In silico approaches in organ toxicity hazard assessment: current status and future needs for predicting heart, kidney and lung toxicities

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Contents

Abstract

The kidneys, heart and lungs are vital organ systems evaluated as part of acute or chronic toxicity assessments. New methodologies are being developed to predict these adverse effects based on *in vitro* and *in silico* approaches. This paper reviews the current state of the art in predicting these organ toxicities. It outlines the biological basis, processes and endpoints for kidney toxicity, pulmonary toxicity, respiratory irritation and sensitization as well as functional and structural cardiac toxicities. The review also covers current experimental approaches, including off-target panels from secondary pharmacology batteries. Current *in silico* approaches for prediction of these effects and mechanisms are described as well as obstacles to the use of *in silico* methods. Ultimately, a commonly accepted protocol for performing such assessment would be a valuable resource to expand the use of such approaches across different regulatory and industrial applications. However, a number of factors impede their widespread deployment including a lack of a comprehensive mechanistic understanding, limited *in vitro* testing approaches and limited *in vivo* databases suitable for modeling, a limited understanding of how to incorporate absorption, distribution, metabolism, and excretion (ADME) considerations into the overall process, a lack of *in silico* models designed to predict a safe dose and an accepted framework for organizing the key characteristics of these organ toxicants.

1. Introduction

Chemical safety assessment of substances encompasses the assessment of acute and chronic toxicities, which in turn often includes examination of the adverse effects induced on different organs (e.g., kidney, heart, lung). In repeated-dose toxicity studies, organs and tissues are investigated to monitor changes (e.g., physiological, functional and morphological), leading to an adverse effect and to identify organs that are most affected (i.e., target organs) by a particular chemical [1,2]. Adverse effects on target organs are also relevant in the context of acute systemic toxicity [3]. Whereas, dysregulations and alterations of complex biological pathways result in organ toxicity that can occur as a primary effect on a given organ, they can also be a result of secondary effects in organs and tissues that have a physiological dependence on the primary target [4].

Use of *in silico* toxicology (IST) methods to predict organ toxicity can be sustained and advanced by development of IST protocols that are formulated to offer a standardized way to exploit *in silico* methods [5]. Such a standardization process promotes acceptability of both the methods and the corresponding predictions by end users, colleagues, collaborators, and regulators as well as provides a means to support a more transparent analysis of the results. Protocols that describe the integration of *in silico* methods with existing experimental data to identify potential genotoxicants [6] and skin sensitizers [7] have been developed based on the experience of a cross-industry consortium comprising many organizations.

An IST protocol is a description of the *in silico* prediction workflow within a consistent and well-documented structure and includes [5]:

- identification of adverse effects or mechanisms to predict alongside the corresponding experimental data and/or *in silico* methodologies and approaches to use;
- recommendation on generation of the predictions and on assessment of relevant experimental data;
- indications on the performance of the *in silico* analysis to generate results including expert review;
- recommendation on the reporting formats to share the results and the corresponding uncertainties.

An IST protocol then consists of the definition of experimental data and *in silico* methodologies associated with each effect or mechanism, the definition of rules underlying the combination of information, the definition of expert review guidelines, and the definition of a documentation guideline (see Figure 1). Hence the development of an IST protocol first requires the definition of an assessment framework that outlines how to integrate data originating from different sources, e.g., *in vivo* and alternative methods including *in silico* predictions. A basic assessment framework has been drafted and proposed for liver toxicity and this is shown in Figure 2 [8]. The current work is a preparatory step for the development of IST protocols for other organ toxicities, and more specifically for the development of a framework that integrates *in silico* methods predicting potential adverse effects from the molecular structure of chemicals. The focus is on toxicity to specific organ systems, namely kidney toxicity (i.e., nephrotoxicity or renal toxicity), heart toxicity (i.e., cardiotoxicity or cardiac toxicity), and lung toxicity (i.e., pulmonary toxicity). It was recently noted that the

term kidney should be preferred over the use of either “renal” or the prefix “nephro-” to generally describe kidney disease and function especially in non-technical contexts [9].

The review material collected here provides the basis for identification of endpoints and definition of their relationships in a mechanistically-informed framework that constitutes the basis for the development of the IST protocol. These organ systems are reviewed, and this includes a description of organ toxicity along with processes and endpoints. These are outlined to provide context to what needs to be predicted. Current *in vivo* and *in vitro* methods are generally discussed, as this information is essential to incorporate within the weight of evidence (WoE) in any hazard assessment in addition to supporting the development of *in silico* methods. An outline of the current state of the art in predicting organ toxicity is provided together with a discussion on what progress is needed to improve such predictions. Finally, the discussion summarizes key issues to address across all organ systems highlighted.

2. Kidney toxicity

Kidney toxicity (nephrotoxicity) is defined as disease or dysfunction of the kidney caused by chemical insult following acute or chronic exposure to drugs or xenobiotics [10]. It relates to toxicity to the nephron, the functional unit of the kidney. The primary functions of kidneys are clearance of waste products from the blood, maintenance of electrolyte and acid-base balance, regulation of extracellular fluid volume, and endocrine activity [11–13]. Vulnerability of this organ to chemical injury is related to its specialized role in the filtration, metabolism, and excretion of exogenous compounds [14,15] resulting in high local concentration of potentially toxic substances and/or formation of reactive metabolites.

A number of physiological and biochemical factors contribute to renal liabilities. First, the small mass of the kidney as compared to the resting cardiac output that it receives exposes this organ to high levels of circulating xenobiotics and of corresponding metabolites mainly produced in the liver [12,16]. Second, the renal processes of glomerular filtration, tubular reabsorption, and secretion contribute to concentrate toxicants in the kidneys; glomerular filtration is the first step of production of urine and results in an ultrafiltrate of the plasma; during tubular reabsorption and secretion, glomerular filtrate passes through the different segments of renal tubules where filtered solutes and water are reabsorbed, allowing the elimination of waste products [17]. Importantly, transport proteins play a critical role in concentrating potential toxicants [16]. Third, kidneys have high energy requirements to maintain their reabsorptive and secretory functions and this makes them susceptible to oxidative stress, resulting in an imbalance between free radical production and antioxidant defense [10,11]. This effect is particularly exacerbated in patients with common systemic diseases such as hypertension, diabetes mellitus and hypercholesterolemia [18,19]. Fourth, the renal system includes enzymes such as CYP450 and flavin-containing mono-oxygenases that mediate the metabolic degradation of xenobiotics possibly leading to the formation of reactive metabolites that are nephrotoxic [12].

Given the central role of this organ in the filtration and active elimination of foreign compounds, kidney toxicity may arise from exposure to a wide variety of substances including pharmaceuticals, agrochemicals, and industrial and environmental chemicals; growing concern is also posed by substances such as herbal remedies, natural products, and nutritional supplements [12,20,21]. After hepatotoxicity, toxicity to kidney significantly accounts for drug candidate failure in drug discovery and development; it is also a rather common problem in standard clinical care [22,23] and it contributes to acute or chronic functional changes of kidneys [24]. Prolonged cumulative lifetime exposure to chemicals in conjunction with age factors may accelerate the deterioration of kidney function and lead to chronic kidney disease (CKD) [25]. Exposure to pesticides has been clearly linked to kidney adverse effects [26,27]. As such, kidney toxicity is a specific concern in the context of occupational health too.

2.1 Kidney toxicity - Processes and endpoints

The spectrum of kidney toxicity manifestations is wide, and it reflects the diverse damage that can occur along the different segments of the nephron. Each nephron consists of glomerulus, proximal tubule, loop of Henle, distal tubule, and collecting duct; the different segments of the nephron comprise cells designed to perform specific functions and express various transporters and receptors. Notably, drug-induced kidney injuries frequently affect the proximal tubules, and it results in acute or chronic functional changes as a consequence of their key function in glomerular filtrate concentration and drug transport [24,28].

How toxicants cause injury to the nephron has been extensively studied in the context of drug-induced kidney injury, highlighting that different mechanisms of toxicity exist with drugs selectively targeting specific cell types, or non-selectively injuring multiple cell types [29]. Chemically induced kidney injury specifically depends on the intrinsic nephrotoxic potential of the chemical and the corresponding exposure (dose, route of administration, duration). A simplistic way to picture progression of kidney toxicity involves a first step where the foreign substances can undergo metabolic degradation that potentially forms reactive metabolites; toxic compounds can interact with organelles in the cells, interfere with signaling pathways, and ultimately lead to cell death and inflammation [30]. Kidney injury may progress to specific diseases including glomerulonephritis (injury to the glomeruli), acute kidney injury (AKI), CKD, and kidney failure. While AKI entails an abrupt change in kidney function, CKD is characterized by lasting structural and functional abnormalities. Kidney failure is defined as the final stage of chronic kidney disease (i.e., the disease stage where kidneys cannot function on their own).

Notably, oxidative stress is known to play an important role in the development of kidney injury or diseases, where an imbalance between the generation and elimination of reactive oxygen species can elicit damaging processes including inflammation, cell death (necrosis or apoptosis), fibrosis, tissue damage, and finally abnormal kidney function [11,30–32].

Extensive studies on kidney toxicity for pharmaceuticals have linked the adverse effects of kidney toxicants to general pathogenic mechanisms (see Table 1) that may be further related to specific molecular and biological events within the Adverse Outcome Pathway (AOP) construct (see Table 2) [33].

