxicity testing. This redefinition will require a series of prototypes to show the process in practice (Boekelheide and Andersen, 2010). Such a redefinition of adversity requires a mechanistic understanding of toxicity at the molecular scale as it will rely on perturbation of pathways rather than description of effects on apical endpoints. In a phase of transition it is, however, reasonable to harmonize the current definition of adversity at the organ level (as apical endpoints) with the new pathway-based paradigm. This describes the positioning of SEURAT-1 in the field of repeated dose systemic toxicity, with a focus on organ toxicity (mainly on the liver) that needs to be linked with the development of understanding of molecular-scale processes. The guiding principle of the SEURAT research strategy is, therefore, to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health. This knowledge will then be used to develop complementary theoretical, experimental (*in vitro*) and computational models that predict quantitative points of departure needed for safety assessment. Finally, SEURAT-1 is targeting a proof-of-concept level, showing how the scientific tools and knowledge developed can be combined to deliver decision support systems for safety assessment. These fundamental considerations for SEURAT-1 and beyond are summarized in Fig. 2.

The mode-of-action framework is based on the premise that any adverse human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key "events" that result in a patho-

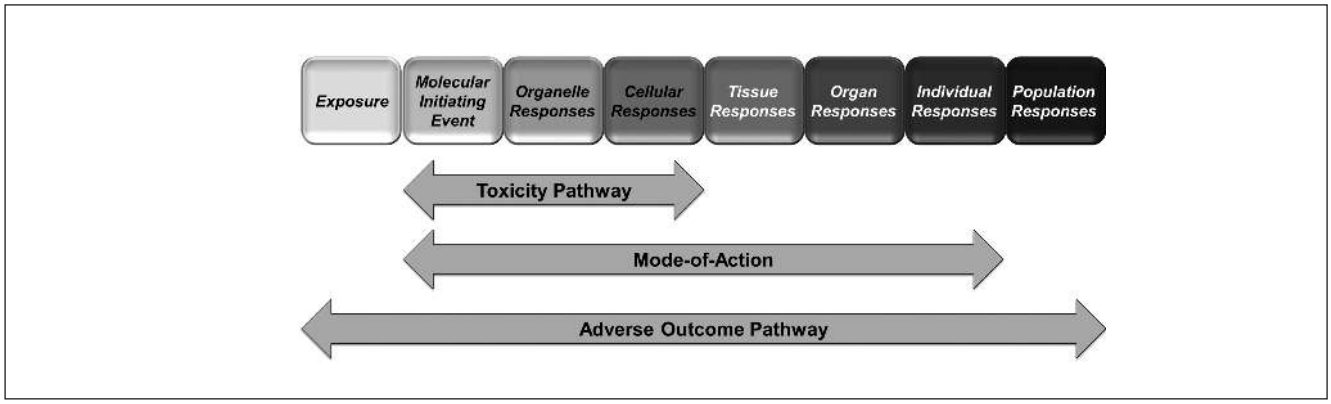

Fig. 3: Schematic illustration of a sequence of events contributing to an Adverse Outcome Pathway, including the Mode-of-Action and Toxicity Pathways as sub-sequences

logical endpoint or disease outcome (Boobis et al., 2008). An "adverse outcome pathway" is a similar concept proposed by the computational toxicology community (Ankley et al., 2010), where the linking of a chemical with a pathway that leads to an adverse human health or ecological outcome is determined by its ability to trigger the associated "molecular initiating event." Another related framework is that of "toxicity pathways" introduced by the NRC (Krewski et al., 2010), where the description of toxicological processes tends to focus on early events at the molecular and cellular level. Thus, one can consider toxicological pathways as critical upstream elements of a more expansive mode-of-action or adverse outcome pathway description of how a chemical can compromise human health (Kleensang et al., 2014; Fig. 3). In addition, looking at stress response pathways may provide insights into toxicological mechanisms. Conceptually, stress response pathways are not perturbations but responses to perturbations and, as such, their activation by chemicals indicates what kind of biological process was disturbed (Jennings, 2013) without looking at downstream apical endpoints that may be very diverse. Hence, stress response pathways (such as the activation of the Nrf2 pathway in the context of the induction of oxidative stress) are valuable targets for toxicity testing (Krewski et al., 2011).

Mode-of-action theory is still emerging, but already a number of important principles have shaped the SEURAT research strategy. The first is that every toxicant can be associated with one or more mode-of-action categories. To facilitate this, however, a suitable ontology that describes all the possible modes of toxicological action needs to be developed by harvesting and organizing the wealth of knowledge and information available from the literature on well-studied chemicals and pharmaceuticals (Hardy et al., 2012). Systematically checking "reference" chemicals against mode-of-action categories will help to challenge and refine the mode-of-action ontology as it emerges, and will identify a wide range of key biological events and pathways that should be represented in relevant experimental (*in vitro*) and computational models. At the level of interactions between chemicals with biological targets, toxicity can be broken down into two categories of selectivity and three categories of reversibility (Fig. 4): (i) chemicals designed to interact selectively with specific biological targets (such as drugs and pesticides) through high affinity receptor interactions that are in general reversible; other "non-selective" chemicals that can interact with many biological targets, either (ii) irreversibly (such as alkylating agents) or (iii) reversibly (e.g., low affinity receptor interactions that can perturb multiple cellular targets within a narrow concentration range). Correctly binning the chemicals into each of the three categories will serve as the first step for evaluating the potential mode-of-action of a chemical (Thomas et al., 2013).

This framework assumes that many modes-of-action share common key molecular or biological events. Thus, it is the particular chain of causally linked events that makes a mode-of-action unique. In the case where a substance is promiscuous and could trigger multiple modes-of-action, the concentration and persistence of the substance at the initiation sites will dictate the modes-of-action that will tend to dominate. Thus, in many cases chronic low-dose effects may be quite different from high-dose

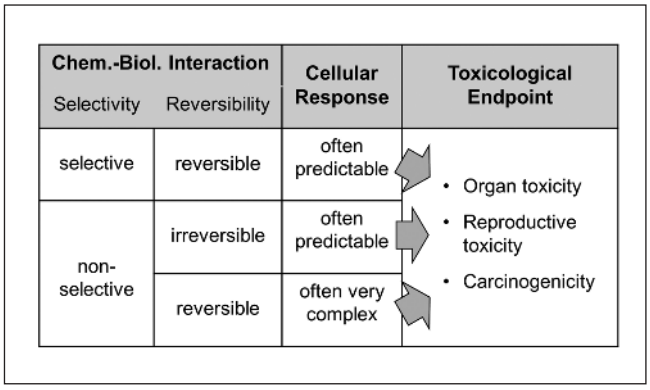

Fig. 4: Chemical reactivity triggers initial interactions with biological targets and downstream modes-of-action
Note that the toxicological endpoint is not indicative for the type of the initial interaction.

