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Review

Safer chemicals using less animals: kick-off of the European ONTOX project

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ABSTRACT

The 3Rs concept, calling for replacement, reduction and refinement of animal experimentation, is receiving increasing attention around the world, and has found its way to legislation, in particular in the European Union. This is aligned by continuing high-level efforts of the European Commission to support development and implementation of 3Rs methods. In this respect, the European project called "ONTOX: ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment" was recently initiated with the goal to provide a functional and sustainable solution for advancing human risk assessment of chemicals without the use of animals in line with the principles of 21st century toxicity testing and next generation risk assessment. ONTOX will deliver a generic strategy to create new approach methodologies (NAMs) in order to predict systemic repeated dose toxicity effects that, upon combination with tailored exposure assessment, will enable human risk assessment. For proof-of-concept purposes, focus is put on NAMs addressing adversities in the liver, kidneys and developing brain induced by a variety of chemicals. The NAMs each consist of a computational system based on artificial intelligence and are fed by biological, toxicological, chemical and kinetic data. Data are consecutively integrated in physiological maps, quantitative adverse outcome pathway networks and ontology frameworks. Supported by artificial intelligence, data gaps are identified and are filled by

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targeted *in vitro* and *in silico* testing. ONTOX is anticipated to have a deep and long-lasting impact at many levels, in particular by consolidating Europe's world-leading position regarding the development, exploitation, regulation and application of animal-free methods for human risk assessment of chemicals.

1. Introduction

European citizens care about safety of chemicals. According to a Eurobarometer survey performed in 2017, 65 % of the Europeans is concerned about being exposed to hazardous chemicals and as much as 50 % feels that the current level of regulations in Europe should be increased (EU, 2017). At present, risk assessment of chemicals still heavily relies on animal testing. This is not only rather poorly predictive of human safety towards chemicals, but equally poses a serious ethical problem. In this respect, 90 % of the European population underscores the importance of establishing high animal welfare standards that are recognized worldwide, while 89 % states that Europe should increase awareness of the importance of animal welfare internationally (EU, 2016). Nevertheless, considerable progress has been made in the past few decades in Europe regarding the development of animal-free approaches for risk assessment of chemicals, thereby fully embracing the 3Rs concept, which calls for replacement, reduction and refinement of animal experimentation (EURL ECVAM, 2020). For most acute and local toxicity endpoints, such as skin and eye irritation, a variety of non-animal methods is available. However, for chronic and systemic toxicity endpoints, such as organ-specific repeated dose toxicity, animal-free methods are unfortunately still lacking (https://ec.europa.eu/jrc/en/eurl/ecvam), which poses a ubiquitous scientific problem (Mahony et al., 2020). The project called ONTOX (*i.e.* "ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment") intends to tackle these ethical and scientific problems (https://ontox-project.eu/). ONTOX was conceived in the context of the project call "advancing the safety assessment of chemicals without the use of animal testing" published between July 2019 and June 2020 as part of the European Horizon2020 research and innovation framework program. The ONTOX consortium consists of 18 partners with a background in academia, industry, consultancy or health institution based in 8 European countries and the USA (Table 1). The ONTOX consortium is supported by a stakeholder advisory board composed of 7 individuals from diverse chemical industries, regulatory agencies and universities, all that have expertise relevant to one or more aspects of the project. In addition to providing general recommendations on a frequent basis, the stakeholder advisory board advises the ONTOX consortium on specific actions, such as the selection of chemicals, *in vitro* assays and *in silico* tools, and method validation. ONTOX kicked off in May 2021 for a 5-years period with a budget of €17.2 million (https://cordis.europa.eu/project/id/963845).