The AOPs associated with kidney toxicity as included in the AOP-Wiki are instead listed in Table S1 of the supplemental material [34,35], which shows that all of the mechanisms need to be finalized. The AOP-Wiki is a platform overseen by the Organisation for Economic Co-operation and Development (OECD).

Histopathology-related findings included in preclinical toxicity study reports for regulatory submissions can be organized in two-level clusters of terms (see Table 3) related to similar findings (and, possibly, similar mechanisms) *[36]*. As demonstrated in our sister publication on liver *[8]*, such organization is important for the development of an assessment framework for kidney toxicity (as outlined in Figure 2), where the consistent use of defined terminology and ontologies is crucial to map actual data.

Table 1. Pathogenic mechanisms of kidney toxicity [12,33,37–40]. It should be noted that rhabdomyolysis and thrombotic microangiopathy are two forms of kidney toxicity that have a systemic origin [33].

Pathogenic mechanisms	Details
Altered intraglomerular hemodynamics	Regulation of intraglomerular pressure is mediated by circulation of prostaglandins (vasodilation) and the action of angiotensin-II (vasoconstriction). Alteration of glomerular pressure and a decrease of the glomerular filtration rate can be promoted by substances with antiprostaglandin activity (e.g., nonsteroidal anti-inflammatory drugs) or with antiangiotensin-II activity (e.g., inhibitors of ACE receptor or blockers of ARB receptor).
Tubular injury (proximal and distal)	Tubules, especially the proximal segments, are vulnerable to toxicants that can elicit cytotoxicity by affecting mitochondrial function, impairing tubular transport, increasing oxidative stress, or favoring free radical formation.
Nephritis (tubular, interstitial, and glomerular)	Nephritis is inflammation of the kidneys that occurs in glomerulus, renal tubular cells, and/or the surrounding interstitium to promote regeneration and repair of the kidney injury; unresolved inflammation can progressively lead to renal fibrosis and impairment of the kidney function. Nephritis involves both cells of the immune system and activation of intrinsic renal cells.
Tubular obstruction	Insoluble crystals are formed in the nephron tubules, primarily in the distal segments, obstructing urine flow and driving disorder in kidney function.
Rhabdomyolysis	Rhabdomyolysis is a syndrome caused by skeletal muscle injury leading to death of muscle fibers and release of intracellular contents (myoglobin and creatine kinase) into the plasma that in turn induce adverse effects in the kidneys.
Thrombotic microangiopathy	Thrombotic microangiopathy is a vascular issue, where platelet thrombi in the microcirculation induce kidney damage.

Table 2. Molecular initiating events identified for the pathogenic mechanisms of kidney toxicity [33]. The table shows mechanisms involving enzymes such as cyclooxygenase (COX) and ornithine aminotransferase (OAT).

Pathogenic mechanism	Molecular Initiating Event in the AOP
Hemodynamic alteration	COX-1 and/or COX-2 inhibition leading to reduced prostaglandin synthesis and uncontrolled renal vasoconstriction [41,42].
Proximal and distal tubular cell toxicity	Mitochondrial toxicity pathways: a) Mitochondrial DNA incorporation [43]. b) Mitochondrial DNA polymerase gamma inhibition [43]. c) Depletion of SH-groups leading to reactive oxygen species (ROS) induction [44].

	Metabolization by oxidase in hepatocyte to benzoquinoneimine, followed by formation of GSH (glutathione) S-conjugates [45].
Tubular, interstitial, tubulointerstitial and glomerular nephritis	Interaction with hOAT1 and 3, accumulation within proximal tubule cells, followed by uncoupling/inhibition of mitochondrial oxidative phosphorylation and tubular/papillary necrosis [41].
Tubular obstruction	OAT interaction causing secretion via proximal tubule cells, accumulation and crystal formation in urine leading to concentration in renal tissue/tubule and obstructive nephropathy [43].

Table 3. The hierarchical organization used to group histopathology terms of similar findings (and mechanism) for kidney toxicity; findings were extracted from preclinical toxicity study reports for regulatory submissions [36].

KIDNEY TOXICITY	
General clusters	**Specific clusters**
Tissue damage	Necrosis
	Degeneration
	Nephropathy
Inflammatory changes	Inflammation
	Infiltration
Structural alterations	Dilation
	Adaption cell size / number
Accumulative lesions	Accumulation
	Vacuolation
	Mineralization

2.2 Kidney toxicity - *In vivo* and *in vitro* methods

Identification of kidney toxicity traditionally relies on *in vivo* testing. For pharmaceuticals, kidneys do not fall within the safety pharmacology core battery and supplemental studies on the renal system are required when there is cause of concern not addressed by the core battery [46] or repeated-dose toxicity studies [47].Together with histopathological observations, changes in the kidney function are detected by assessing clinical markers such as glomerular filtration rate (GFR), blood urea nitrogen (BUN) and serum creatinine (sCr) [11]. Much effort is underway to identify novel biomarkers that could ideally allow for an early detection of chemically induced kidney toxicity, differentiate it from other causes, and predict long-term kidney outcome and mortality; some promising biomarkers include Kidney Injury Molecule-1, Beta-2 Microglobulin, and albuminuria [16,48].

Animal models have been challenged by the insufficient level of prediction of kidney failure in humans and their inadequacy has been linked to the significant differences in expressions of transport proteins and metabolizing enzymes between species [11,29]. Kinetics needs to be evaluated in a human-relevant system (including a human-based mathematical model) to adequately assess internal exposure and dose-response relationships over time.

In vitro screens are also being used to evaluate chemically induced kidney injury, but a standardized approach is not currently available and existing models are found to be poorly predictive of human kidney toxicity

[29,49,50]. Advanced 3D *in vitro* models such as organoids and kidney-on-a-chip platforms are emerging to overcome the limitations of the 2D *in vitro* assays including and improve kidney safety assessment [51].

2.3 Kidney toxicity - Molecular targets

In vitro safety pharmacology profiling panels are used by pharmaceutical companies to investigate organ toxicity [52].In the safety panel by Bowes and co-workers [53], cyclooxygenase 1 (Cox1) and vasopressin V1A receptor (Table S2 of the supplemental material) are associated with kidney adverse effects. Additional molecular targets have been associated with [54], and Tables S3 and S4 of the supplemental material provide lists of targets derived from a genetic and pharmacological phenotype analysis [55] or other data curation processes [56], respectively.

2.4 Kidney toxicity - *In silico* methods

An IST protocol for the identification of potential kidney toxicants needs to account for a draft assessment framework that includes several types of data as depicted in Figure 3. In terms of hazard identification, available IST approaches for kidney toxicity are based both on statistical-based (or QSAR) methods [36,57–61] and expert rule-based (or expert/structural alerts) methods [36,62,63]. Such methods are usually built on either *in vivo* data (e.g., rat and mouse) or human data, the latter originating in the pharmaceutical sector from clinical trials or post-marketing surveillance reports. The resulting *in silico* models must be expected to be generalistic in their predictive capabilities as the underlying broad database will be based on many mechanisms of action and potentially many different effects. As such, they may identify compounds with the potential for kidney toxicity, but the type of adverse effects and quantitative identification of the Point of Departure (PoD) will be difficult to determine unless detailed analysis is undertaken. In addition, in terms of risk assessment, since animal models have been challenged as to their ability to adequately predict kidney adverse effects in humans, particularly if these are driven by kinetics, integrating human data in predictive models is vital.

The prediction of the general endpoint (i.e., “kidney toxicity”) can be combined with the prediction of other toxicity subcategories to gain a better understanding of specific adverse effects. An illustration of this was reported by Matthews and coworkers, who constructed QSAR models based on the adverse events retrieved from FDA post-market reports. Their models predict six composite endpoints of the urinary tract: acute kidney disorders, nephropathies, bladder disorders, kidney function tests, blood in urine, and urolithiases [60]. Even within these groupings, whilst there will be greater homogeneity of mechanisms of action, there will be variability. It is likely that these QSARs for “groups” of effects will be more localized models, with less applicability. An “ontology” of some form, which organizes mechanisms linked to effects in a hierarchical manner, may be required to gain a more comprehensive overview of kidney toxicity and associated mechanisms. For instance, a good example of this approach is provided by an appropriate hierarchical clustering of histopathology data (see Table 3) [36]. The advantage of setting out adverse effects related to

the kidney (or any organ level toxicity) is that Amberg and coworkers developed a number of models (i.e., structural alerts, fragment-based, molecular descriptor-based machine learning approaches) to predict specific kidney toxicity findings. This modeling approach, also applied in the context of other target organ toxicities (i.e., liver and heart), indicates that a proper clustering process, and hence grouping endpoints/effects in a meaningful way, is crucial for a good predictivity.

A number of structure-activity relationships (SARs) are available for kidney toxicity, as well as focusing on specific biological pathways [33] such as protein binding [64] and mitochondrial toxicity [65,66]. At the current time, a comprehensive, publicly available, *in silico* profiler for kidney toxicity is lacking. However, lists of alerts for kidney toxicity, e.g. from data mining approaches, are available [63]. These alerts are very useful starting places, although to allow for greater applicability, especially for regulatory approaches, they require adequate definition and linkage to mechanisms of action.