acute effects. For example, carbon tetrachloride induces fully reversible massive necrotic cell death in perivenous hepatocytes upon a single high dose, but irreversible damage (fibrosis) upon repeated doses (Hoehme et al., 2010). A second example is phenobarbital, a barbiturate that causes a locus-specific change in the DNA-methylation pattern of mice after several weeks of exposure, which is not seen after short-term exposure (Thomson et al., 2013). This change in the DNA methylome causes a corresponding change in the transcriptome, triggering the tumor promoting effect of the barbiturate seen after chronic treatment but not seen after acute exposure, which only causes adaptive and fully reversible liver growth and an enzyme induction response aimed at facilitating elimination of the drug from the body (Thomson et al., 2013). However, according to Haber's Rule, such discrepancies do not occur for the so-called "c x t-compounds," for which a toxicological effect is the result of the total dose over a period of time, such that even very small doses, given for prolonged periods of time, will produce the same toxic effect as a high dose given for only a short period of time. Special consideration needs to be given, therefore, to characterizing dose-response relationships, to describing how and when mode-of-action transitioning may occur for a single substance, depending on factors such as exposure dynamics, site of action, genetic and epigenetic predisposition, and inherent phenotypic vulnerabilities. Consequently, establishing an animal-free testing paradigm requires careful consideration of both the *in vitro* biokinetics of the substance of interest and its toxicodynamics as a prerequisite for quantitative *in vitro* to *in vivo* extrapolation (Blaauboer et al., 2012; Groothuis et al., 2013).

Even though, *a priori*, it is not clear whether the biological key events relevant for repeated dose systemic toxicity differ from those relevant for acute toxicity, it is reasonable to assume that there is at least some mechanistic overlap between both exposure scenarios. As most current mechanistic information stems from the acute exposure scenario, a pragmatic first step when putting the mode-of-action theory into practice in the context of repeated dose systemic toxicity is the clarification of differences regarding key events between both exposure scenarios. These differences may be fully controlled by the biokinetics (accumula-

tion in the cells versus transformation rates; the latter may cause metabolic activation) leading to: (i) repeated hits (of the parental compound or metabolites) on the same molecular target; (ii) overload of defense/repair mechanisms through accumulation of a chemical (parental compound or metabolites) at certain initiation sites; (iii) progressive change in the epigenome; (iv) effects on the immune system, such as proliferation of memory cells and progressive activation and transformation of, e.g., hepatic stellate cells; and finally (v) induction of a sequence of adverse reactions involving different cell types (and organs).

Another principle to be considered concerning mode-of-action theory is that many key events and pathways are common to many cell types throughout the human body (for example, the Nrf2-pathway; Krewski et al., 2011). Thus, although the same substance can cause different pathological outcomes in different tissues, the upstream event, such as inhibition of mitochondrial function or generation of reactive oxygen species, may be common to the modes-of-action triggered at each site. Conversely, certain modes-of-action involve key events or pathways that are associated with specific biological functions expressed by particular cell types. For example, the presence of metabolizing enzymes in liver cells may bioactivate exogenous chemicals to produce toxic metabolites, or the presence of cell membrane transporters required for the uptake of certain toxicants. Similarly, the presence of receptors for neurotransmitters in neuronal cells can be targeted by toxicants. This is another example of cell-specific properties that can be implicated in a toxicological mode-of-action.

2.2 Implications for *in vitro* and *in silico* toxicity testing: The SEURAT-1 approach

Establishing a comprehensive description of the mode-of-action domain is a challenging element of the strategy and requires the use of advanced discovery and modelling tools to identify the key biological events and biomarkers that comprise a particular mode-of-action. Elucidating the relationship between these events can benefit greatly from high content functional analysis tools such as transcriptomics and proteomics (Wilmes et al., 2013). These data are used to guide the definition of systems biology models which capture the process dynamics and allow quantitative prediction of biological pathway perturbation. As the mode-of-action framework is refined, and more key biological events are identified, new biomarkers of effect can be incorporated into assay systems.

In order to overcome any shortcomings regarding translation of mode-of-action theory into test system development through inter-species variability, SEURAT-1 researchers are using human-based cell lines as biological models (established cell lines, primary cells and stem cell-derived cell lines). Stem cell-derived cell lines were thoroughly characterized to evaluate for which exact purpose they are fit (Pistollato et al., 2012). Bioreactor technology is being employed to engineer 3D tissue constructs *in vitro* in an attempt to capture the intricate interactions between different cell types present in an organ that must work in unison to maintain homeostasis and function. Furthermore, genetically engineered stem cell-derived models are being generated that are equipped with reporters which are suitable for indicating changes in the activity of selected signaling or metabolic pathways. Such tailor-made reporter cell lines allow the capture of specific key events within particular modes-of-action (Wink et al., 2014).

Complementing the cell and tissue models, computational chemistry, quantitative structure-activity relationships (QSARs) and chemoinformatics tools, such as software made available through the QSAR toolbox (OECD, 2014a), provide the means to understand and predict key biochemical events such as protein binding and metabolic transformation. The attention within SEURAT-1 focuses on finding associations between the structural features of a chemical and its ability to trigger the key biomolecular events that initiate toxicological responses that may lead to adverse health outcomes (Ellison et al., 2011). Forming chemical categories based on combined structure-activity descriptors will ultimately facilitate more rapid and robust hazard profiling of chemicals and read-across between chemicals that have similar modes-of-action.

Another important aspect of the SEURAT strategy is the emphasis placed on understanding and predicting the *in vivo* biokinetics of exogenous chemicals. Quantifying the dose in different target tissue compartments as a function of time and exposure conditions is a fundamental requirement of any predictive toxicology paradigm (NRC, 2007a). In particular, the establishment of an *in vitro* testing paradigm requires methods to extrapolate *in vitro* concentrations to the *in vivo* situation, taking into consideration the *in vitro* biokinetics (Groothuis et al., 2013) and the high sensitivity of omics data to the dosing protocol as well as to the time point of the respective analyses (Blaauboer et al., 2012). First models calculating *in vivo* concentrations and exposure based on *in vitro* data were already developed for this purpose (Wetmore et al., 2012; Yoon et al., 2012). These models are adapted to the research work within SEURAT-1 (Péry et al., 2013).

In summary, SEURAT-1 has a work program that aims to demonstrate a proof-of-concept for the scientific and technological underpinning of the SEURAT strategy. The overall empha-

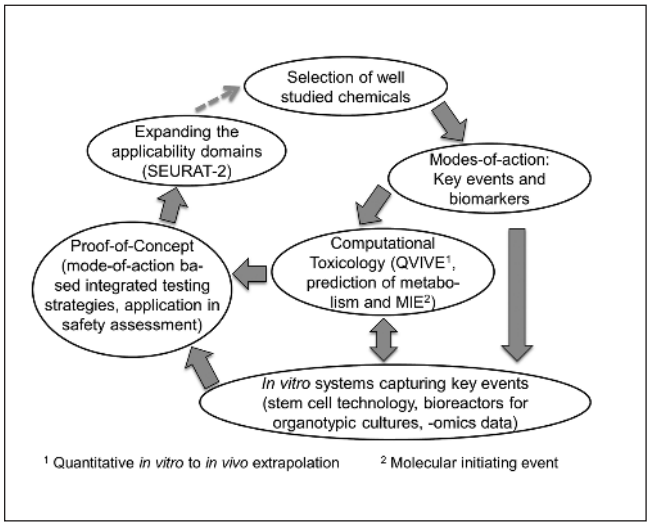

Fig. 5: Key elements of SEURAT-1 and perspectives for a possible second phase of SEURAT

sis is on the identification and elucidation of modes-of-action related to repeated dose systemic toxicity in humans, and the development of experimental and computational models that effectively capture the related pathways and key biological events (Fig. 5). A set of reference chemicals has been compiled from chemicals that have been thoroughly investigated regarding their chronic toxicological action in animals and, if possible, in humans. This information was used to propose an initial mode-of-action framework to which the various research activities can refer (see below).