2. Goal and objectives

The overarching goal of ONTOX, which itself encompasses 8 specific objectives (Table 2), is to deliver a general strategy to create innovative new approach methodologies (NAMs) to predict systemic repeated dose toxicity effects of chemicals that, upon combination with customized exposure assessment, will enable human risk assessment. A NAM generally refers to any non-animal technology, methodology, approach or combination thereof that can be used to provide information on chemical hazard and human risk assessment (Dent et al., 2018). In the specific context of ONTOX, a NAM denotes an ontology-driven and artificial intelligence-based strategy linked with a battery of *in vitro* assays and *in silico* tools for hazard prediction to be combined with tailored exposure assessment for the purpose of human risk assessment. For proof-of-concept purposes, focus is put on 3 organs frequently involved in systemic repeated dose toxicity, each with 2 specific types of adversity, namely the liver (*i.e.* steatosis and cholestasis), the kidneys (*i.e.* tubular necrosis and crystallopathy) and the developing brain (*i.e.* neural tube closure and cognitive function defects). This will result in 6 NAMs in total. Chemicals from different application domains are considered, including from, but not limited to, the pharmaceutical, cosmetics, food and biocide sectors. The 6 NAMs will each consist of a computational system based on advanced artificial intelligence technology, and will be primarily fed by available biological, toxicological, chemical and kinetic data. These data are consecutively integrated in physiological maps, quantitative adverse outcome pathway (qAOP) networks (https://aopwiki.org/) and ontology frameworks. Supported by an artificial intelligence system, data gaps are identified and are filled by targeted *in vitro* and *in silico* testing (Piersma et al., 2019). By doing so, ONTOX fully implements the 21st century toxicity testing vision, which intends to move away from reliance on animal testing to *in vitro* assays and *in silico* tools mainly designed to detect toxicity pathway perturbations (NRC, 2007). Furthermore, ONTOX aligns with the principles of next generation risk assessment, being a safety evaluation approach centered around a hypothesis on a biological mechanism and driven by exposure considerations, which integrates NAMs to ensure

Table 1
ONTOX consortium.

Partner	Country
Vrije Universiteit Brussel (coordinator)	Belgium
Université de Liège	Belgium
Leibniz-Institut für umweltmedizinische Forschung an der Heinrich-Heine-Universität Düsseldorf	Germany
Istituto di Ricerche Farmacologiche Mario Negri IRCCS	Italy
Universidad de Valencia	Spain
Hogeschool Utrecht-University of Applied Sciences Utrecht	The Netherlands
Universiteit Maastricht	The Netherlands
Universiteit Utrecht	The Netherlands
Johns Hopkins Bloomberg School of Public Health	USA
Center of Experimental Medicine-Slovak Academy of Sciences	Slovakia
Altertox	Belgium
3Rs Management and Consulting ApS	Denmark
EsqLABS GmbH	Germany
Molecular Networks GmbH	Germany
Norwegian Institute of Public Health	Norway
ProtoQSAR SL	Spain
Bayer AG	Germany
ToxTrack Inc.	USA

Table 2
ONTOX objectives.

To generate ontologies for systemic organ-specific repeated dose toxicity testing by collecting data, mainly already available information, from the biological, toxicological, chemical and kinetic domains.
To develop, optimize and apply artificial intelligence for data collection, integration and prediction of chemical hazard.
To implement the developed NAMs in daily risk assessment practice in different chemical sectors.
To set up batteries of *in vitro* assays and *in silico* tools to fill data gaps and assist the artificial intelligence system to predict systemic repeated dose toxicity effects of chemicals in the liver, kidneys and developing brain.
To support transparency, interpretability and sustainability by re-using quality-assessed data and generate novel data that are readily findable, accessible, interoperable and re-usable.
To collaborate with industry and regulatory agencies in order to generate impact as well as to secure end-user acceptance and regulatory confidence.
To trigger innovation and competitiveness by identifying commercialization opportunities.
To train end-users, mainly in industrial settings, and regulators to apply the developed NAMs.

human risk assessment of chemicals (Dent et al., 2018).

3. Pillars

3.1. Ontology

The first (conceptual) pillar of ONTOX is the ontology, which is defined as a framework to qualitatively and quantitatively integrate and structure relevant data from various sources (Desprez et al., 2019). The ontology is composed of 4 critical features for hazard prediction related to the biological domain (*i.e.* molecular and cellular mechanisms), toxicological domain (*i.e.* clinical and epidemiological data), chemical domain (*i.e.* structural and physico-chemical properties) and kinetic domain (*i.e.* absorption, distribution, metabolism and excretion properties, and external and internal exposure). As such, 3 tools are used in ONTOX for well-rationalized and manageable data integration in a consecutive way, including physiological maps, qAOP networks and ontologies (Fig. 1).

The physiological maps are generated by combining all pertinent information based on a broad analysis of available biological data. The widest scope of existing databases and scientific literature are mined to map organ function and homeostatic processes at the molecular and cellular level. Existing mathematical models of organ homeostasis are included as quantitative information on regulation of organ physiology.