SAR based alerts can be used in a variety of ways but are generally useful either as direct predictions of toxicity, i.e., a qualitative estimation, or as a means of grouping to allow for read-across. There are a small number of published reports of attempting read-across for kidney toxicity and repeated dose toxicity driven by effects to the kidney. For instance, Fowles and co-workers identified adverse effects to the kidney as a significant factor in the toxicological assessment of ethylene glycols [67]. Use of metabolomics was demonstrated to support read-across for organ level toxicity including that to the kidney [68].

In conclusion, there are a variety of *in silico* approaches that predict kidney toxicity. At the current time there is no unified approach to toxicity prediction, for instance that may apply generalistic broad QSAR type models supplemented by more mechanistic models or confirmation through the use of structural alerts. In addition, little has been performed in terms of ensuring the toxicokinetic component of kidney toxicity is included [69]. Whilst the current models are satisfactory for prioritisation and possibly hazard identification, an integration of approaches (including ADME predictions) will be needed for risk assessment.

2.5 Kidney toxicity - *In silico* approaches: data gaps and issues

In silico models for kidney toxicity bring with them a number of problems and issues to overcome, some of which are general for all organs, others are specific to kidney. There is no easy way to approach the topic of modeling kidney toxicity for two fundamental reasons: (1) the complexity of the endpoint and (2) the quality and relevance of the data to model. Starting with the complexity of the endpoint, clear guidance, or definition within a model, is required as to what constitutes kidney toxicity, e.g., general toxicity to the kidney, specific effects within the nephrons or kidney structure, or related adverse effects such as to the urinary tract or bladder. As noted above, there are a variety of means to obtain information relating to kidney toxicity from both *in vitro* and *in vivo* methods. It is crucial to decide for the modeling approach, what endpoint is to be predicted. Thus, a general *in silico* model for the presence of kidney toxicity from *in vivo* test results, for instance from a repeat dose experiment, may include a variety of mechanisms of action and apical effects.

Such models should not be discounted, but they may be most appropriate for screening and prioritisation purposes, i.e., to identify those compounds with a strong probability of causing kidney toxicity. The use of *in vivo* data is also made more complex in that it will be difficult to prove a negative test, i.e., there is no adverse effect on the kidney. This may be because the test was not performed at a sufficiently high dose, or that other toxicities were observed at lower doses and no account was taken of adverse effects to kidneys. Thus, the use of such data must be considered for generalistic models. The biomarker and histopathology data are likely to be important to gain a more detailed approach of potential kidney toxicity. In other words, it is probable that there will be models based on localised areas of chemistry which may be suitable for risk assessment provided the quality of the original data is acceptable. The problem of predicting accurately Points of Departure (PoD) is particularly relevant for kidney toxicity. As noted above, kidney toxicity is largely driven by toxicokinetics and the ability to accumulate within the kidney. In terms of modeling, to obtain a PoD predictions will be required not only for relative hazard but also for bioavailability in the relevant compartment of the kidney, for which data are currently scarce. The use of techniques such as physiologically-based mechanistic modeling, an extension of PBK, is likely to become increasingly important to perform adequate risk assessment. There is also an opportunity for physiologically-based mechanistic modeling to assist in the proper incorporation of inter-species differences, e.g., for the extrapolation of rodent data to humans.

3. Lung toxicity

The lung is a primary target organ for potential chemically induced damage caused by inhaled material, such as gases and particles [70–72]; it acts as portal of entry for airborne chemicals into the human body facilitating gas exchange between blood and air. While pulmonary toxicity refers to toxicity to the lung as target organ, inhalation toxicity refers to the route of exposure through the respiratory system that includes the upper respiratory tract (mouth, nose, and pharyngeal region) and the lower respiratory tract (tracheobronchial region and the pulmonary parenchyma or alveolar region) [70,73]. Since the lung is highly perfused and receives the total cardiac output to be replenished with oxygen [74], this organ may also be injured through the vascular system, namely by xenobiotics entering the systemic circulation irrespective of the route of absorption [75].

Toxicity to the lungs may be caused by a great variety of chemical agents from intentional or non-intentional exposure including natural products, industrial chemicals, pesticides, environmental pollutants, combustible cigarettes, and drugs. Notably, evaluation of the adverse effects to the lungs is of paramount importance in the acute inhalation studies for hazard identification and characterization of chemicals, including classification and labelling [76,77]. Lungs are also a prominent target organ for occupational diseases caused by accidental or prolonged inhalation of xenobiotics. In the context of pharmaceuticals, drug-induced lung diseases are reported to be a significant subset of adverse drug reactions [78,79] with the most common

form being the so called drug-induced interstitial lung disease (DILD), which is mainly caused by oral and parenteral administration [80]. Additionally, in the drug discovery and development of inhaled therapies, toxicity to the lungs represents a challenging hurdle to overcome [81].

3.1 Lung toxicity - Processes and endpoints

Lung toxicity following inhalation of airborne chemical agents concerns gases and vapors, as well as aerosols and particulate matter. Central to inhalation toxicity is the concept of dosimetry (rather than exposure concentration), that seeks to define the amount, rate, and form of a substance delivered to the target tissue [76,82]. Dosimetry involves evaluation of the deposition, clearance, and translocation patterns within the respiratory tract, and two key elements have been singled out to influence these patterns: a) respiratory anatomy and physiology that differs among species; and b) the physico-chemical characteristics of the inhaled chemical agents [76]. Deposition, clearance, and translocation patterns of particles are affected by properties such as size, shape, density, hygroscopicity, and surface characteristics [83]. For gases and vapors, solubility is critical in determining the depth of penetration of the substance; generally, low-water soluble substances penetrate lower in the respiratory tract [70].

Toxicity to the pulmonary tissue following inhalation exposure or systemic circulation of xenobiotics frequently depends on the metabolizing capability of this organ; phase I and II enzymes are involved in the lung disposition processes and they can catalyze biotransformation reactions resulting in the formation of toxic metabolites [71,84]. Potential bioactivation of parent compounds in highly reactive intermediates together with other factors (e.g., preferential exposure or accumulation of the xenobiotics or metabolites in given sites, specific cellular defense mechanisms) affect the types of lung cells that are injured by chemicals [71].

3.1.1 Irritation

Chemically induced transient effects to the lung are referred to as irritation. Irritation is a nonimmunological state of the respiratory tract that follows inhalation of substances at doses that cause inflammation [85]. Within the EU classification and labelling (C&L) perspective, the European Chemicals Agency (ECHA) states that respiratory tract irritation is “a transient target organ effect, i.e. an effect which adversely alters human function for a short duration after exposure and from which humans may recover in a reasonable period without leaving significant alteration of structure or function” [86]. For the U.S. Occupational Safety and Health Administration (OSHA), irritant chemicals cause a reversible inflammation in contrast to corrosive damage that is permanent and irreparable [87]. Under the Specific Target Organ Toxicity (Single Exposure) (STOT-SE) of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), respiratory tract irritation falls in category 3, namely in the transient target organ effects category, where respiratory irritant effects (characterized by localized redness, edema, pruritis, and/or pain) impair function with symptoms such as cough, pain, choking, and breathing difficulties [88]. The OSHA implementation of the GHS

emphasizes that such adverse effects are of short duration after exposure, and do not result in significant alterations of structure or function following recovery [89].

Adverse effects related to respiratory tract irritation are grouped into two different forms [85,86,90]: local cellular damage and effects caused by airborne chemicals that stimulate the peripheral nerve fibers innervating the respiratory tract from the nose to the alveoli (sensory irritation) [91]. Inhaled substances interacting with the nerve endings of the respiratory tract have been classified by Alarie (2014, 1973) according to the "first level" of the respiratory tract at which they act as the exposure concentration increases from zero [90]. Sensory irritants, when inhaled via the nose, stimulate the trigeminal nerve endings, evoke a burning sensation of the nasal passages, and inhibit respiration. Bronchoconstrictors act on the conducting airways of the lung and induce an increase in resistance to air flow within the airways. Pulmonary irritants stimulate the nerve endings within the lung, increase the respiratory rate, and decrease tidal volume (rapid shallow breathing). According to the "by-first-level-of-action" classification by Alarie, respiratory irritants interacting with peripheral nerve fibers can act as a sensory irritant, a bronchoconstrictor, and a pulmonary irritant and they are capable of all three actions; there is little difference between the concentrations at which they induce an effect at the three levels: nose, conducting airways, and deep lung. Physico-chemical properties of the inhaled substances play a role in the Alarie classification: highly water soluble and/or reactive chemicals (e.g., formaldehyde) affect the upper airways while less water-soluble compounds deeply penetrate the lung and affect the lower respiratory tract [90].