Aiming at a more quantitative description of a mode-of-action requires mathematical models of sufficient complexity. Systems biology theory and tools provide a strong basis for these models (Kohonen et al., 2014) that needs to be integrated into a multi-scale modelling framework connecting subcellular events with effects at the organ scale (Niklas et al., 2013). As the mode-of-action framework becomes more established and the range of validated models grows, an increasing number of chemicals can be profiled to establish in which mode-of-action categories they belong. This will then facilitate read-across within categories and provide the basis for ultimately predicting hazard threshold values, akin to *in vivo* no-effect levels.

3 Implementation of the research strategy

3.1 Chemical selection

The first sign of how the guiding principles of the research strategy outlined above have influenced the research cluster is reflected in the approach adopted for the selection of standard reference compounds to be used across the projects. The selection of standard reference compounds is a critical issue in any research program and a cross-cluster working group led by the servicing project ToxBank was established within SEURAT-1 for defining selection criteria according to the overarching research strategy and, ultimately, providing the research projects with a list of reference compounds. The selection was governed by the following basic considerations: (i) The standard reference compounds should be associated with well-known modes-of-action; (ii) the standard reference compounds should be relevant for repeated dose toxicity; (iii) promiscuity, i.e., lack of structural specificity in ligand binding, should be considered; and (iv) extrapolation from well-studied reference compounds to a broader chemical space, taking into account different uptake routes, should be possible.

Consequently, the working group first identified and described a range of known modes-of-action more commonly cited in repeated dose toxicity studies, and then picked molecules for which there is ample mechanistic evidence of association with toxicological effects or pathways underpinning those modes-of-action. The resulting list of standard reference compounds is now publicly available as an online resource (ToxBank, 2014) along with extensive descriptions with respect to the above-mentioned criteria, including further considerations regarding their applicability in cell-based *in vitro* assays (e.g., long shelf-life, soluble in buffer, commercially available, etc.). Not surprisingly, many of the reference chemicals are pharmaceuticals or failed drugs since these molecules typically have specific mechanisms or modes-of-action that are extensively described in the literature. It is precisely these mode-of-action-related properties that make them reliable candidates for nomination as reference compounds, rather than their actual origin or commercial use.

3.2 The proof-of-concept case studies

3.2.1 Why case studies?

The SEURAT-1 Research Initiative aims at delivering many important computational and experimental tools and related knowledge that will be critical components in predictive toxicology approaches. To demonstrate the potential of these tools and how they can be assembled in an integrated manner, the cluster undertakes a proof-of-concept exercise, separated into three distinct areas: theoretical (mode-of-action descriptions), methodological (development of integrated testing strategies according to modes-of-action) and application (aiming at improved, animal-free safety assessment approaches).

In this way, SEURAT-1 is following a case study approach. Adopting a mode-of-action toxicological framework as outlined above means that one needs to learn by doing, with the starting point being identifying some "prototype" modes-of-action that could be elaborated. Covering all potential modes-of-action based on existing knowledge is simply not feasible. Hence, being selective is important at the beginning of this endeavor – "selective" in terms of chemicals (see above, chemical selection), modes-of-action, and definition and design of case studies to prove the underlying concepts. The following quote, taken from an article that discussed some fundamental considerations about the way forward in predictive toxicology, summarizes the motivation behind the SEURAT-1 case study design:

> *"... in the near-term, however, what is most needed is a demonstration of the feasibility of these new approaches and their ability to be both reliable and predictive. These near-term goals ... can most effectively be met with demonstration projects using a case study approach" (Andersen et al., 2011).*

3.2.2 Mode-of-action descriptions

Proof-of-concept at the theoretical level aims to show how toxicological knowledge concerning modes-of-action can be mined, or perhaps generated, and then reconciled, consolidated and explicitly described in a format that can be managed and communicated in an effective and harmonized manner. For this purpose, the International Programme on Chemical Safety (IPCS) of the World Health Organisation (WHO) has published guidance (Boobis et al., 2008) on what type of information should be provided to describe a mode-of-action (MoA) and, just as importantly, how the relevant evidence should be presented to demonstrate the validity of the proposed description. More recently, the OECD has followed this direction by proposing an analytical tool termed "adverse outcome pathway" (AOP, see above), and published a template for capturing the relevant information (OECD, 2013). This template clearly indicates which information should be provided, both to describe the toxicological process itself and the evidence that supports

the postulation of the associated key events, including the molecular initiating event (Fig. 6A). Examples of AOP descriptions based on this concept were published recently (Vinken, 2013; Vinken et al., 2013).

The ongoing selection of modes-of-action (MoA) within SEURAT-1 is performed in the context of the development of such AOP descriptions. Note that SEURAT-1 is entirely focusing on the replacement of animal tests by the application of human cells and, thus, will not conduct *in vivo* experiments. Therefore, the development process relies heavily on a systematic review of the literature and publicly available toxicity study databases to extract the mechanistic knowledge applicable to the MoA in