The qAOP networks are built, or, if already (partly) available (https://aopwiki.org/), further optimized based on the physiological maps. The qAOP networks are generated by additionally considering toxicological and kinetic data, the latter supporting the quantitative aspect together with dose-response relationships. This strategy guarantees that known essential molecular and cellular information of organ function is covered. This has been the main issue with classical approaches starting from available individual *in vitro* assays or combinations thereof attempting to cover as many mechanisms of toxicity as possible without the ability of being certain to what extent the (patho)physiological domain is adequately covered. In this approach, results of individual assays are difficult to interpret, which hampers implementation of alternative approaches. The ONTOX strategy offers the opportunity to select from the physiological map the specific part that is especially responsive to chemicals, leading to adverse outcomes when perturbing homeostasis, which can be defined as the qAOP network.

While qAOP networks are chemical-agnostic (Knapen et al., 2018), the ontologies additionally take physico-chemical properties of the chemicals that trigger the selected specific systemic repeated dose toxicity effects into consideration. The ontology therefore combines qualitative and quantitative biological, toxicological, chemical and kinetic data in a single structured framework. The well-structured and pragmatically developed ontologies streamline data input for the artificial intelligence system. In addition, the ontologies, and in particular their qAOP network core, allow selection of a combination of *in vitro* assays and *in silico* tools that cover the relevant mechanistic spectrum. Against this background, the ontology framework and the associated testing strategy are by definition comprehensive, or if not, data gaps and missing methods are at least identified. This can then lead to dedicated filling of existing gaps by targeted *in vitro* and *in silico* experimentation.

3.2. Artificial intelligence

The second (methodological) pillar of ONTOX is artificial intelligence, thus the simulation of human intelligence processes by computational systems. Artificial intelligence is applied at 2 levels, namely (*i*) to retrieve relevant heterogeneous big data from different sources to feed in the ontology, and (*ii*) to predict chemical hazard based on machine learning. ONTOX produces a cutting-edge artificial system to predict hazard qualitatively (*i.e.* hazard identification) and quantitatively (*i.e.* hazard characterization). The real strength of artificial intelligence lies in its ability to automatically extract relevant features from its environment that are suitable for solving a specific task. Training the algorithms on what is relevant information on the basis of associated features of interest related to hazard helps to integrate them for making predictions (Hartung, 2016). In some instances, this information suffices to come to a weight-of-evidence conclusion on the chemical of interest. Going a step further, for data-poor or untested chemicals, a read-across structure-activity relationship (RASAR) approach is followed. The RASAR concept originally used structural similarity combined with supervised learning (Luechtefeld et al., 2018). Chemical similarity is hereby done by generating a binary fingerprint for each chemical and using Jaccard distance on fingerprints (Luechtefeld and Hartung, 2017). Supervised learning methods then provide a statistical model of the input variables (*i.e.* labels) and from chemical similarity. Due to automation, the RASAR approach can be applied to large datasets and hence validated according to common statistical methods, such as cross-validation. The power of the RASAR approach lies in using the information available on all similar chemicals for prediction in addition to anything known about the chemical itself. The RASAR approach is expanded in ONTOX in order to take also physico-chemical and toxicological similarity into consideration. The ontologies developed in ONTOX are used to organize the available information of the chemicals in training labels. For each chemical, the property and the degree of similarity of the most similar chemical with or without this property is used to train the machine learning algorithm. This is combining the advantages of quantitative structure-activity relationships and the robustness of local read-across between similar chemicals. At the same time, it makes comprehensive use of information available on similar chemicals, thus exploiting transfer learning and network effects.

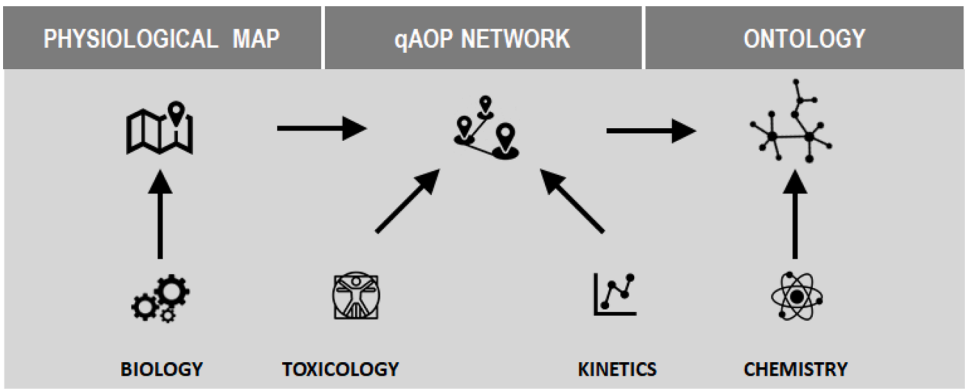

Fig. 1. Tools to integrate data in ONTOX.

