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The evolving role of investigative toxicology in the pharmaceutical industry

Francois Pognan [1] ✉, Mario Beilmann[2], Harrie C. M. Boonen[3], Andreas Czich[4], Gordon Dear[5], Philip Hewitt[6], Tomas Mow[7], Teija Oinonen[8], Adrian Roth[9], Thomas Steger-Hartmann[10], Jean-Pierre Valentin [11], Freddy Van Goethem[12], Richard J. Weaver [13] & Peter Newham [14] ✉

Abstract

For decades, preclinical toxicology was essentially a descriptive discipline in which treatment-related effects were carefully reported and used as a basis to calculate safety margins for drug candidates. In recent years, however, technological advances have increasingly enabled researchers to gain insights into toxicity mechanisms, supporting greater understanding of species relevance and translatability to humans, prediction of safety events, mitigation of side effects and development of safety biomarkers. Consequently, investigative (or mechanistic) toxicology has been gaining momentum and is now a key capability in the pharmaceutical industry. Here, we provide an overview of the current status of the field using case studies and discuss the potential impact of ongoing technological developments, based on a survey of investigative toxicologists from 14 European-based medium-sized to large pharmaceutical companies.

Sections

[1]Discovery and Investigative Safety, Novartis Pharma AG, Basel, Switzerland. [2]Nonclinical Drug Safety Germany, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany. [3]Drug Safety, Dept of Exploratory Toxicology, Lundbeck A/S, Valby, Denmark. [4]R&D Preclinical Safety, Sanofi, Frankfurt, Germany. [5]In Vitro In Vivo Translation, GlaxoSmithKline David Jack Centre for Research, Ware, UK. [6]Chemical and Preclinical Safety, Merck Healthcare KGaA, Darmstadt, Germany. [7]Safety Pharmacology and Early Toxicology, Novo Nordisk A/S, Maaloev, Denmark. [8]Preclinical Safety, Orion Corporation, Espoo, Finland. [9]Pharmaceutical Research and Early Development, Roche Innovation Center Basel, Basel, Switzerland. [10]Investigational Toxicology, Bayer AG, Berlin, Germany. [11]Non-Clinical Safety Evaluation, UCB Biopharma SRL, Braine-l'Alleud, Belgium. [12]Predictive, Investigative & Translational Toxicology, Nonclinical Safety, Janssen Research & Development, Beerse, Belgium. [13]Innovation Life Cycle Management, Institut de Recherches Internationales Servier, Suresnes, France. [14]Clinical Pharmacology and Safety Sciences, AstraZeneca R&D, Cambridge, UK. ✉e-mail: francois.pognan@novartis.com; peter.newham@astrazeneca.com

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Introduction

New tools and strategies in investigative toxicology are continually being implemented in pharmaceutical companies to reduce safety-related attrition in drug development[1,2]. A key objective of investigative toxicology is the prediction of clinical safety, which is still a high hurdle. The chemical and biological uniqueness of each drug candidate (which limits the predictive value of knowledge from previous drugs) and the limited translational relevance of many standard preclinical toxicity models to humans contributes to unforeseeable late-stage attrition. Current investigative toxicology strategies therefore adopt a tiered approach, employing progressively more complex in silico, biochemical, cellular in vitro assays and in vivo safety experiments as drug candidates progress through the pipeline to build a strong platform for identification and mitigation of hazards, and finally a risk assessment for the drug candidate. Computational approaches and prospective assays aim to predict toxicities that could lead to the termination of a drug candidate or an entire development programme, and bespoke retrospective assays are used for issue resolution and management if target-organ toxicities are identified in in vivo 'toxicology signal-generation' studies or later chronic studies (Fig. 1). Crucially,

the goal of toxicology in the discovery phase is not to simply 'front-load' attrition, but to increase the likelihood of success by optimizing the safety dimension of drug design and candidate selection such that subsequent non-clinical programmes and clinical trials can successfully test clinical hypotheses.