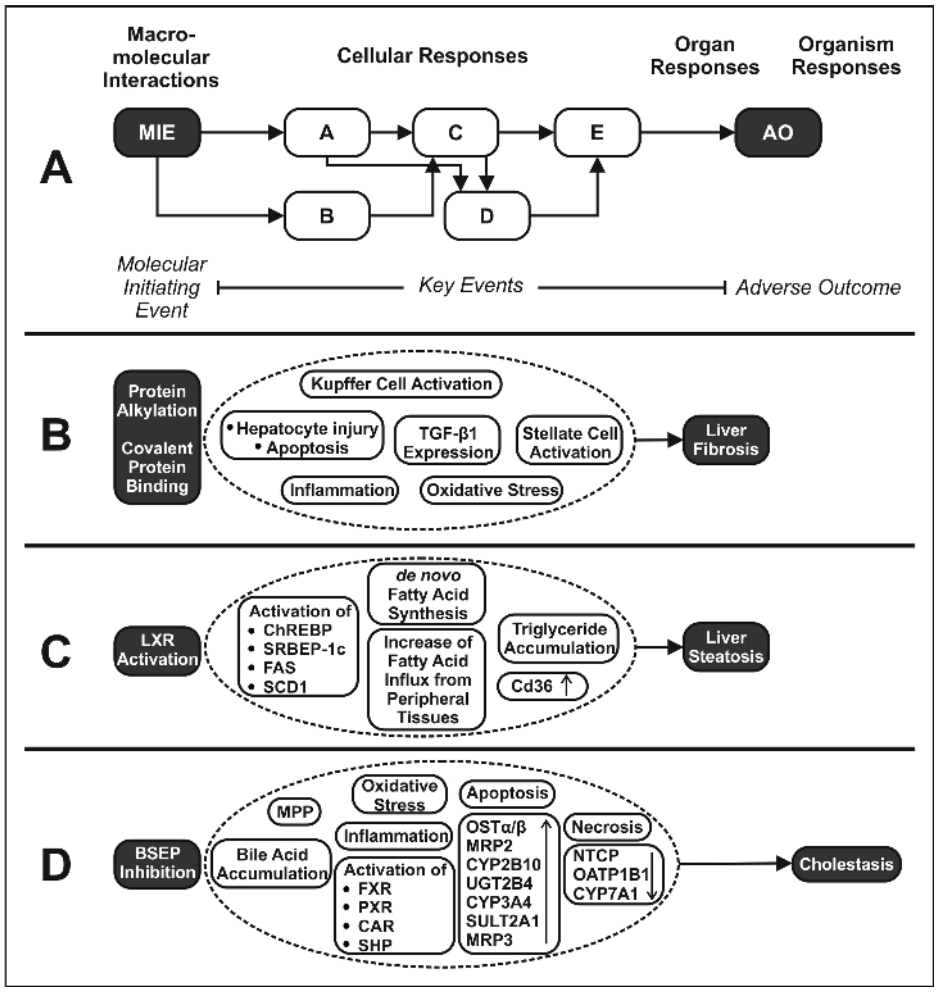

Fig. 6: Prototype mode-of-action descriptions developed within SEURAT-1

A: Generic mode-of-action description causally linking a molecular initiating event (MIE) with an adverse outcome (AO) at the organ or organism level by identifying intermediate key events and connecting them with biological plausibility with the MIE and AO, respectively (OECD, 2013). The key events are the basis for hypothesis development and testing and should be experimentally quantifiable.

B: Collection of key events connecting chemical-induced liver fibrosis (AO) with protein alkylation (MIE) to be used as anchors for toxicity testing (TGF-β1: transforming growth factor beta 1). A mechanistic description assembling the key events in a biologically plausible way can be found elsewhere (Landesmann et al., 2012; Vinken, 2013).

C: Collection of key events (ChREBP: carbohydrate response binding protein; SREBP-1c: sterol response element binding protein; FAS: fatty acid synthase; SCD1: stearoyl-coenzyme A desaturase 1; CD36: fatty acid translocase) connecting chemical-induced liver steatosis (AO) with the activation of the liver X receptor (MIE; Landesmann et al., 2012; Vinken, 2013).

D: Collection of key events (PXR: pregnane X receptor; CAR: constitutive androstane receptor; FXR: farnesoid X receptor; SHP: small heterodimeric partner; OSTα/β: organic solute transporter α/β; MRP2, 3: multidrug resistance associated proteins 2 and 3; CYP2B10, 3A4, 7A1: cytochrome P450 2B10, 3A4, 7A1; UGT2B4: uridine 5'-diphosphate-glucuronosyltransferase 2B4; SULT2A1: dehydroepiandrosterone sulfotransferase; NTPC: sodium/taurochlorate cotransporter; OATP1B1: organic anion transporter 1B1; MPP: mitochondrial permeability pore) connecting chemical induced cholestasis (AO) with the inhibition of the bile salt export pump (BSEP, MIE). Further mechanistic explanations can be found elsewhere (Vinken et al., 2013).

question. Considering the wealth of information already available, a MoA description can be typically brought to a relatively mature state of development by studying relevant review papers and reported studies. However, at some point the process plateaus since the finer mechanistic detail specific to the MoA is often lacking in the description. Thus, more extensive development of a MoA can require the undertaking of prospective experimental (*in vitro*) investigations that are specifically designed to shed light on the less understood aspects of the MoA.

Given the high number of hepatotoxins in the list of SEURAT-1 standard reference compounds as a result of the chemical selection strategy (ToxBank, 2014), it was reasonable to start the exercise with MoA descriptions that are of relevance to the liver and to try to define related pathways based on the identification of interactions of a chemical with known targets. As a result, the following three prototype MoA descriptions were assembled (Fig. 6B-D):

From protein alkylation to liver fibrosis, Fig. 6B
Liver fibrosis is a potentially reversible wound healing response to a variety of chronic as well as acute injuries including those due to toxic insults. It results from a complex interplay between various cell types (hepatocytes, Kupffer cells and stellate cells) and signaling pathways with transforming growth factor beta 1 (TGF-β1) expression and activation of hepatic stellate cells as key events (Brenner, 2009). This MoA description was assembled by Landesmann et al. (2012) and is summarized by Vinken (2013) with further information about the interplay between the key events.

From liver X receptor activation to liver steatosis, Fig. 6C
Liver steatosis (fatty liver) is characterized by the micro- or macrovesicular accumulation of lipid droplets in the liver (Amacher, 2011). The development of steatosis can be attributed to many different causes, including interactions of chemicals with nuclear receptors that are involved in hepatic lipid metabolism. Among others, the activation of the liver X receptor (LXR), which is involved in fatty acid homeostasis, cholesterol metabolism and in the control of inflammation (Zelcer and Tontonoz, 2006), was identified as one important molecular initiating event triggering liver steatosis (Ducheix et al., 2013). LXR activation induces transcriptional changes (activation of the expression of the carbohydrate response binding protein (ChREBP), the sterol response element binding protein 1c (SREBP-1c), fatty acid synthase (FAS) and stearoyl-coenzyme A desaturase 1 (SCD1)), and up-regulation of the fatty acid translocase (CD36) production. More details and a flow chart summarizing this MoA can be found elsewhere (Landesmann et al., 2012; Vinken, 2013).

From bile salt export pump inhibition to liver cholestasis (Fig. 6D)
Liver cholestasis results from obstructed bile flow from the liver to the duodenum. Besides mechanical reasons (gallstones), the inhibition of transporters such as the bile salt export pump (BSEP) plays a key role in this liver injury. Various chemicals can directly inhibit the BSEP (Padda et al., 2011), which serves as the molecular initiating event in this MoA description. Subsequently, bile salt accumulates in the cytosol, inducing both adaptive and deteriorative cellular responses: cytosolic bile acid accumulation triggers transcriptional changes (activation of the pregnane X receptor (PXR), the constitutive androstane receptor (CAR) and the farnesoid X receptor (FXR), which induces the gene silencer small heterodimeric partner (SHP)), leading to deregulation of a number of proteins (up-regulation: organic solute transporter α/β (OSTα/β), multidrug resistance associated proteins 2 and 3 (MRP2, 3), cytochrome P450 2B10, 3A4 (CYP2B10, 3A4), uridine 5'-diphosphate-glucuronosyltransferase 2B4 (UGT2B4), dehydroepiandrosterone sulfotransferase (SULT2A1); down-regulation: sodium/taurochlorate cotransporter (NTCP), organic anion transporter 1B1 (OATP1B1) and cytochrome P450 7A1 (CYP7A1)), which is considered as an adaptive cellular response counteracting the primary cholestatic insults. This is distinguished from a deteriorative cellular response (formation of the mitochondrial permeability pore (MPP), oxidative stress and inflammation) leading to necrosis and apoptosis. A complete overview and mechanistic interpretation of this MoA description along with a weight of evidence assessment discussing the confidence in this construct was published recently (Vinken et al., 2013).