4. Organization

The specific objectives of ONTOX are addressed in 14 interactive work packages (WP). The actual research is performed simultaneously and in parallel for the cases studies of hepatotoxicity, nephrotoxicity and developmental neurotoxicity (WP7−9), each for which 2 NAMs for the 2 selected specific types of adversity are developed (WP1−5), validated and implemented (WP6). The research core of ONTOX is framed by a number of general and specific activities aimed at coordination, data management and quality control, dissemination, exploitation and communication (WP10−14) (Fig. 2).

WP1 gathers biological information as input for the ontologies. In first instance, and as a unique feature, mechanistic maps of the physiological functioning of the liver, the kidneys and the developing brain are generated. This helps to guide subsequent development of the 6 qAOP networks for the specific systemic repeated dose toxicity effects, in particular by serving as a compass for covering the entire spectrum of mechanisms pertinent to the (patho)physiology concerned. WP1 equally delivers molecular and cellular data critical for the establishment of the 6 qAOPs networks in WP2.

WP2 gathers toxicological information as input for the ontologies. While WP1 focuses on molecular and cellular information, WP2 specifically aims at data at the tissue, organism and population (*i.e.* clinical and epidemiological) level for qAOP network development purposes. All data, both from WP1 and WP2, are captured in 6 AOP networks. The AOP networks are quantified by consideration of kinetics and dose-response relationships, yielding 6 qAOP networks.

WP3 gathers chemical information as input for the ontologies. Critical physico-chemical parameters of the chemicals that induce the specific systemic repeated dose toxicity effects are identified. Quantitative structure-activity relationship and read-across approaches are set up and applied as the basis for generating batteries of *in silico* tools that pick up molecular initiating events and key events for the specific systemic repeated dose toxicity effects.

WP4 gathers kinetic information as input for the ontologies. WP4 develops *in silico* models of *in vivo* distribution kinetics for characterizing the absorption, distribution, metabolism and excretion properties to predict tissue exposure for the selected chemicals and their systemic repeated dose toxicity effects. WP4 also generates *in silico* models of *in vitro* distribution kinetics for estimating cell-associated *in vitro* effect concentrations for quantitative *in vitro* to *in vivo* extrapolation in absorption, distribution, metabolism and excretion, and qAOP network development. Furthermore, WP4 performs quantitative *in vitro* to *in vivo* extrapolation of selected chemicals and the 6 ontologies for risk assessment purposes.

WP5 is among the most critical parts of ONTOX, as it deals with data integration as well as with the establishment of the 6 ontologies and artificial intelligence system. WP5 establishes a big data platform and performs data gap filling for integration of data collected in WP1−4. Several sources for data collection are considered, including, but not limited to, curated toxicological legacy information from European and other projects, scientific literature and dedicated public databases, and safety evaluation reports from advisory committees and regulatory agencies. As a key task, WP5 develops the 6 quantitative ontologies for the selected systemic repeated dose toxicity effects. Most importantly, machine learning, including deep learning, approaches are set up and applied to hazard prediction. This is achieved by relying on the RASAR approach.

WP6 performs custom-fit external exposure assessments with consideration of the threshold of toxicological concern, and reliance on epidemiological data and biomonitoring data. The outcome of the exposure assessments is evaluated together with the hazard prediction resulting from WP5 to culminate into actual human risk assessment. WP6 equally focuses on end-user acceptance and implementation of the 6 NAMs. In order to do so, WP6 maintains intensive interaction with industrial and regulatory stakeholders.