3.1.2 Sensitization

In contrast to respiratory irritants, respiratory sensitizers lead to hypersensitivity of the airways following inhalation [88], an immune-mediated response to an otherwise innocuous antigen [94]. The immune-mediated hypersensitivity reactions are referred to as chemical respiratory allergy [95], and include two steps. The first phase is sensitization (induction) involving the development of specialized memory cells in the immune system of an individual following initial exposure to the respiratory sensitizer. The second phase is elicitation when, on repeated exposure, the heightened immunological responsiveness can provoke allergic reactions resulting in clinical manifestations such as asthma and rhinitis [95]. The number of chemicals confirmed with the potential to cause allergic sensitization of the respiratory tract are relatively low (less than a hundred) in contrast to the hundreds to thousands of confirmed dermal sensitizers [96]. Many dermal sensitizers have not been regarded as respiratory sensitizers and there are examples of respiratory allergens that have no potential to cause skin sensitization [97]. Commonly it was assumed that inhalation exposure was necessary for respiratory sensitization, but there is evidence that respiratory sensitization might also result from dermal exposure [98,99]. While there are similarities in the biological pathways that lead to the acquisition of dermal and respiratory sensitization, the differences are important to note. Respiratory sensitizers preferentially bond lysine and result in a cytokine profile that favors the generation of a T helper 2 (Th-2) response as opposed to a T helper 1 (Th-1) response with skin sensitization [99]. Furthermore, the

Th-2 response promote IgE antibody production but the role of the IgE antibody in respiratory sensitization remains uncertain [100].

3.1.3 AOP

In the context of inhalation toxicity, a field tightly bound to lung toxicity, important global efforts are being undertaken to advance the use of alternatives methods and promote their global regulatory acceptance [76,77] and mechanistically-informed Integrated Approaches to Testing and Assessment (IATA) are being developed using the Aggregate Exposure Pathway (AEP) and AOP frameworks. Table S5 of the supplemental material lists some AOPs specifically targeting the lung as listed in the AOP-Wiki [34,35]. All of these AOPs needs further development.

3.2 Lung toxicity - *In vitro* and *in vivo* methods

Toxicity to lung can be induced by inhaled substances and several OECD Test Guidelines (TGs) provide the framework to measure the adverse effects in the upper part and lower part of the respiratory tract following inhalation exposure. More specifically, inhalation studies are conducted in animals and include tests for acute inhalation toxicity with death as endpoint (TG 403 [101], TG 436 [102], tests based on clear signs of toxicity as endpoint (TG 433 [103]), and repeated-dose inhalation testing (TG 412 [104], and TG 413 [105]) [76,77,106]. For pharmaceuticals, adverse effects to the respiratory tract are identified at a relatively late phase during the comprehensive pre-clinical assessment undertaken during *in vivo* toxicity studies [81]. Inhalation *in vivo* studies using rodents must account for significant species differences (e.g., different nasal/pharyngeal anatomy and obligate nose breathing) and translation of results to humans needs to be critically evaluated [107].

Inhalation toxicology studies are increasingly taking advantage of the 3D *in vitro* models (e.g., organ-on-chip, organoids) that better reflect cell interactions in their natural environment as compared to traditional 2D *in vitro* assays [108–111]. However, it is important when using these more sophisticated 3D tissues to mimic the *in vivo* exposure route with a more relevant exposure system to dose at the air/liquid interface [112,113].

3.2.1 Irritation

Whilst inhalation studies provide information related to respiratory irritation, *in vitro* methods that address lung irritation are limited compared to other organs (skin and eye). The use of cytotoxicity as a surrogate to investigate irritation is widely accepted in the development of *in vitro* models to predict irritation potential of chemicals [114,115]. Neilson and co-workers took this approach to develop an *in vitro* 3D airway tissue model to assess the potential irritancy of e-cigarette aerosols compared to cigarette smoke [116]. The Alarie test assesses the sensory irritation potential by measuring the inhaled concentration of a substance necessary to cause a 50% reduction in the respiratory rate in mice allowing for the quantification of irritating concentrations and ranking of chemicals for their sensory irritancy potential [117]. Sensory irritation is

frequently identified as a critical endpoint for setting occupational exposure limits [118,119]. Notably, there is no generally accepted *in vitro* model for assessing respiratory irritation [119].

3.2.2 Sensitization

To date, no *in vitro*/*in vivo* test methods have been validated for the assessment of respiratory sensitization and test methods used for skin sensitization hazard assessment are employed as a surrogate for respiratory sensitization [86,98,120]. Of these, the Direct Peptide Reactivity Assay (DPRA) and Amino Acid Derivative Reactivity Assay (ADRA) both assess activation of the molecular initiating event (MIE), covalent modification of proteins. While respiratory sensitizers preferentially bind to lysine (a comparatively hard nucleophile), this selectivity is not absolute, and reactivity with cysteine also occurs with some respiratory sensitizers [121]. However, in more recent studies [122], the preference for lysine binding was not as apparent, but the use of the DPRA assay was still deemed useful within the testing strategy. The Local lymph Node Assay (LLNA) and Guinea Pig Maximization Tests (GPMT) also supports the weight of evidence assessment of respiratory sensitization [98,123]. Dermal exposure to a respiratory sensitizer triggers an immunological effect that could be detected in methods that assess skin sensitization; it is not possible to distinguish between the respiratory and dermal effects using standard methods. As such, a negative LLNA result is part of the evidence in support of a negative assessment for respiratory sensitization [100,123], although the possibility of false negatives needs to be considered carefully [124]. Modifications to the LLNA allow for cytokine profiling which can distinguish between the Th2 versus Th1 response types following either dermal or inhalation exposures [86]. Total IgE measurements have also been used to support an assessment of respiratory sensitization. None of these approaches, however, are validated or standardized. Additional experimental approaches that may support a weight of evidence assessment of respiratory sensitization could be found in the ECHA guidance [86].

3.3 Lung toxicity - Molecular targets

The molecular targets associated with lung toxicity as derived from the *in vitro* safety pharmacology profiling panel of 44 targets discussed by Bowes and coworkers [53] are listed in Table S6 of the supplemental material. Additional molecular targets associated with liabilities to the respiratory system have been discussed in the literature [54], and Tables S7 and S8 of the supplemental material report some collections as derived from the analysis of human genetics and pharmacology data [55] and other data curation processes [56].

3.4 Lung toxicity - *In silico methods*

An IST protocol to predict lung toxicity will be based on a draft assessment framework that accounts for different types of endpoints such as irritation and sensitization and that integrates information from several sources, e.g., human data, animal *in vivo* data, specific biologic responses (**Error! Reference source not found.**) [5,8] and ADME information (Figure 2).

In silico methods to predict lung toxicity can be sorted according to the type of adverse effects they predict and thus according to the type of data they are built on, including sensitization, irritation (i.e., cellular damage, sensory effects), other acute lung injury, and chronic effects (i.e., asthma, fibrosis, chronic obstructive pulmonary disease). Examples of *in silico* models are given in Table 4. *In silico* methods for the prediction of GHS classes based on acute inhalation toxicity studies address systemic toxicity rather than specifically pulmonary toxicity.

Several *in silico* systems have been developed to predict respiratory sensitization including both expert systems and QSAR models [125–131] with some respiratory sensitization models specifically built on a dataset of asthmagenic chemicals [132]. Enoch et al. defined structural alerts which describe covalent protein binding in the lung; each structural alert is associated with a mechanistic domain, which could be used to support a read-across assessment [127]. Similarly, Mekenyan et al. reported a mechanistic approach for the assessment of respiratory sensitization potential or for grouping chemicals for subsequent read-across application [133]. Other efforts have resulted in similar profilers [134].

Within the project "Respiratox", models for pulmonary irritation have been developed to predict the potential to induce tissue damage and/or sensory irritation effects [135]. Some other models have been developed using lung injuries data [59]. Jeong et al. reported the development of an adverse outcome pathway (AOP) to better define the linkage of PPARγ antagonism to the adverse outcome of pulmonary fibrosis using the ToxCast Database and a Deep Learning Artificial Neural Network Model-Based Approach [136].

Table 4. Some models for lung toxicity. Models for the prediction of inhalation toxicity are not included.

Endpoint	Endpoint details	References
Irritation (sensory)	Model based on set of 145 diverse volatile organic compounds as sensory irritants	[137]
Irritation (pulmonary)	Data (either sensory irritation or tissue damage) on 1997 organic compounds	[135]
Respiratory sensitization	Training and validation sets have been built from chemicals that are negative for human sensitization potential (Graham et al 1997), tested negative in the LLNA, non-sensitizers based on occupational exposure limits (OELs) and no cases of occupational asthma (OA); in addition to, chemicals that are identified as respiratory sensitizers through case-reports, and asthmagens that cause OA	[126–130,132]
Inflammation	IL-8 gene expression: *in vitro* data on gene expression in A549 cells of IL-8, a well-known inflammatory cytokine	[138]
Drug-induced respiratory toxicity	Dataset with a series of toxicological end points of mouse intraperitoneal respiratory toxicity including: focal fibrosis (pneumoconiosis), acute pulmonary edema, bronchiolar constriction, bronchiolar dilation, changes in pulmonary vascular resistance, chronic pulmonary edema, cyanosis, dyspnea, pleural thickening, respiratory depression, respiratory obstruction, respiratory stimulation, structural or functional change in trachea or bronchi, and other changes	[59]

3.5 Lung toxicity - *In silico* approaches: data gaps and issues

As noted for other organ toxicities, the complexity of the pathways leading to adverse effects on the lung poses an obstacle for the development of *in silico* models, as does the heterogenous nature of compound properties (and their interplay) which can lead to lung toxicity. Such biological pathways that may lead to different types of adverse effects (e.g., sensitization, sensory irritation, tissue damage) need to be accounted for in the development of *in silico* models.