3.2.3 Systems to predict toxicity

At the systems level, the intention is to demonstrate how test systems can be produced by integrating various *in vitro* and *in silico* tools emanating from the SEURAT-1 projects, in order to assess the toxicological properties of chemicals using modes-of-action as an analytical basis. These systems can then be used to develop a robust and predictive data integration approach for safety evaluation, while minimizing uncertainties in the prediction through mechanistic understanding. Such systems include a combination of computational chemistry models with a battery of *in vitro* assays to generate a mixed set of chemical structure and bioactivity descriptors that can be used to group chemicals into MoA-based categories. The approach for designing MoA-based integrated testing strategies is currently under development and will be reported separately. In essence, the identification of key events in the process of developing a MoA description provides the backbone of an MoA-based integrated testing strategy. Test systems focus on certain key events and their sensitivity and specificity will be assessed by a sophisticated selection of standard reference compounds demonstrating that the test system is indeed predictive for the mechanism addressed (which follows a strategy of "mechanistic validation"; Hartung et al., 2013b).

The overall aim of this exercise is to predict certain aspects of toxicity of a chemical that are primarily related to the above-described modes-of-action for liver toxicity. Other organs are also represented in the selection of case studies, which will allow differentiation between organ-specific and more general pathways. Note that the MoA description need not be complete to be used as a blueprint in the design of the integrated testing strategy, and may be further developed iteratively when results from the testing strategy addressing a particular MoA become available. By these means, further experimental elucidation of the MoA will lead to confirmation and refinement or reformation of

the theoretical model. Conversely, to select methods for toxicity prediction, it might be sufficient to select one dominating key event rather than looking into a detailed MoA description.

Two main questions should be addressed when planning the development of respective *in vitro* test systems: (i) What is the appropriate biological model (based on the requirements, i.e., the prediction goal of the system, that needs to be defined beforehand); and (ii) what is the most relevant *in vitro* treatment protocol to relate to the human *in vivo* exposure (concentrations, dosing frequency, time interval after exposure when the endpoint should be measured)? There is no general approach, but recommendations to address these issues appropriately based on a decision tree that considers the properties of the chemical of interest, the assay setup and the MoA measured in the assay were published recently (Groothuis et al., 2013).

Although experimental investigation to elucidate mechanisms and modes-of-action is an important activity within SEURAT-1, there is widespread acknowledgement that mining publicly available databases that report both *in vitro* and *in vivo* toxicity studies (e.g., the "Open TG-GATEs" database established by the Toxicogenomics Project in Japan, or the DrugMatrix database of the U.S. National Toxicology Program) can provide an invaluable source of MoA information. In particular, there is an enormous amount of toxicogenomics data that, with the appropriate analysis, could uncover hidden mechanisms and key events and provide supporting evidence for a MoA during its development and evaluation (Kongsbak et al., 2014). SEURAT-1 case studies use data mining approaches for the elucidation of pathways through integrated analysis of omics data as well as for the validation of biomarkers for toxicity, which were discovered within the case studies.

3.2.4 Application in safety assessment

At the highest level, proofs-of-concept address the desire to show how the data and information derived from the tools and methods developed within SEURAT-1 can be used in specific safety assessment frameworks and scenarios. This could include the assessment of hazardous properties to screen a large set of substances to select groups with particular characteristics or to assess a specific substance. Again, the selection of chemicals is key for the formulation of case studies in the context of safety assessment and will strongly influence the case study design, simply due to the lack of validated animal-free testing methods addressing repeated dose systemic toxicity. From this perspective, the chemicals selected for the formulation of mode-of-action descriptions and the design of respective integrated testing strategies can be considered to be data-rich chemicals. Indeed, these chemicals were selected because of their well-known toxicological properties (see Section 3.1). The general problem in safety assessment scenarios, however, is the prediction of the toxicological effects of chemicals with a very limited amount of available information (data-poor) and to find appropriate data-rich candidate chemicals to be used as counterparts or indicators for assessing data-poor chemicals. Furthermore, the prediction must comprehensively evaluate all possible effects (MoA) for a chemical to ensure its safety. Two case studies, supported by the test systems to be developed within SEURAT-1 (see Section 3.2.3 above), are being undertaken as key contributions to the proof-of-concept of the SEURAT-1 approach at the application level: an *ab initio* approach, in which the point-of-departure (POD) for safety assessment will be mechanistically derived from *in silico* and *in vitro* tools, i.e., with the information combined in a rational manner; and a read-across approach, in which the toxicological properties (in effect the POD) will be predicted by "reading across" from analogue substances of known toxicological properties also using *in silico* and *in vitro* tools to support the prediction.

Ab initio case study

This case study will translate findings and data from the integration of relevant case studies from the systems level (see Section 3.2.3) for quantitative mechanistic safety assessment. The ambition is to demonstrate the feasibility of the SEURAT-1 approach to support the prediction of human health. Ultimately, the prediction goal is to determine a safe dose of an ingredient within a consumer use scenario, but this quantitative mechanistic safety assessment approach is the most ambitious scenario and represents the long-term perspective of putting tools of predictive toxicology into practice rather than demonstrating complete application cases within SEURAT-1. This is mainly due to the fact that it would require either complete knowledge about toxicological modes-of-action (including methods to evaluate the most sensitive pathways for non-selective chemicals that could affect multiple pathways), or a test system that covers all these potential pathways. Both are far beyond the scope of SEURAT-1, and the strategy of using prototype modes-of-action as a basis for case study design intends to produce "islands of knowledge", from which safe doses of chemicals can be derived only with respect to these particular modes-of-action. These shortfalls will, in the near-term, limit the application of this approach; i.e., it will not be adequate to assess a specific substance for regulatory purposes. Nevertheless, it explores how far the tools and methodology that will be available by the end of SEURAT-1 can be extended. Of course, the exercise is also designed to highlight major knowledge gaps and thereby provide a clear indication on where future research and development efforts in the field of safety assessment need to focus, bearing in mind that compiling a comprehensive list of all toxicity pathways as sub-sequences of toxicological modes-of-action is the goal of projects focusing on mapping the human toxome (Hartung and McBride, 2011).

The obvious way of starting this endeavor is to select a few of the SEURAT-1 standard reference compounds (see Section 3.1 and ToxBank, 2014) with known modes-of-action to evaluate the predictive toxicity systems (see Section 3.2.3) supported with additional methods developed within SEURAT-1. Further data-rich test chemicals can then be selected from existing databases or published surveys which classify chemicals according to target organs and suspected modes-of-action following repeated exposure to chemicals (e.g., Vinken et al., 2012).