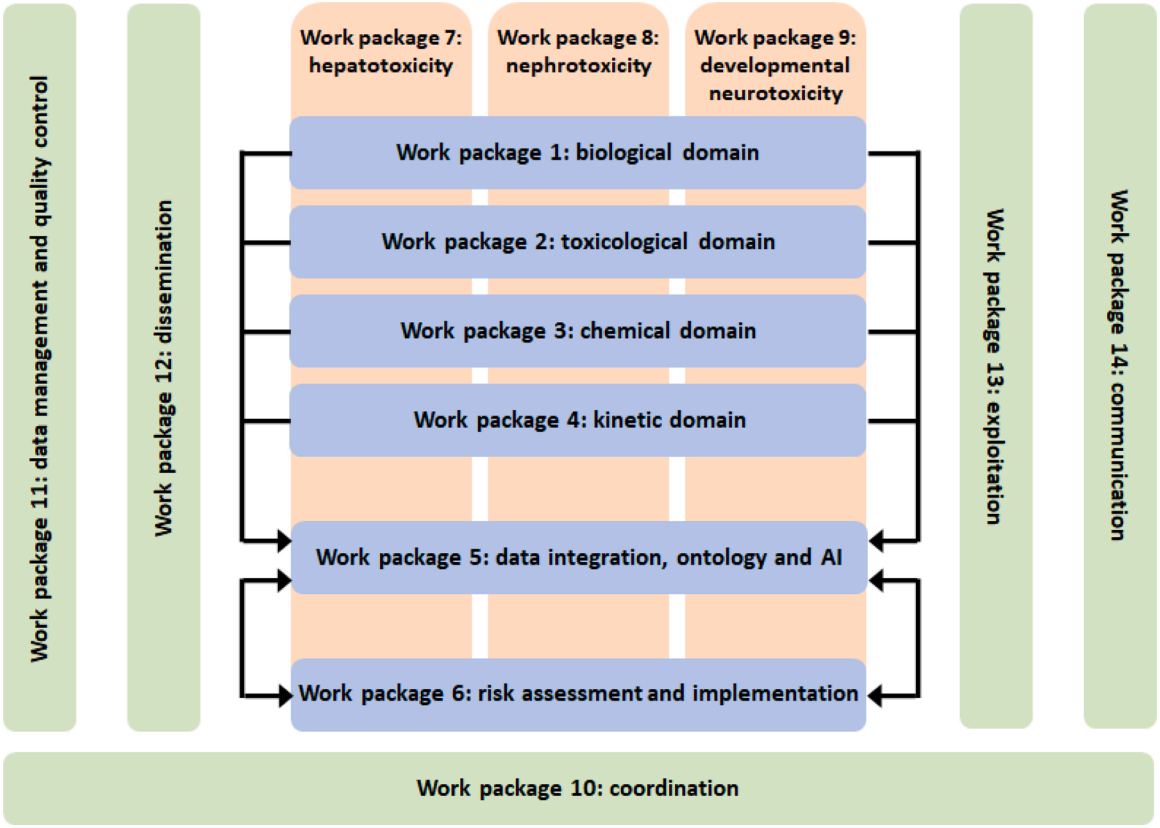

Fig. 2. Organization of ONTOX.

WP7–9 run in parallel through WP1–6 with focus on hepatotoxicity, nephrotoxicity and developmental neurotoxicity, respectively. WP7–9 set up and apply existing *in vitro* assays to detect specific molecular initiating events and key events in the qAOP networks resulting from WP2. These *in vitro* assays are evaluated for parameters, such as reproducibility and chemical applicability domain, by selecting and testing specific chemicals. These 6 *in vitro* test batteries are scientifically validated by appraising their coverage of the spectrum of mechanisms relevant for the specific systemic repeated dose toxicity effects. Furthermore, WP7–9 test chemicals identified by the artificial intelligence approach for which specific data are lacking.

WP10 is devoted to coordination, thereby overseeing data management, dissemination, exploitation and communication activities. WP11 fully focuses on data management and quality control as well as on sustainability. Furthermore, WP11 establishes the infrastructure for data storage, and coordinates data sharing, archiving and preservation according to the FAIR (*i.e.* findable, accessible, interoperable, re-usable) principles. WP11 also watches over compliance with Good *In Vitro* Method Practices/Good Cell Culture Practice guidelines (OECD, 2018) as well as with Quantitative structure-activity relationship Model Reporting Format (QMRF) and Quantitative structure-activity relationship Prediction Reporting Format (QPRF) guidelines (ECHA, 2008). WP12–14 aim at dissemination, exploitation and communication, respectively, in order to provide the means to end-users, in particular industry and regulators, to become well acquainted with the newly developed NAMs in daily risk assessment practice.