In relation to respiratory sensitization, it can be noted that the limited acceptance of *in vitro*/*in vivo* approaches for respiratory sensitization presents a challenge to the standardization of a robust training set. A tiered *in silico* approach using two SAR models could not conclude with a reliable classification on 65% of the chemicals tested in an external evaluation set [126]. While read-across could be used to fill these gaps, the overall conclusion points to the need for standardized testing methods for respiratory sensitization.

Future assessment of chemical respiratory sensitizer potential should take advantage of multiple lines of evidence to draw conclusions. While not the sole method to assess hazards, the weight of evidence approach remains the only available option within the absence of validated methods for hazard assessment.

It should then be noted the close relationship between toxicity to the lung and inhalation toxicity with the latter mostly referring to systemic toxicity rather than lung toxicity. An ontology that unifies existing knowledge on lung adverse effects may facilitate the advancement of *in silico* methods and their corresponding applications.

4. Heart toxicity

Toxicity affecting the heart, namely cardiac toxicity or cardiotoxicity, is particularly important in the context of pharmaceuticals, where it significantly contributes to the attrition of drug candidates in the pre-clinical phase of drug discovery and development. It is one of the major causes of human adverse drug reactions occurring both in the clinical phase and post-market approval phase [139,140]. Not only do cardiac safety liabilities remain a major obstacle for the pharmaceutical industry, they also pose an increasing concern in the context of environmental risks [141] as they have been associated with exposure to environmental chemicals [142] such as pesticides [143,144], flame retardants [145,146], and polycyclic aromatic hydrocarbons (PAHs) [147,148]. Cardiac safety is also becoming recognized as an issue with dietary supplements and herbal products [149].

4.1 Heart toxicity - Processes and endpoints

Chemical insults initiate a series of events in cardiac cells (see Figure 5) that may manifest as functional and/or structural perturbations of the heart [150–152]. Functional effects correspond to alteration of the mechanical (contractility) or electrical (ECG) function whereas structural effects correspond to morphological

damage or loss of cellular/subcellular components. Structural change may precede dysfunction, or occur as a result of it [153–155]. In contrast, chemically-induced changes on myocardial contractility can arise from both electrophysiological and structural elements [150,153,156].

Pharmaceutical companies have summarized cardiac key liabilities faced throughout drug discovery, drug development and clinical practice (see Table 5). These include proarrhythmic potential, myocardial ischemia, myocardial necrosis, heart failure, coronary artery disorders, cardiac valve disorders and endocardial disorders [139].

Drug-induced QT prolongation (delayed ventricular repolarization of the cardiac action potential) is one of the most investigated cardiac safety concerns. QT prolongation is a surrogate marker of proarrhythmia, for example Torsades de Pointes (TdP), a rare form of arrhythmia that is potentially lethal and has caused the removal from the market of several drugs [139,157–159]. QT prolongation has thus been extensively studied and the understanding of the underlying biological mechanism led to the development of a successful cardiac safety assessment paradigm for use in drug discovery and development. This paradigm was formalized in 2005 by the International Council on Harmonization (ICH) through the release of the S7B- and E14 regulatory guidelines [160,161]. From a mechanistic point of view, QT prolongation is associated with prolonged ventricular cardiac action potential, with the potassium channel encoded by the human ether-à-go-go related gene (hERG) being responsible for cardiac repolarization [162–165]. QT prolongation and associated arrhythmia arising from the inhibition of the hERG potassium channel is an example of functional cardiotoxicity.

The safety paradigm for cardiotoxicity defined by the ICH S7B and E14 guidelines focuses on the assessment of QT interval as marker of proarrhythmia [157]. ICH S7B addresses the nonclinical evaluation of the QT interval prolongation, recommending a testing strategy that includes both an *in vitro* assay to assess whether a compound or its metabolites block the repolarizing ionic current through inhibition of hERG and an *in vivo* animal assay to assess ventricular repolarization (it should be noted that hERG assessment is not appropriate for all pharmaceuticals). ICH E14 establishes the quality of the clinical evaluation required to understand drug-induced QT prolongation.

In contrast to pharmaceuticals, the hERG channel activity of dietary supplements and herbal products is not routinely assessed nor have regulatory guidelines been developed that specifically address this issue, despite their widespread use and evidence that some are potent hERG blockers [166].

Table 5. Key cardiac liabilities reported by pharmaceutical industry (adapted from Laverty et al., 2011b).

Toxicity	Common standard assessment strategies[§]
Proarrhythmic potential	Drug discovery: hERG screening, other cardiac ion channel screening, i*n vitro* profiling in cardiac tissue, *in silico* hERG and cardiac action potential assessment

	Drug development: QT interval determination in telemetry and or toxicology studies and mechanistic investigations (e.g., hERG trafficking, metabolites effects) Clinical practice: QT interval determination including concentration QTc modeling and assessment of other ECG parameters
Myocardial ischemia	Drug discovery: assessment of ECG morphology changes, histological examinations and functional assessments (e.g. LVEF) Drug development: observation of clinical signs, assessment of ECG morphology changes, histological examinations and functional assessments (e.g. LVEF)
Myocardial necrosis	Drug discovery: few predictive *in vitro* methods, histological examination from early repeated-dose toxicity studies Drug development: some reflective biomarkers available (e.g., troponin), histological examinations, imaging (e.g., echocardiography)
Heart failure	Drug discovery: assessment of some functional endpoints *in vitro* and *in vivo* (e.g., contractility), histological examinations and cardiac biomarkers (e.g. pro NT-BNP) Drug development: observation of clinical signs, imaging and cardiac biomarkers
Coronary artery disorders	Drug development: observation of clinical signs and imaging
Cardiac valve disorders	Drug discovery: evaluation of alerts from receptor (e.g., 5-HT2B) binding data Drug development: imaging, histological examinations
Endocardial disorders	Drug development: histological examinations

[§]hERG (human ether-à-go-go related gene); ECG (electrocardiogram); LVEF (left ventricular ejection fraction); pro-N terminal B-type natriuretic peptide; QT (duration of ventricular depolarization and repolarization); QTc (corrected QT interval); 5-HT2B (5-Hydroxytryptamine receptor 2B).

Regarding structural cardiotoxicity, this may be described by a continuum of progression of cardiac cell injury spanning through degeneration, necrosis, responding inflammatory changes (inflammatory cell infiltrate) and eventually fibrosis, with the latter being a repair process which does not generate functional contractile tissues [152]. The number and distribution of the injured cells determines the ultimate effects on the myocardial contractile function [152].

Histopathological observations included in preclinical toxicity study reports for regulatory submissions have been organized in groups of similar findings (and mechanism) [36]; as in the case of other organ toxicities (i.e., toxicity to liver and kidney) [8], heart-related histopathology data can be structured in two-level clusters (i.e., tissue damage, inflammatory changes, structural alterations), that can be further separated into more specific groups of terms as shown in Table 6. As in case of other organs, the consistent use of terminology is key for later re-use of the data generated.

Table 6. The hierarchical organization used to group histopathology terms of similar findings (and mechanism) for heart toxicity; findings were extracted from preclinical toxicity study reports for regulatory submissions [36].

HEART TOXICITY	
General clusters	**Specific clusters**
Tissue damage	Necrosis
	Degeneration
	Myopathy
Inflammatory changes	Inflammation
	Infiltration

Structural alterations	Dilation
	Adaption cell size / number

In contrast to ion-channel mediated mechanisms, other biological pathways leading to heart toxicity are in general poorly understood, particularly those underlying cardiac contractility and structural cardiotoxicity. Efforts are underway to elucidate such mechanisms possibly within an AOP framework [155]; this offers a means to organize the existing knowledge of adverse outcomes and to advance the mechanistic understanding of heart toxicity [140]. However, data coverage in both the chemical and biological domain is a limiting factor in the field.

Information on biological pathways that are associated with cardiac liabilities is being collated in the AOP-Wiki, and Table S9 of the supplemental material lists several AOPs as included in this repository [34,35]. The AOPs cited in the AOP-Wiki focus on ion channel activity.

4.2 Heart toxicity - *In vivo* and *in vitro* methods

In drug discovery and development, functional and structural cardiotoxicity is assessed using a variety of *in vitro* (e.g., over expressing cell lines, primary cardiomyocytes, stem cell derived cardiomyocytes), *ex vivo* (e.g., isolated heart, ventricular wedge) and *in vivo* (e.g., single and repeat-dose rodent and non-rodent species) models. *In vitro* approaches can be divided into molecular- and phenotypic-based assays. Phenotypic-based assays are primarily used to identify a potential cardiac safety risks (hazard detection) that can be further characterised in a more complex model system. These approaches allow the investigation of multiple cardiac effects, for example the assessment of cardiac contractility via measurement of calcium transients or impedance and cardiac structure via high content biology imaging. The phenotypic endpoints typically use integrated *in vitro* models, such as human induced pluripotent stem cell-derived cardiomyocytes that contain a milieu of kinases, ion channels, enzymes and receptors present within the heart facilitating the detection of potential adverse cardiac effects where the molecular understanding is limited [156].

Molecular *in vitro* approaches mainly focus on prediction of electrocardiogram abnormalities and QT-interval prolongation by ion channel screening and measurement of cardiac action potentials [152]. The assessment of QT interval prolongation and hERG inhibition has proven to be very sensitive and thus successful in eliminating drug candidates at risk of causing TdP. On the other hand, assessment of hERG block and QT prolongation is an imperfect biomarker for predicting proarrhythmia risk since it is known that multiple drugs inhibit hERG and/or prolong QT, albeit, not leading to TdP [159,167].