Read-across case study

The read-across approach is probably best known for hazard and safety assessment of industrial chemicals under REACH, but is often employed in other sectors too (Patlewicz et al.,

2013). Traditional read-across relies on the concept that chemical similarity leads to similar chemical and physical properties, and thus to similar toxicity; i.e., the basis is primarily on chemical similarity, but with biological similarity as a supplementary consideration. Such predictions can be confounded due to the underlying complex mechanisms of toxicity. The credibility of the scientific argument to support read-across can be supported by other information including test data. Hence, information from *in vitro* molecular screening, omics assays and computational models can be used to improve the robustness of the read-across justification (OECD, 2014b). The SEURAT-1 case study on read-across aims to demonstrate that the robustness of read-across of repeated-dose oral toxicity from a source substance of known toxicology to target substance(s) can be improved using SEURAT-type evidence. In effect, in this new approach SEURAT-1 data is used as supporting evidence to improve the confidence in read-across based on similarity in chemical structure and is equivalent to adding an examination of biological similarity (as modelled by multiple short-term assays). Indeed, integrated chemical-biological read-across arguments appear to be more robust as compared to the traditional approaches focusing on chemical similarity alone (Low et al., 2013). Following this principle, an advanced two-step procedure that combines chemical and biological considerations as well as toxicological data as search criteria for the identification of the analogues (first step) with an approach to categorize the identified analogues (second step) was implemented (Wu et al., 2010). This framework was successfully applied in read-across case studies (Blackburn et al., 2011). The process did not always result in surrogate values for risk assessment and also highlighted cases where read-across could not be performed; thus, data gaps to be addressed in future work were identified. In addition, a decision tree for the application area of developmental and reproductive toxicity emerged from these exercises, which was then further developed and can now be used as a screening tool to identify chemicals with a potential for developmental and reproductive toxicity (Wu et al., 2013). Furthermore, as the chemical groupings generated in this process rely on chemical and biological principles, they may be useful starting points for the formulation of hypotheses regarding respective modes-of-action and the development of corresponding *in vitro* test methods (Wu et al., 2013). This procedure can act as a model and the framework adapted to other fields such as repeated dose systemic toxicity and, through the delivery of additional mode-of-action descriptions, may potentially advance the overall SEURAT1 approach.

It should be noted that the read-across approach is fundamentally different from the *ab initio* case study. The primary goal here is to show a real case of how non-animal methods can now be used to support decision-making in the regulatory context of safety assessment. Specifically, the aim of the read-across case study is to meet the standard for filling a REACH registration information requirement. Conceptually, this means that the complete set of results and findings of a 28-/90-day repeated-dose oral rat toxicity study on the "source" substance should be able to be "read across" to the target substance (which has not been studied in animals). The idea is that this prediction is essentially equivalent to the omitted standard animal study and it must be adequate for classification and risk assessment. The basic idea of the case study is to strengthen traditional read-across approaches with additional new *in vitro* data to reach the necessary REACH standard when the structural similarities would be insufficient without this supporting evidence. An important aspect of the case study is to decide how to determine the added value of the SEURAT-1 information. This could be by expert judgment of the case before and after the extra evidence is added to give a qualitative assessment of the robustness of the toxicity prediction. In some cases classical animal toxicity data on the target substance may exist against which the "read-across" predictions may be tested (with and without SEURAT-1 evidence). Hence, chemicals for the read-across case study were selected "top-down", independent of the criteria for the selection of SEURAT-1 standard reference compounds (see Section 3.1), but considering: (i) indications for an association with repeated dose toxicity, and (ii) relevance to industry. The selected chemicals fall into four read-across scenarios: (i) direct-acting toxicants (no metabolism); (ii) chemicals involving metabolism; (iii) chemicals with general low or no toxicity: and (iv) chemicals with high toxicity and specific modes-of-action. Further practical guidelines about how to perform read-across studies can be found elsewhere (ECHA, 2013a,b).

This case study is a realistic target within SEURAT-1 that will be of practical regulatory use within the short term and, hence, reduce animal testing. It will also be a practical outcome from SEURAT-1 that demonstrates a particular application of the approach of the "conceptual framework" (Section 4), thus giving reassurance that the broader application of the approach to *ab initio* prediction of toxicological properties will be feasible.

4 Conceptual framework for a rational integrated assessment strategy

Following earlier work in the field of regulatory toxicology (e.g., Gubbels-van Hal et al., 2005), the intention of the safety assessment case studies (see Section 3.2.4) is to bridge the gap between safety assessment decision-making and innovative predictive systems being developed within SEURAT-1. The aim is to harness the mechanistic outputs with an emphasis on how emerging science can best impact and reshape current safety assessment practice, focusing on the application level to identify pragmatic ways to use information derived from predictive tools to support safety assessment processes and decisions. While pulling together the various elements of SEURAT-1 in order to formulate the case studies and provide context for where the various strands of work being undertaken can be aligned and incorporated, a flexible "conceptual framework" has emerged that can be used as a basis for the rational combination of information derived from predictive tools to support a safety assessment process or decision that achieves a stated protection goal in the context of repeated dose systemic toxicity (Fig. 7). Similar to previous schemes that were developed to implement *in vitro* and *in silico* approaches into regulatory safety assessment (e.g., Blaauboer et al., 1999), this framework is intended to set

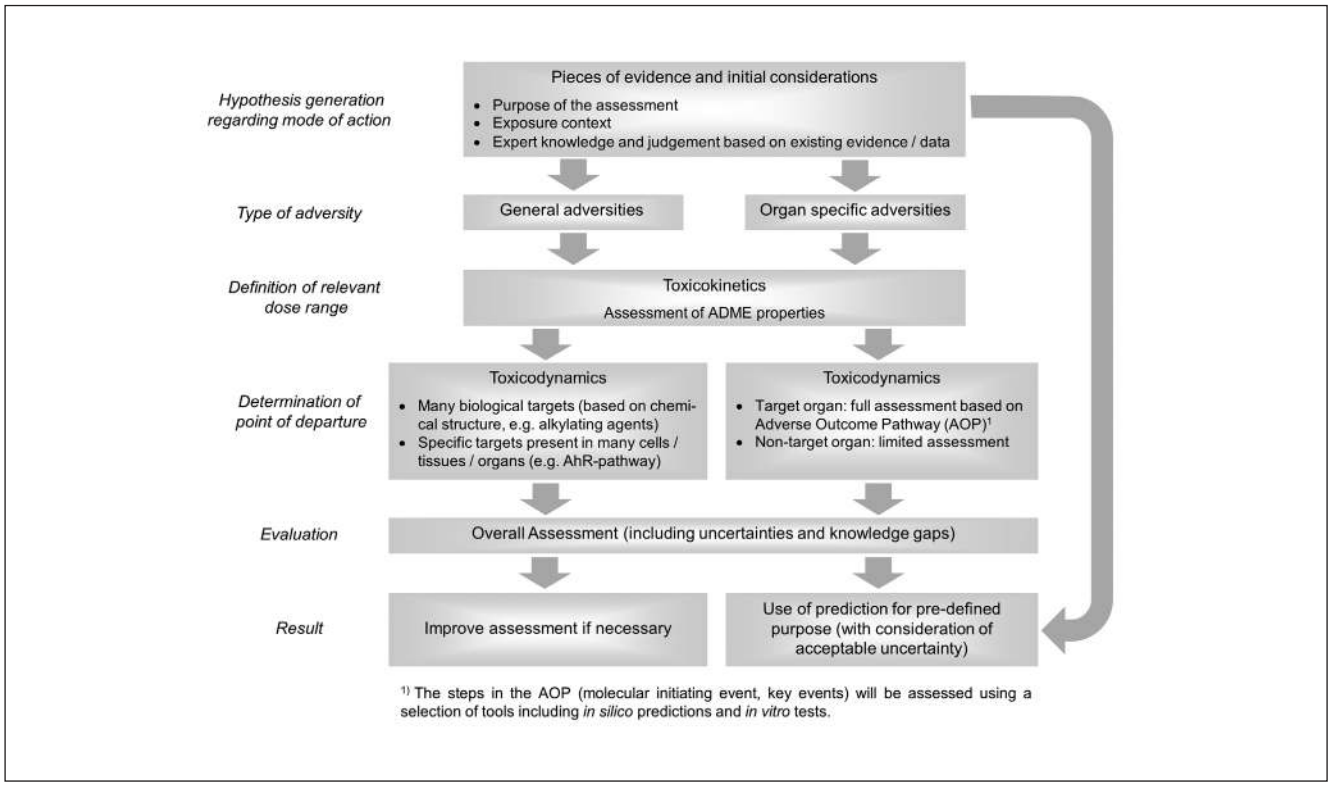

Fig. 7: The SEURAT-1 conceptual framework as a structure for safety assessors in devising a fit-for-purpose bespoke integrated assessment strategy for a particular case