5. Workflow

The overarching goal of ONTOX is achieved by following an agile workflow that consists of 3 tiers, namely (*i*) data collection from the biological, toxicological, chemical and kinetic domains (WP1–4), (*ii*) data integration, including creating the ontologies and artificial intelligence system (WP5), and targeted *in vitro* and *in silico* testing (WP3 and WP7–9), and (*iii*) risk assessment, including exposure assessment, and implementation to leverage end-user acceptance and regulatory confidence (WP6). Tier 2 implies a series of repetitive steps with continuous feedback processes, which allows to concomitantly optimize and validate the ontology-driven and artificial intelligence-based NAMs. First rounds of chemical testing are performed with chemicals mainly from the pharmaceutical, cosmetics, food and biocide sectors to evaluate the reproducibility, chemical applicability domain and (patho)physiological coverage of the methods. The same chemicals as well as additional ones that are identified by the artificial intelligence system are considered in subsequent rounds of testing for data gap filling, mainly dynamic and kinetic information, and overall validation. This entire workflow is followed individually yet simultaneously for the 6 specific systemic repeated dose toxicity effects (*i.e.* NAMs) (Fig. 3).

The specific 11 steps in the workflow are (*i*) collection of biological information as ontology input, including generation of physiological maps (WP1), (*ii*) collection of toxicological information as ontology input, including generation of qAOP networks (WP2), (*iii*) collection of chemical information as ontology input (WP3), (*iv*) collection of kinetic information as ontology input (WP4), (*v*) establishment of the ontology that triggers the set-up of the *in vitro* and *in silico* test battery, and that serves as input for the artificial intelligence system (WP5), (*vi*) establishment and application of the *in vitro* and *in silico* test battery for further optimization of the ontology and for data gap filling identified by the artificial intelligence system (WP7–9), (*vii*) establishment and application of the artificial intelligence system that guides targeted *in vitro* and *in silico* testing, identifies any missing elements in the ontology and serves as the basis for hazard prediction (WP5), (*viii*) hazard prediction, including hazard identification and hazard characterization (WP5), (*ix*) exposure assessment (WP6), (*x*) risk assessment (WP6), and (*xi*) implementation and maximizing end-user acceptance and regulatory confidence through dialogue with industrial and regulatory stakeholders (WP6).

6. Ambition

The ambition of ONTOX is to strengthen Europe's world-leading position regarding the development, exploitation, regulation and application of NAMs for human risk assessment of chemicals by empowering (*i*) scientific excellence (*i.e.* to move beyond the state-of-the-art by delivering an unprecedented set of NAMs), (*ii*) industrial innovation (*i.e.* to generate new value creation opportunities and market potential), (*iii*) regulatory pioneering (*i.e.* to introduce a paradigm shift in the validation and use of non-animal methods), and (*iv*) societal prioritization (*i.e.* to increase human chemical safety while fully replacing the use of animals).

ONTOX will deliver a set of NAMs able to perform chemical hazard prediction and human risk assessment. These NAMs optimally combine the most relevant cutting-edge developments in research and technology in order to allow to move beyond the current state-of-the-art. The ontology cornerstone foresees integration of all critical aspects of repeated dose toxicity starting from basic biology. The artificial intelligence cornerstone facilitates data collection, yet first and foremost forms the methodological basis for highly innovative computational systems to predict chemical hazard. ONTOX focuses on specific systemic toxicity repeated dose toxicity in the liver, the kidneys and the developing brain. Chemicals from different areas are considered, including from the pharmaceutical, cosmetics, food and biocide sectors. Nevertheless, the

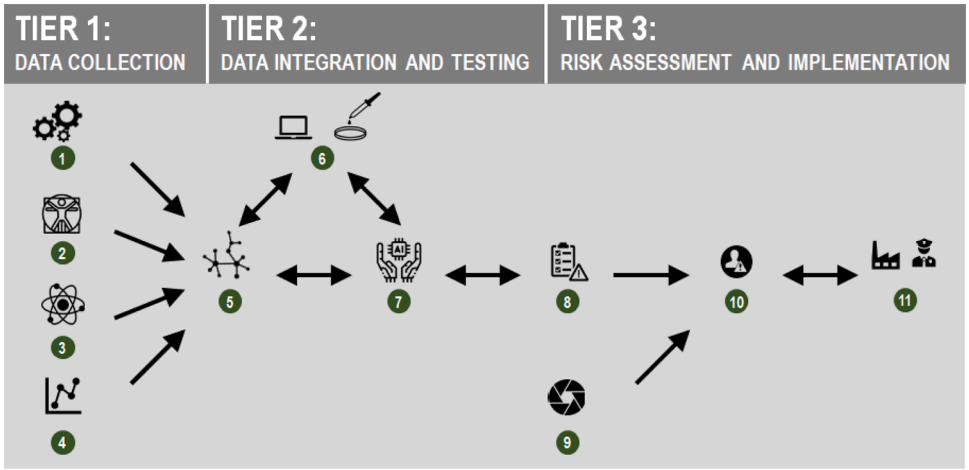

Fig. 3. Workflow of ONTOX.