The key to successful investigative toxicology in drug discovery is to efficiently combine safety data with other compound-specific features, such as absorption, distribution, metabolism and excretion (ADME) and physicochemical properties, which requires timely and integrated experimental approaches to address the risks inherent in the potential target and in potential lead candidates. This involves a paradigm shift from the use of classical (and generally low-throughput) in vivo toxicology methods towards a focus on translatable mechanistic in vitro assays that can act as reliable predictive surrogates for specific aspects of in vivo studies. Such assays rely heavily on the development of human physiologically relevant model systems in combination with appropriate toxicity end points.

In recent years, there have been major advances in areas such as the use of induced pluripotent stem cells (iPSCs), 3D tissue models, advanced microphysiological systems (MPS) and imaging techniques
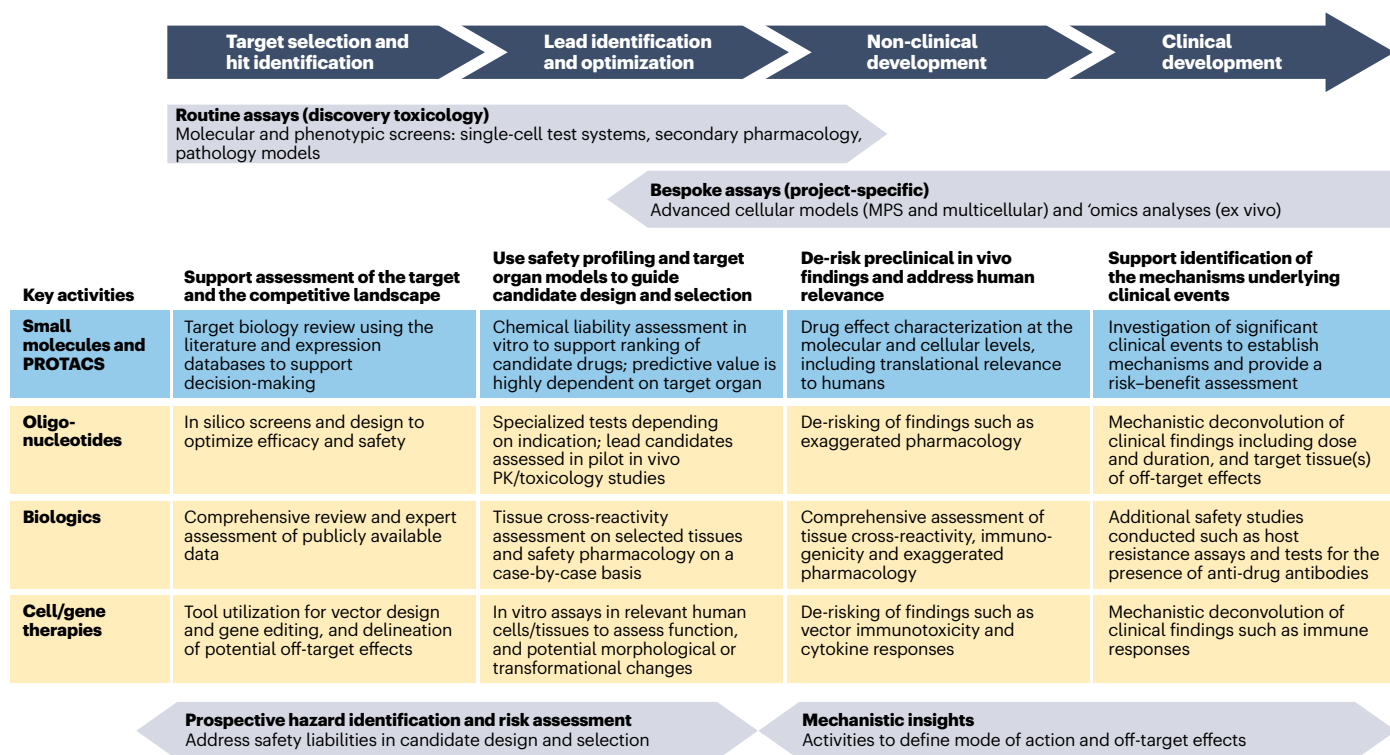
Key activities	Support assessment of the target and the competitive landscape	Use safety profiling and target organ models to guide candidate design and selection	De-risk preclinical in vivo findings and address human relevance	Support identification of the mechanisms underlying clinical events
Small molecules and PROTACS	Target biology review using the literature and expression databases to support decision-making	Chemical liability assessment in vitro to support ranking of candidate drugs; predictive value is highly dependent on target organ	Drug effect characterization at the molecular and cellular levels, including translational relevance to humans	Investigation of significant clinical events to establish mechanisms and provide a risk–benefit assessment
Oligo-nucleotides	In silico screens and design to optimize efficacy and safety	Specialized tests depending on indication; lead candidates assessed in pilot in vivo PK/toxicology studies	De-risking of findings such as exaggerated pharmacology	Mechanistic deconvolution of clinical findings including dose and duration, and target tissue(s) of off-target effects
Biologics	Comprehensive review and expert assessment of publicly available data	Tissue cross-reactivity assessment on selected tissues and safety pharmacology on a case-by-case basis	Comprehensive assessment of tissue cross-reactivity, immunogenicity and exaggerated pharmacology	Additional safety studies conducted such as host resistance assays and tests for the presence of anti-drug antibodies
Cell/gene therapies	Tool utilization for vector design and gene editing, and delineation of potential off-target effects	In vitro assays in relevant human cells/tissues to assess function, and potential morphological or transformational changes	De-risking of findings such as vector immunotoxicity and cytokine responses	Mechanistic deconvolution of clinical findings such as immune responses