out a structure to guide assessors in devising a fit-for-purpose bespoke integrated assessment and testing approach (IATA) for the particular circumstances and case. The overall outcome is anticipated to be robust as it is not based on single pieces of evidence, but rather on a weight of evidence combined in a biologically rational manner.

Before beginning the assessment, the degree of confidence needed for the prediction is decided. There may be an exposure context that enables the acceptance of a moderate or low degree of confidence in the prediction, possibly due to well-controlled and low human exposure resulting from the particular use of the substance. Then the idea is to begin with examining existing knowledge from different lines of evidence. In particular, it is important to consider if the compound is a "general chemical" expected to be unselective in interacting with biological targets or a drug/pesticide designed to be selectively biologically active. Other evidence could include toxicological studies on the substance or "read-across" from chemical or biological analogues, QSARs and structural alerts and expert judgment. Following this step there are two parallel lines of consideration: (i) "general" adverse effects not associated with a particular organ and (ii) organ-based adverse effects. Toxicokinetics/toxicodynamics must be considered in both lines of investigation. Effects on organs can be assessed by one or more AOPs with the molecular initiating event (MIE) and intermediate events (IEs) incorporating existing knowledge and with new data as a combination of *in vitro* assays (omics data, HPT data, etc.) and *in silico* predictions in a battery of tools.

The aim within SEURAT-1 is to set the framework and show how mechanistic safety assessment could proceed through specific case studies and make a start in filling the details of the strategy and identify knowledge gaps and areas for further development. The need to develop guidance on the use of physiologically-based models in chemical risk assessment that includes toxicokinetic and toxicodynamic models incorporating *in vivo*, *in vitro* and *in silico* data was highlighted recently in a report published by the European Food Safety Authority (EFSA, 2014). The SEURAT-1 standard reference compounds identified provide an initial basis for work to proceed as they comprise known human relevant toxicants that we can benchmark the framework against. Nevertheless, we need to be able to generalize the approach to more generic chemistries, and the flexible conceptual framework may provide the basis for doing so.

The SEURAT-1 toxicological mode-of-action approach incorporates the overall principles of this framework, taking into account the particular chemical and its properties, as well as the (regulatory) purpose to deliver a fit-for-purpose prediction. SEURAT-1 focuses, however, on chemicals interacting selectively with a limited number of biological targets, for which the assessor needs to identify the respective modes-of-action (i.e., following the sequence on the right-hand side in Fig. 7). The next step is to determine the ADME-properties for the chemical of interest to predict the internal dose at the target organ. A battery of *in silico* and *in vitro* tools combined in an integrated testing strategy will then validate the suspected mode-of-action and, based on methods of reversed dosimetry (Yoon et al.,

2012), convert the *in vitro* dose response to *in vivo* values and respective environmental concentrations (exposure) as a point-of-departure (POD) for safety assessment.

For non-selective chemicals that affect multiple biological pathways, toxicity profiles need to be developed based on the identification of structural alerts and read-across studies. Some general, sensitive and organ-unspecific modes-of-action (which are currently unknown) need to be tested to derive a POD for safety assessment. High-throughput screening tools distinguishing between selective and non-selective chemicals using a high number of assays are being developed within the U.S. Tox21 research program – the pragmatic strategy to derive the POD would be to use the most sensitive assay as a starting point (Thomas et al., 2013). The overall outcome of an assessment based on this framework would be robust as it is not based on single pieces of evidence; nevertheless the type and degree of uncertainty in the predictions would need to be validated as "fit-for-purpose."

Other schemes for evaluating chemicals using the new tools for toxicity testing are also under development. A data-driven framework based on data from a broad range of high-throughput *in vitro* assays from the Tox21 program (see Section 5 below), which invokes successive tiers of testing with margins-of-exposure as the primary metric, was proposed recently (Thomas et al., 2013). This framework provides a risk-based approach to the evaluation of chemical safety, drawing broadly from previous experience and incorporating technological advances to increase efficiency. Even though emerging from a high-throughput screening approach, it is fully complementary with the above-described conceptual framework of the SEURAT-1 approach.

The discussion about these concepts and frameworks highlights the fact that SEURAT-1 is operating in a very dynamic field of research, and a number of related research projects in different parts of the world are active in parallel, advancing the field rapidly. This is briefly outlined in the following section.

5 SEURAT-1 in the international context

5.1 Related international research activities

Related, ongoing research activities in Europe are mainly those under the umbrella of the European Commission's RTD Framework Programmes and the Innovative Medicines Initiative Joint Undertaking (IMI JU), a pan-European public-private partnership between the European Commission and the European Federation of Pharmaceutical Industries and Associations (EFPIA). Based on common interests, concrete interactions are planned with *HeCaToS* (Hepatic and Cardiac Toxicity Systems Modelling, European Commission's 7th Framework Programme; http://www.hecatos.eu/) and MIP-DILI (Mechanism-based integrated systems for predicting drug-induced liver injury, IMI; http://www.mip-dili.eu/). The overall goal of *HeCaToS* is to develop an integrated framework for modeling toxic perturbations in the liver and heart across multiple scales. Advances in computational chemistry and systems toxicology will be combined for this purpose and case studies based on biopsies from patients suffering from liver injuries or cardiomyopathies due to adverse drug effects will be developed. Particular attention will focus on adverse outcome pathways related to mitochondrial deregulations and immunological dysfunctions. The aim of *MIP-DILI* is to develop improved tools for liver toxicity testing in the early stages of the drug development process. This will require a deepened understanding of the science behind drug-induced liver injury and the application of this knowledge to overcome the many drawbacks of the tests currently used. The relationships of these two initiatives with SEURAT-1 are obvious: *HeCaToS* and *MIP-DILI* can build on the case study approach of SEURAT-1 and develop it further, taking the existing adverse outcome pathway descriptions and the respective test systems into account to assess their suitability for the purposes of: (i) assessing specific diseases (*HeCaToS*) and (ii) improved toxicity testing in drug development (*MIP-DILI*).