underlying ontology-driven and artificial intelligence-based strategy is generic and can as such be applied to any type of chemical and systemic repeated dose toxicity effect. ONTOX thus provides a fully functional and sustainable solution for advancing human risk assessment of chemicals without the use of animals.

ONTOX introduces industrial innovation due to the unique combination of the conceptual ontology cornerstone with the methodological artificial intelligence cornerstone. In turn, this provides ample opportunities for economic valorization, in particular regarding relevant, reliable and cost-effective NAMs to perform chemical hazard prediction and human risk assessment. The reduction in cost and time will allow the front-loading of toxicity assessments in product stewardship. The ONTOX outcome will be high value for money, which will support exploitation and end-user acceptance. This ambition is realized through a set of specific exploitation actions in ONTOX. Furthermore, training of industrial entities and regulators is organized to maximize implementation and exploitation potential.

ONTOX aims to be a game changer in the regulatory arena. The idea is introduced to start risk assessment from human biology in order to avoid the current detour of the animal experiment. By subsequently mapping human biology (*i.e.* physiological maps) and toxicology (*i.e.* qAOP networks), all possible adversities and mechanisms of toxicity are covered, resulting in more reliable human risk assessment, which does not necessitate interspecies extrapolation and that can be personalized. In fact, ONTOX steps away from the classical 1-to-1 replacement of animal studies with single non-animal methods towards full replacement of animal studies for toxicity testing by using batteries of human-based *in vitro* assays and *in silico* tools encompassing the full (patho) physiological spectrum and hence reflecting the *in vivo* complexity of adverse effects.

ONTOX has the ambition to become a historic milestone regarding animal-free human risk assessment of chemicals and will build further on previous collaborative initiatives supported by the European Commission, including the EU-ToxRisk project (*i.e.* "an integrated European flagship program driving mechanism-based toxicity testing and risk assessment for the 21st century) (https://cordis.europa.eu/project/id/681002). Europe has always played on the forefront at a global scale regarding the protection of animals for experimental and other scientific purposes as well as regarding the development of non-animal methods. In this respect, the European chemicals strategy will foster innovations for advanced tools, methods and models, and data analysis capacity to move away from animal testing (EU, 2020). ONTOX allows to consolidate Europe's key role in this area and therefore sets an example for other parts of the world. This ambition is realized in first instance by banning animal experimentation in ONTOX. Furthermore, the ontology framework relies as much as possible on human data, and any new *in vitro* and *in silico* testing is fully human-based. This aligns with the principles of 21st century toxicity testing (NRC, 2007) and next generation risk assessment (Dent et al., 2018), which are intertwined with ONTOX.

ONTOX has teamed up with the 2 other projects granted within the European Horizon2020 project call "advancing the safety assessment of chemicals without the use of animal testing", namely RISK-HUNT3R (*i.e.* "risk assessment of chemicals integrating human centric next generation testing strategies promoting the 3Rs") (https://cordis.europa.eu/project/id/964537) and PrecisionTox (*i.e.* "toward precision toxicology: new approach methodologies for chemical safety") (https://cordis.europa.eu/project/id/965406). More specifically, ONTOX, RISK-HUNT3R and PrecisionTox have formed a project cluster, which has as the main goal to strengthen and facilitate collaboration between the 3 consortia in view of boosting outcome and impact. As part of the latter, the project cluster intends to set up collaboration with similar initiatives across the globe, including at the USA Environmental Protection Agency.

CRediT authorship contribution statement

All authors have contributed to the concept of the paper. The paper has been written by **Mathieu Vinken** and **Aldert H. Piersma**. All authors have revised the paper at least twice and have provided recommendations for improvement.

Author contributions

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Declaration of Competing Interest

Thomas Hartung consults Underwriters Laboratories on computational toxicology, especially read-across, and has a share of their respective sales. He also holds stock options in and consults ToxTrack LLC. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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