Fig. 1 | Key goals of investigative toxicology in drug discovery and development. The figure shows a roadmap of investigational toxicology activities that can be applied at each stage of the pipeline. In the earlier stages, these activities typically involve computational assessments to support target selection and batteries of routine in vitro molecular and cellular assays to support lead identification and optimization. These assays evaluate broad characteristics such as cytotoxicity, mitochondrial and genetic toxicity, as well as effects on specific cell types such as hepatocytes and cardiomyocytes to evaluate the risk of toxicity to particular organs. At later stages of the process, regular in vivo toxicology studies that support progression into clinical development may be complemented by bespoke project/target-organ-specific assays — for example, with more advanced cellular models (microphysiological systems (MPS) and

multicellular assays) — to address the human relevance of preclinical in vivo findings and gain mechanistic insights (for example, on off-target effects). It is important to stress that approaches may vary significantly between companies based on internal drivers, priorities, resources and history. The focus of the figure is on approaches for small molecules, with selected activities specific for other modalities shown below. It is currently unclear whether the roadmap of toxicology activities for small molecules adequately covers the safety evaluation needs for targeted protein degraders such as proteolysis-targeting chimeras (PROTACS). There are several PROTAC-specific attributes that must be considered, including off-target degradation, intracellular accumulation of the natural substrates for the E3 ligases used in the ubiquitin–proteasome system and proteasome saturation by ubiquitylated proteins[185–188]. PK, pharmacokinetic.

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Box 1

Investigative toxicology: a pharmaceutical industry survey

To obtain a detailed assessment of the adoption, range and impact of investigative toxicology in supporting drug discovery and development in the pharmaceutical industry, a survey of 14 medium-sized to large pharmaceutical companies was conducted by the authors of this article (Supplementary information). These companies are engaged in therapeutic areas that include oncology, neurology, cardiovascular and immunology (50–80% of portfolio), but also infection, and metabolic and respiratory disease. Although the predominant focus of investigational toxicology is small-molecule drugs, six of the companies have a portfolio of biologic-based therapeutics, while other modalities such as oligonucleotides make up only a small fraction. Thus, the focus of investigational toxicology so far is on small-molecule drug discovery and development, with a growing need for experimental support of newer drug modalities.