The current proarrhythmia testing paradigm relies on the predictive link between drug-induced hERG block and *in vivo*/clinical QT interval prolongation and TdP [157,159]. It provides a valuable example of a screening approach for hazard identification and elimination of compound with predicted toxicities on humans based on the AOP concept. Given the observation that blockade of multiple cardiac ion channels might be predictive

of torsadogenic potential [167,168], the scientific community is moving towards an updated proarrhythmia paradigm promoted by the CIPA (Comprehensive *in vitro* Proarrhythmia Assay) initiative. This initiative is based on the integration of data from *in vitro* testing of multiple cardiac ion channels with mechanistic *in silico* electrophysiology modeling to predict proarrhythmic risk [159,169–173]. The ongoing improvements of the assessment strategy [157,159] through the CIPA initiative is expected to lead to further refinements via an ICH S7B-E14 Questions and Answers process enabling a more efficient, comprehensive and mechanism driven process with greater emphasis of non-clinical data [174–176].

Improved *in vitro* models are required to further enhance the ability to detect and risk assess heart toxicity *in vitro*,. 3D *in vitro* models are attracting interest and attention in drug discovery as promising approaches to investigate both structural and functional toxicity affecting the heart [177–179]. For example, human 3D cardiac microtissue is proposed as a model to capture drug-induced structural cardiotoxicity and gain mechanistic insights [180]; it is noted that this type of model overcomes some of the limitations of current *in vitro* preclinical testing that predominantly focuses on the prediction of functional changes.

4.3 Heart toxicity - Molecular targets

Heart toxicity is investigated by pharmaceutical companies using panels of safety molecular targets that have been associated with different adverse effects [52]. The molecular targets associated with cardiac liabilities as derived from the safety panel by Bowes and co-workers [53] are listed in Table S10 of the supplemental material. This target list is complemented with other off-target panels (see for example Tables S11 and Table S12 of the supplemental information) derived from different studies such as the analysis of human genetic and pharmacology data [55] or other data curation processes [54,56,181]. Associations between molecular targets and structural cardiotoxicity have also been investigated by mining data from FDA Adverse Event Reporting System and assay outcomes from ToxCast leading to the formulation of mechanistic hypotheses of toxicity [155].

4.4 Heart toxicity - *In silico* methods

The schema for the development of an IST protocol for the prediction of potential cardiotoxicants is shown in Figure 6, which combines different types of information and where *in silico* methods can be integrated.

Current *in silico* models for the prediction of cardiac toxicity mainly address hERG inhibition, a surrogate marker for proarrhythmia, and they build on the *in vitro* hERG-related data from early screening in drug discovery and development. The most popular approaches for predicting pharmacological hERG blockade are ligand-based methods that correlate the biological activity to the structural information of chemicals [182,183]. Such methods use approaches such as QSAR (based on different techniques including machine learning), pharmacophore, and 3D QSAR methodologies. Most of these models are classification-based QSARs, but regression-based QSARs have also been proposed to predict activity [184]. *In silico* models using multiple ion channel data (hERG, Cav1.2 and Nav1.5) have been shown to more accurately predict TdP than

models based on hERG effects alone [167,185]. Improving chemical space coverage and quantitative activity prediction remain areas of current research.

Structure-based approaches (e.g., docking) that make use of structural knowledge of the biological target (i.e., hERG) have also been applied to identify hERG blockers [164,186,187].

In silico predictions of inhibition of ion channels were integrated in the CIPA approach [188], the paradigm for the assessment of ventricular proarrhythmic liabilities based on *in vitro* methods and mathematical models simulating cellular cardiac electrophysiologic activity [169,172]. QSAR models based on human data [189–191] have been developed for the prediction of several cardiac adverse effects such as: arrhythmia, hypertension, bradycardia, conduction disorder, electrocardiogram, palpitations, QT prolongation, rate rhythm abnormality, tachycardia, Torsades de pointes, coronary artery disorders, heart failure, myocardial disorders, and myocardial infarction. One of the strengths of using post-market data is that idiosyncratic toxicities can be identified and incorporated into a QSAR model. Unfortunately, such databases suffer from various reporting biases and confounding factors and have been said to be more suitable for signal detection rather than validation (other types of data would be needed to draw reliable conclusions on the observed effects) [192]. Nonetheless, development of QSAR models using these data have been shown to provide useful predictions [190].

Using the hierarchical organization of similar findings (and mechanisms) collated from preclinical toxicity study reports for regulatory submissions (see Table 6), *in silico* models built on different methodologies (e.g., statistical fragment/fingerprint-based models, molecular descriptor-based machine learning models, expert-rule based models) were developed by Amberg and co-workers [36]. It was noted that the initial clustering of the effects affected the resulting predictivity of these models.

4.5 Heart toxicity - *In silico* approaches: data gaps and issues

Currently, alternative approaches for heart toxicity prediction focus on cardiac electrophysiological effects, that pharmaceutical research investigates through an integration of *in silico* and *in vitro* methods; this is then supported by short-duration *in vivo* studies [152]. Regarding structural cardiac toxicity, integrated tiered approaches that exploit predictivity of *in vitro* and *in silico* models are instead generally limited [151].

Development of alternative methods (e.g., *in silico* and *in vitro*) that accurately predict the entire spectrum of cardiac toxicity must rely on robust understanding of the cellular and molecular mechanisms leading to cardiac liabilities. The AOP framework sustains the advance of such understanding (see Table S9 of the supporting material addressing AOPs related to cardiotoxicity).

Available *in vitro*, *in vivo*, and human data on which *in silico* model can be constructed are sparse. As observed by Laverty and coworkers [139], the majority of the adverse effects reported in the FDA's Adverse Event

Reporting System are often not described in detail, and the causal relationship between an adverse effect and a drug is generally not established in the reports provided.

5. Discussion

Different computational methods (e.g., statistical-based methods, rule-based methods) can exist to identify chemicals that potentially induce organ toxicity. These methodologies can be used in a complementary manner, e.g., a statical-based method together with structural alerts. They can also be linked to the AOP framework. For example, structural alerts can be applied to categorize chemicals potentially linking a given class of compounds to a specific mechanism or even MIEs [193].

Application of *in silico* approaches should account for the specific use case, context and thus purpose (e.g., screening, prioritization, classification and labelling, risk assessment, and product development) [193,194]. For example, for consumer safety, a missed hazard may be crucial and lead to subsequent risks; in product development*, in silico* predictions may be used for flagging organ toxicity and prompting scientists to monitor the corresponding liability as the compound advances through discovery.

The present mechanistically-driven analysis of *in silico* methods to predict organ toxicity highlights a number of areas for further research that would enhance such predictions.

It is noted how organ toxicity involves a multitude of biological pathways associated with a plethora of endpoints, and how the underlying molecular mechanisms are often poorly understood. This complicates the development of predictive *in silico* models that are mechanistically-informed. Advances in the understanding of biology at a molecular level would fuel strategies for organ toxicity prediction, based on the integration of different alternative approaches and combination of information in a quantitative manner, such as through defined approaches or on the transcriptomics, proteomics, and metabolomics levels, that are currently lacking to a large extent.

As most *in silico* modeling approaches require a database of historically performed experimental *in vivo* or *in vitro* test results to build such models, the lack of appropriate experimental tests in certain areas provides some challenges. For example, a number of MIEs or key events (KEs) within existing organ toxicity AOPs do not have a corresponding experimental assay or the available assays have limitations such as the lack of metabolic competency. In some situations, as in the case of pulmonary toxicity, the *in vivo* models have strong limitations themselves, which are being addressed with the development of next generation *in vitro* models. Subjective grading (and terminology) of histopathology endpoints represents one of the problems with existing data; however, current digital pathology developments may help come up with more objective and consistent assessments in the future.

Databases containing appropriately annotated information are essential to support any *in silico* model building as well as to support an expert review of the results. There is currently a lack of large *in vivo*

databases covering organ toxicity that (1) are linked to chemical structures, (2) are annotated with the necessary experimental design information, and (3) document both positive and negative (i.e., no treatment related findings) results on findings at tested timepoints and concentrations. These findings should also be linked to the endpoints within the assessment frameworks. Ontologies, standardized terminology, and other technology to support integration and linking of information from different sources are critical. The use of SEND and documents produced through the INHAND working groups will be important to support these databases [195,196]. Toxicogenomic databases are emerging tools that can be used to develop predictive approaches for the classification of chemicals in terms of their toxicogenomic signatures which are, in turn, related to the mechanisms underlying their toxicity. Toxicity is directly linked to gene expression data in databases [197] such as DrugMatrix [198], Open TG-GATEs (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System) [199] and the Comparative Toxicology Database (CTD) [200].

The number of *in silico* models being developed, as discussed in this paper, is rapidly expanding; however, a limited number of models fit in specific areas outlined in the proposed assessment framework, and this limitation concerns models to predict MIE's or *in vivo* models for certain major toxicological endpoints such as kidney toxicity. The training sets used to build any models may also limit the chemical space that such models may predict (i.e., applicability domain consideration).