SEURAT-1 is not only serving other projects, but also benefits from others: Of utmost relevance are the Tox21 and ToxCast research programs in the United States (US). In 2005, the US government launched Tox21 (http://www.epa.gov/ncct/Tox21/), an initiative to use *in vitro* high-throughput screening (HTS) to identify what proteins, pathways, and cellular processes chemicals interact with and at what concentration they interact. Currently, the Tox21 effort has screened ~10,000 chemicals across nearly 50 *in vitro* HTS assays. Tox21 is a consortium that pools the resources and expertise of US Environmental Protection Agency, National Institutes of Environmental Health Sciences/National Toxicology Program, National Institute of Health/National Center for Advancing Translational Sciences, and the Food and Drug Administration. In parallel with Tox21, the US Environmental Protection Agency launched the ToxCast program (http://www.epa.gov/ncct/toxcast/). ToxCast also utilizes *in vitro* HTS of chemicals, but has screened fewer chemicals (~1,800) over an expanded set of 700 biochemical and cell-based *in vitro* assay endpoints (Kavlock et al., 2012). Both the Tox21 and ToxCast programs aim to develop high-throughput decision support tools for prioritizing the thousands of chemicals that need toxicity testing. The experimental work is being accompanied by the development of models that can be used to more effectively predict how chemicals will affect biological responses. The different methods should be effectively combined as a toolbox of innovative chemical testing methods. Finally, the challenge of being able to provide the data generated from the innovative chemical testing methods to risk assessors for making decisions about protecting human health and environment is addressed (see above and Thomas et al., 2013).

The common goal of Tox21, ToxCast and SEURAT-1 is to implement state-of-the art technologies emerging from recent scientific advances into safety assessment procedures. All initiatives focus on a combination of *in vitro* methods and *in silico* tools as components of modern, innovative testing methods. However, the approaches of each initiative are fundamentally different: Tox21 and ToxCast, not restricted to any field within the arena of toxicology, follows a screening strategy studying a high number of chemicals in a very diverse set of available test systems. In contrast, SEURAT-1 focuses on repeated dose systemic toxicity and has selected a limited number of well-studied chemicals for the development of mode-of-action-driven test

batteries using only human cells including reporter cell lines derived from induced pluripotent stem cells. Another common interest of all initiatives is the extrapolation of *in vitro* effects to the *in vivo* context. In summary, the research programs are highly complementary: Knowledge about toxicity pathways from Tox21 and ToxCast inspires the construction of mode-of-action descriptions in SEURAT-1, and new assays developed within SEURAT-1 may find their way into Tox21 and ToxCast. Strategies for implementing these tools into safety assessment approaches were discussed above (see Section 4) and all initiatives are well aware that combining their efforts is mutually beneficial to all the parties involved. Exchange activities were commenced during an expert meeting held at the Joint Research Centre in Ispra (Italy) in June 2013.

5.2 On track? The roadmap and SEURAT-1

Developing new strategies for toxicity testing inevitably requires alignment of different areas of science such as chemistry, systems biology, computer sciences and toxicology. The SEURAT-1 research initiative assembles the available resources in a mode-of-action driven case study approach. This was inspired by considerations from international experts in this regard who recommended an initial focus on adverse effects in the liver (Kimber at al., 2011). It is not expected that SEURAT-1 will deliver ultimate solutions, but the consortium has taken up important components from the roadmap for the development of non-animal test methods for the replacement of *in vivo* repeated dose toxicity testing (Tab. 1; Basketter et al., 2012) and, hence, should be well prepared to proceed on the next level.

6 Concluding remarks: Beyond SEURAT-1

The successful completion of SEURAT-1 will lay the foundation for follow-on efforts that will broaden the toxicological, chemical and regulatory domains addressed. The intention of SEURAT-1 is to provide a blueprint for future implementation of knowledge-based test systems into new safety assessment approaches. New approach methods and biological thinking can be used in two ways: (i) for hazard (and risk) assessment adapted into the current regulatory "paradigm", and/or (ii) to incorporate approaches for regulatory science into the new

Tab. 1: Comparison of recommendations from the roadmap for the development of non-animal test methods for the replacement of *in vivo* repeated dose toxicity testing (Basketter et al., 2012) and approaches within SEURAT-1

Recommendations (Basketter et al., 2012)	SEURAT-1 approaches
Joint task force: Toxicity database to gather all current data on a wide variety of compounds	Generation of a curated database for cosmetics (COSMOS); Collection of SEURAT-1 data in a data warehouse (ToxBank)
Tiered testing systems and decision trees (ITS)	Establishment of integrated testing strategies for toxicity prediction based on selected modes-of-action; Development of safety assessment strategies based on integrated data from non-animal methods
Understand signalling pathways: (i) As long as not all pathways of toxicity are known, complex systems addressing more apical endpoints should be used too. (ii) Use modern tools (e.g., RNAi) for pathway elucidation.	(i) SEURAT-1 will establish high-throughput assays for well-known pathways of toxicity (SCR&Tox) as well as complex bioreactors with diverse compositions of cell-types (allowing cell-cell interactions; HeMiBio). (ii) A battery of omics technologies are being used for pathway identification in DETECTIVE and NOTOX.
Considerations for the development and validation of *in vitro* systems: (i) (Functional) limitations of *in vitro* systems (ii) In the near future, focus on 3D-systems and co-cultures. (iii) Selection of appropriate endpoints for each test and test system, or "-omics" readouts for many endpoints (iv) Measure free concentrations in *in vitro* systems as a prerequisite for quantitative *in vitro* to *in vivo* extrapolation. (v) Compound selection must consider positive and negative controls for the evaluation of the test systems. Consider applicability domain, different chemical classes and modes-of-action. Create a list of reference compounds with available information on mechanisms of toxicity and potency	(i) Targeted development of test systems (according to the selected modes-of-action), full characterisation of biological models used in the test systems (ii) Emphasis on organotypic cell cultures in NOTOX and HeMiBio (iii) Development of test systems according to pre-defined prediction goals. Endpoints: Functional readouts and "-omics" (SCR&Tox, HeMiBio, DETECTIVE, NOTOX) (iv) Activities within COSMOS and in the cross-cluster biokinetics working group (v) Selection of reference compounds at the beginning of the project by a cross-cluster working group led by ToxBank. Information on mechanisms of toxicity and potency collected and now publicly available as a wiki (http://wiki.toxbank.net/wiki/)
Considerations for the development and validation of *in silico* models: Data quality	Activities in the cross-cluster working group on integrated data analysis led by ToxBank. Definition of standards for quality control / quality assurance for data compilation in the ToxBank data warehouse (annotation of respective measures as part of the data upload procedure).

"paradigm." Providing such a blueprint is challenging, but implementing a new strategy into regulation with the intention of changing the traditional safety assessment paradigm is probably even more challenging. Incorporating scientific advancements into current regulation is never an easy task. However, a paradigm shift in the redefinition of adversity, moving away from descriptive toxicology towards mechanism-based predictive toxicology, will fail if the regulatory bodies do not recognize the concepts emerging from these scientific efforts. It would simply remain theory without consequences and societal impact. There is no doubt that the implementation of a new safety assessment paradigm is a joint undertaking between scientists and regulators. Therefore, communication and the bridging of gaps between these two communities are of utmost importance. Scientists should listen to the requirements of the regulators, and regulators should pay attention to opportunities emerging from progressing science. In essence, future research consortia in this field, whether it is a SEURAT-1 follow-up or another program, should effectively combine these two communities. This is in the interest of a sustainable, successful implementation of a new safety assessment paradigm. We are just at the beginning, but are not absolute beginners...

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Conflict of interest

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