The survey revealed several crucial attributes of investigational toxicology.

- It is an integral part of non-clinical safety organizations, with dedicated groups performing 55% of all experimental work in-house — consistent with the need for speed and flexibility to address safety questions that arise from drug discovery projects.
- There is a constant need for new experimental models or technologies to address gaps in the predictivity of current assays and models and the ability to translate the data to a clinical setting, including the dose or exposure at which this will occur.
- Common deliverables of pharmaceutical investigative toxicology include target safety assessment, hazard profiling of leads (in silico/in vitro) and in-depth hazard qualifying/quantifying experiments that support lead optimization and drug candidate selection before in vivo studies are initiated.
- In vitro assays are commonly used for hepatotoxicity and cardiovascular toxicity profiling; however, there is a crucial need to improve the in vitro–in vivo translation. Complex 3D models and

sophisticated microphysiological systems (MPS) that combine diverse biochemical, gene expression, high-content imaging, reporter gene and functional readouts are being explored in a bid to improve drug toxicity prediction.

- During initial in vivo studies, investigative toxicology has a key role in terms of understanding the mode of action of toxicities observed and their relevance to humans[2]. Although, historically, dose-limiting toxicity in in vivo studies (in the absence of an acceptable therapeutic index) has often led to termination of a given drug candidate, a deep understanding of the mechanism that underlies this toxicity together with an estimate of its human relevance may allow a project to continue[189].

Although the survey revealed several broad consistencies with regard to the purposes and challenges of investigational toxicology, it also highlighted marked differences in how in vitro toxicity data are used within drug discovery projects. Significantly, we found that although ~50% of pharma companies surveyed used in vitro data to make decisions on compound liabilities, a similar proportion of respondents used such toxicity data "for information only", with a more detailed risk assessment being deferred until the availability of in vivo study data. This reflects different strategies that companies can take: the former decision-making approach aims to steer drug design when there is broader chemical scope to do so, whereas the latter avoids undue influence until a significant issue is detected in preclinical toxicology studies. The risk of the former approach is to unduly reject adequately safe compounds, while issues detected with the latter approach can be challenging to understand and resolve in a timely manner. Both approaches are enhanced by in silico and in vitro models coupled to end points that can provide translationally relevant mechanistic insights into drug effects.

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that have the potential to substantially improve the predictive value of investigative toxicology assays[3]. There are a vast number of new platform technologies, and a comprehensive description of all of them is beyond the scope of this article. However, through a survey of 14 Europe-based medium-sized to large pharmaceutical companies (Box 1 and Fig. 2a), we identify key emerging technologies that are actually having an impact and how they are being practically and more routinely incorporated currently in drug discovery. In addition, we consider which technologies − through appropriate development − have the potential to have a 'game-changing' impact on investigational toxicology and how these have evolved over a 5-year period (Fig. 2b).

In this article, we first summarize the key goals of investigative toxicology and highlight the current approaches used in investigative toxicology using published case studies, mainly from across the group of companies surveyed. We then discuss selected emerging technologies that have the potential to shift the current safety-testing paradigm, with a focus on those considered most likely to have the greatest near-term impact. Finally, we consider the role of collaborations and

pre-competitive consortia in enabling the development, validation and effective application of these next-generation tools.