It is observed that models that predict dose/timepoints are limited in part due to technical limitations and the lack of properly annotated data. Current models for organ toxicity are mainly performing classification, delivering limited information on threshold levels, that, on the other hand, may be evaluated through read-across approaches provided that data on analogues are available (and properly annotated) and that a thorough analysis of the chemicals establishes a sound similarity between the source chemical and its analogues. Ordinal models (based on ranges of toxic concentration) that are technically more tractable may provide a way forward to support necessary risk assessment decisions. Such quantitative models can support the safety evaluation of compounds in different contexts including those frameworks where *in vivo* experiments are limited by regulations (e.g., cosmetics).

The importance of internal exposure and in general of the ADME processes has been highlighted, identifying factors (e.g., formation of reactive metabolites) that need to be accounted for when developing the IST organ toxicity protocol. Metabolism is an important element to evaluate for specific organ toxicity (e.g., lung, kidney). Xenobiotic enzyme activity in different organs should be considered as it affects the rate and extent of formation of reactive metabolites. For example, *in silico* technology to predict metabolites, identify the points of metabolism, or predict binding to CYP enzymes is available and should play a role in the integration of the information as well as incorporated into any expert review. Currently, the prediction of metabolites may result in a high number of many predicted metabolites originating from a multitude of potential pathways, that may need to be critically evaluated. Likewise, it remains difficult to predict absolute

likelihoods (as opposed to relative likelihoods) of metabolism at particular sites. ADME considerations are also important in support of the extrapolation of any *in vitro* experiment data (or models derived from such data) to *in vivo* outcomes, as well as for inter-species extrapolation. Species differences is another important element that need to be critically evaluated (e.g., different nasal/pharyngeal anatomy in the context of lung toxicity) to translate results to humans.

The development of frameworks capturing the key characteristics of toxicants to a specific target organ, similar to the ten key characteristics of carcinogens [201–203], would provide valuable organizational principles for the IST framework. Key characteristics do not necessarily represent mechanisms nor are adverse outcome pathways, but they provide a broad and holistic structure to organize relevant mechanistic data for human health assessments of possible toxicants. This construct was first introduced for carcinogens and it is now under consideration in other contexts such as for hepatotoxicants, neurotoxicants and developmental neurotoxicants and cardiotoxicants [204,205].

6. Conclusion

This work is a mechanistically-driven analysis of the current state of the art with respect to the *in silico* prediction of organ toxicity (with focus on heart, lung and kidney) and it includes an overview of key characteristics/mechanisms and how they contribute to organ toxicity. A summary of the major topics discussed throughout the work is summarized in Table 7.

Table 7. Main topics discussed in the present work.

Main topics
• Overview of key characteristics/mechanisms is presented with reference to the AOP construct. • Importance of mitochondrial dysfunction across different organ toxicities is highlighted. • Relevant endpoints for each target organ are discussed. • Binding to molecular targets that are associated with adverse effects to specific organs (i.e., off-target panels from secondary pharmacology batteries) is discussed. • *In vitro* and/or *in vivo* models for investigating target organ toxicity and detecting corresponding toxic xenobiotics are discussed alongside emerging experimental approaches such as 3D *in vitro* models and toxicogenomics. • An overview is given of computational methods (statistical models, expert alerts, read-across) that can be used to identify chemicals that potentially induce organ toxicity with reference to specific key characteristics/mechanisms, if any. • Data gaps and challenges ahead for the development of computational methods predictive of target organ toxicity are discussed.

Declaration of Competing Interest

Disclaimer

CDC Disclaimer

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry.

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Supplementary data

(see supplementary file)

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Figure Legends

Figure 1. Schematic workflow encoded in the in silico toxicology protocols [5].

Figure 2. Draft outline of potential hazard assessment framework for organ toxicity (adapted from [8]). The draft framework combines information from in vitro approaches (e.g., biological responses from receptor-based assays), in vivo experiments, and human data. Other protocols (e.g., ADME or other organs) may feed a protocol for a given organ. Exposure scenarios (e.g., environmental, drug, consumer, accidental) may also be used to supplement the protocol. Effects (predicted by in silico methods or measured experimentally) are combined for the assessment of a given endpoint.

Figure 3. Types of data in a draft assessment framework that needs to be considered for the development of an IST protocol for the identification of potential kidney toxicants.

Figure 4. Toxicity to lung includes different endpoints such as irritation (transient effects) and sensitization (immune-mediated response). Experimental data on lung toxicity originates from different sources and they are combined in a decision framework for hazard assessment; for example, in vitro data may originate from assays investigating molecular targets associated with lung toxicity, such as TRPA1, an ion channel whose activation is proposed to induce sensory pulmonary irritation (see supplementary material). In silico methods build on available experimental data and they can thus be integrated in the overall hazard assessment framework.

Figure 5. Heart's possible response to toxic injury induced by xenobiotics [150,153]. Functional and structural adverse effects are interrelated: primary functional effects may occur with possible secondary structural effects; similarly, primary adverse effects on cardiac structure may occur with secondary functional changes. Myocardial contractility may be altered by functional effects (effects on contractile proteins, Ca^{2+} or mitochondria) or structural perturbations (loss of cardiomyocytes following apoptosis or necrosis and possible replacement with less contractile fibrotic tissue).

Figure 6. Schema for the assessment framework of heart toxicity. Human data (measured or predicted) include endpoints such as arrhythmia and heart failure. In vitro data may be collected from different types of assays such as binding assays, functional flux assays, patch clamp, Langendorff perfused heart assay, Microelectrode Arrays, impedance assays, high content imaging assays, cytotoxicity assays. Other types of data standardized in different protocols can be integrated such as in vitro ADME profiling and toxicokinetics data.

Figures

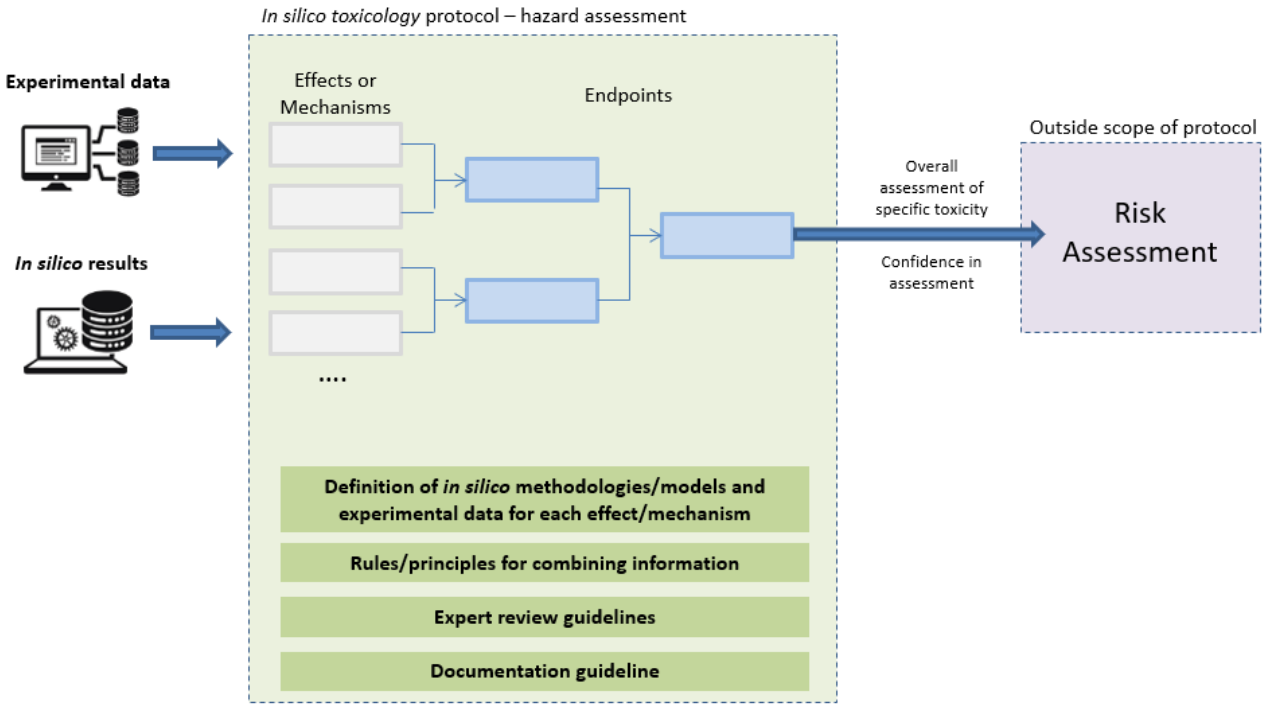


Figure 7. Schematic workflow encoded in the in silico toxicology protocols [5].