Goals and tools for investigative toxicology

Investigative toxicology can be broadly split into two approaches: the prediction of potential toxicity before a compound is tested non-clinically in vivo or clinically (prospective approach), or the provision of mechanistic understanding of non-clinical in vivo or clinical toxicity findings (retrospective approach) (Fig. 1). These approaches have two major goals: in the drug discovery phase, the aim is to guide the identification of the most promising drug candidates to pursue (that is, safe candidates that provide the best therapeutic index) and to deselect the most toxic drug candidates as early as possible. In the preclinical and clinical development phases, the purpose is to provide mechanistic safety data that enable development of a well-characterized hazard and translational risk profile to support clinical trial design (that is, risk assessment, management and mitigation) (Fig. 1). In this section, we summarize the key activities in investigative toxicology as a drug

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Fig. 2 | **Assessment of the 'game-changing potential' of novel assays and technologies in investigative toxicology.** We assessed the perceived immediate, mid-term (<2-year) and longer-term (<5-year) benefit from the routine application of various emerging technologies and assays through surveys of pharmaceutical company experts in 2015 and 2020 (Box 1 and Supplementary information). **a**, Summary of current technologies or methods that offer a step change in investigational toxicology; impact according to consensus and time frame identified by experts from Europe-headquartered pharmaceutical companies ($n = 14$) responding to the question "Which new technologies do you foresee may become game-changers for investigational toxicology?" in 2020. Variations in perceptions across the respondents potentially reflect current practices and usage within their organizations. **b**, Evolution among the respondents of the impact perception for each technology or method from 2015 to 2020. The positive perception of some technologies such as organs-on-chips, genomic profiling and high-content imaging increased, while the perception of others barely changed or the time frame for impact was adjusted (systems toxicology and induced pluripotent stem cells (iPSCs)). Other technologies have clearly lost ground, with the 'no potential game-changer' category becoming more prominent (metabolomics, microRNAs (miRNAs) and mass spectrometry imaging). qPCR, quantitative PCR; RNA-seq, RNA sequencing.

candidate progresses through the pipeline. We focus primarily on small-molecule drugs; however, a high-level comparison with the activities for other therapeutic modalities is provided in Fig. 1.

Support assessment of the target and the competitive landscape

In the traditional approach to regulatory toxicity assessment, before progressing a drug candidate to 'first-in-human' clinical trials, findings of the pivotal regulatory good laboratory practice (GLP) in vivo studies are presented to pharmacologists and clinicians for joint discussion, with the occasional outcome that the toxicological findings could be attributed to effects mediated by the primary target of the drug[4]. Over the past decade, the traditional analysis has been widened to consider the broader physiological role of the target in health and disease, based on a systematic search of the literature and databases. This search should address at least the following topics: description of the main target functions and upstream and downstream signalling

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pathways, description of closely related targets (orthologues and paralogues), analysis of cross-species target homology, function and tissue expression across species, and phenotypic consequences of genetic modifications to the target in animals (knockout and knock-in; conditional or not). Results of competitive landscape analyses, including identification of lead structures from competitors, if possible, should also be incorporated. Target safety assessment may also be important in the evaluation of potential candidates for in-licensing by larger companies (Box 2).

A thorough target safety assessment may contribute to the selection of the most appropriate preclinical safety species; for example, by avoiding non-relevant species that do not express the target. It could also identify potentially affected target organs and tissues that need to be integrated into screening studies, alongside biomarkers for monitoring the occurrence and extent of pharmacodynamic responses[2]. Ultimately, the target safety assessment may lead to a stop/go decision for the particular target based on the target patient population, as well as an experimental plan to quantify risks if the project progresses further[1,5].