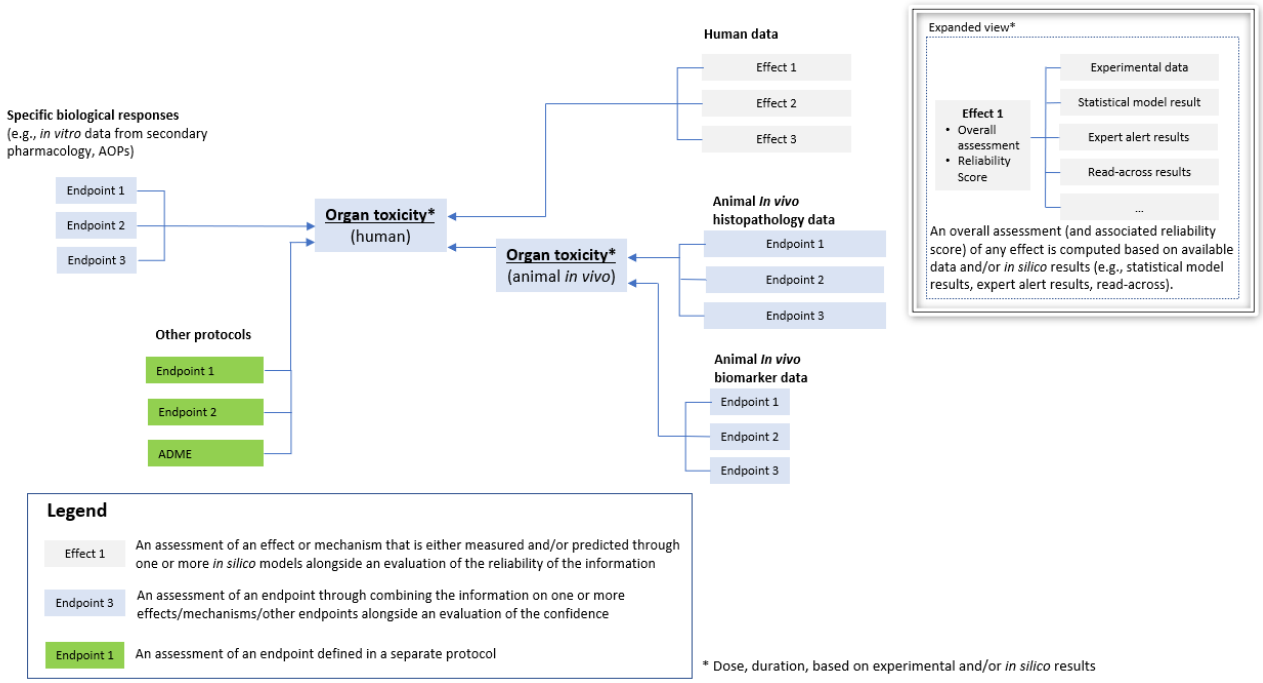


Figure 8. Draft outline of potential hazard assessment framework for organ toxicity (adapted from [8]). The draft framework combines information from in vitro approaches (e.g., biological responses from receptor-based assays), in vivo experiments, and human data. Other protocols (e.g., ADME or other organs) may feed a protocol for a given organ. Exposure scenarios (e.g., environmental, drug, consumer, accidental) may also be used to supplement the protocol. Effects (predicted by in silico methods or measured experimentally) are combined for the assessment of a given endpoint.

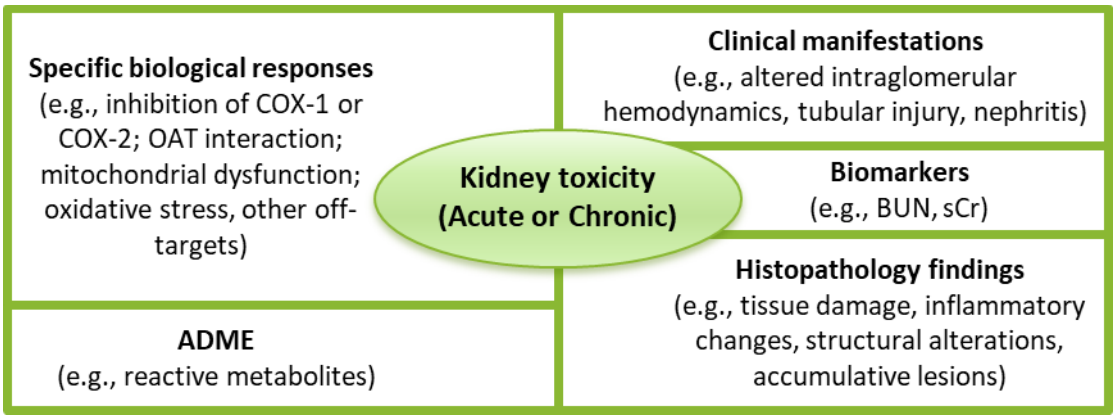


Figure 9. Types of data in a draft assessment framework that needs to be considered for the development of an IST protocol for the identification of potential kidney toxicants.

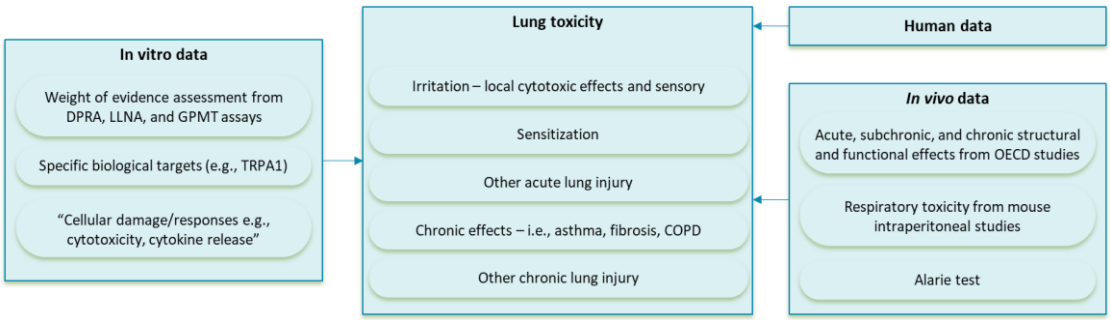
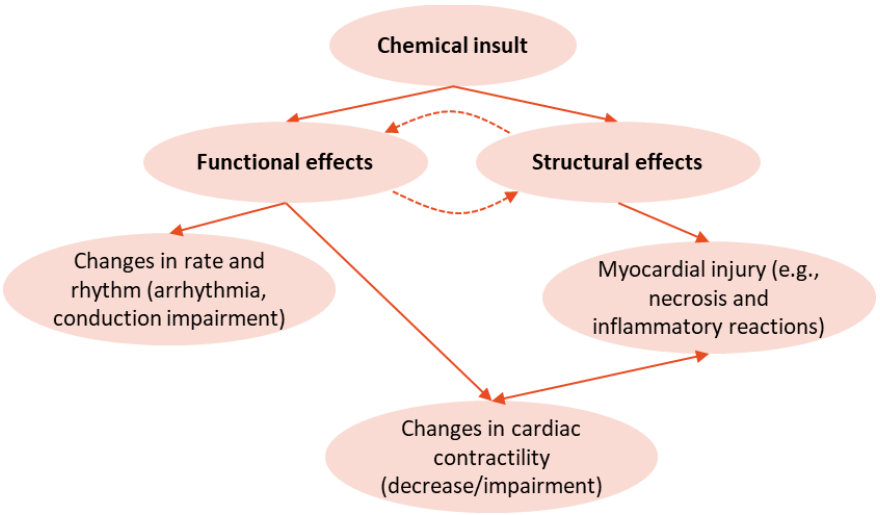


Figure 10. Toxicity to lung includes different endpoints such as irritation (transient effects) and sensitization (immune-mediated response). Experimental data on lung toxicity originates from different sources and they are combined in a decision framework for hazard assessment; for example, in vitro data may originate from assays investigating molecular targets associated with lung toxicity, such as TRPA1, an ion channel whose activation is proposed to induce sensory pulmonary irritation (see supplementary material). In silico methods build on available experimental data and they can thus be integrated in the overall hazard assessment framework.



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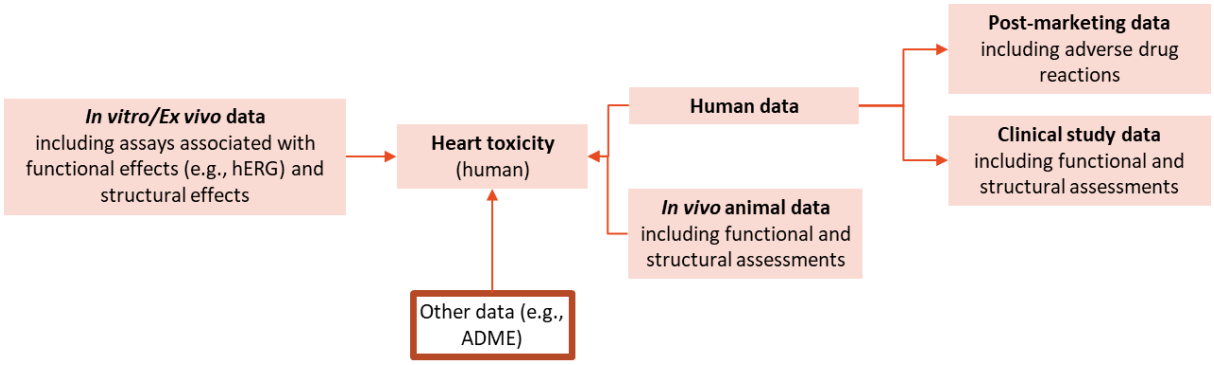

Figure 12. Schema for the assessment framework of heart toxicity. Human data (measured or predicted) include endpoints such as arrhythmia and heart failure. In vitro data may be collected from different types of assays such as binding assays, functional flux assays, patch clamp, Langendorff perfused heart assay, Microelectrode Arrays, impedance assays, high content imaging assays, cytotoxicity assays. Other types of data standardized in different protocols can be integrated such as in vitro ADME profiling and toxicokinetics data.