For example, the family of aspartic proteases from mammals (such as cathepsins, napsin A, pepsin renin and β-secretases) and non-mammals (such as plasmepsins) are an important drug target family[6,7]. However, inadequate or suboptimal selectivity for some aspartic proteases may hamper the development of safe and effective therapies. Evaluation of data collated from in vitro investigations, in vivo animal studies and adverse drug reactions reported in patients treated with approved aspartic protease inhibitors highlighted functional and structural toxicities associated with inhibition of mammalian aspartic proteases[8]. Of these, cathepsins D and E may be of most toxicological relevance, as cathepsin D is a ubiquitous lysosomal enzyme present in most cell types, and cathepsin E is found in the gut and in erythrocytes; that is, tissues likely to be exposed to high drug concentrations. Of particular toxicological relevance, human cathepsin D deficiency has been reported as a cause of congenital human neuronal ceroid-lipofuscinosis, which is a severe lysosomal storage disease characterized by neurodegeneration, developmental regression, visual loss, epilepsy and premature death owing to excessive tissue accumulation of lipopigments (lipofuscin)[9,10]. Moreover, several structurally distinct β-secretase (BACE1) inhibitors have been withdrawn from development after inducing ocular toxicity following chronic treatment in animal models. Quantification of cathepsin D target engagement in cells has been shown to be predictive of ocular toxicity in vivo, suggesting that off-target inhibition of cathepsin D was a principal driver of ocular toxicity for these agents[7]. This example illustrates the need for a thorough assessment of the risks of inhibiting closely related targets when developing drugs for targets in families such as aspartic proteases.

Use safety profiling and target-organ models to guide candidate design and selection

Prediction and/or understanding of organ-specific drug toxicities can be achieved with varying levels of confidence using in vitro cell models. Simple cell death monitoring in vitro, as a rough surrogate of toxicity potential in vivo, is widely used to eliminate compounds early in the candidate selection process[2,11,12]. This works adequately for phototoxicity and genotoxicity, for which there is a simple and close correlation between in vitro and in vivo outcomes. However, there are usually no such relationships between in vitro cytotoxicity and organ function alterations, and so more sophisticated models have been developed

to assess compound-induced perturbations of cellular functions and thus provide information on the potential for specific tissue and organ adverse effects[13].

Initially, work towards this goal focused on measuring simple end points such as cell survival through ATP content or other cytotoxicity end points in organ-derived cell lines that are accessible and amenable to high-throughput screening (HTS), which are still used heavily in industry. Primary cells with improved tissue or organ phenotypes are also used, but sourcing primary cells of suitable quality remains challenging, especially in quantities sufficient to support larger screening programmes and consistent testing over many years. In addition, the ethical and legal issues for human tissue sources require careful consideration. More recently, 2D and 3D cultures and MPS have been developed that attempt to mimic micro-environmental conditions by incorporating multicellular tissues, scaffolding and mechanical factors based on cells such as cardiomyocytes derived from iPSCs or extended pluripotent stem cells (ePSCs)[14–17]. Models for most major toxicologically relevant organ systems have been described[18,19] (Fig. 3). These have been reviewed elsewhere and so are not covered in depth here[17–20].

Each in vitro target-organ approach − from simple cell lines through to MPS − has intrinsic challenges and opportunities (summarized in Fig. 3), and so the use of multiple 'fit-for-purpose' assays best represents the current practice. Simple cell cultures with relatively straightforward end points can provide throughput and robustness for screening and specific mechanistic end point studies (for example, mitochondrial toxicity), but not the complexity often required to reflect the in vivo situation. These may be complemented with

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Box 2

Investigative toxicology in due diligence for externally sourced drug candidates

Over the past 10 years, the pharmaceutical innovation model has shifted towards externalizing innovation[190,191], and a side effect of this shift has been the downsizing of internal safety departments in most companies[192]. This leads to a key challenge: how to integrate investigative toxicology expertise in the early assessment of new external innovations, particularly for new assets in early development stages for which insights into the potential off-target and safety aspects are often limited because the academic institutions or biomedical start-up companies from which they originate have limited financial resources and internal capacities for an in-depth safety assessment. Furthermore, the due diligence team from the company assessing the asset for potential acquisition cannot easily conduct additional studies until a contractual agreement has been reached. A solution to this dilemma is comprehensive use of in silico and read-across approaches to evaluate both the target and the compound internally, leveraging the broad chemical and target experience of the company performing the due diligence. Identified questions and potential liabilities could then be integrated into contract design (for example, via milestone payments).

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	Cell lines	Primary cells	Stem cells	3D systems and organoids	MPS
Heart	• Limited availability of cardiomyocyte phenotypes • Limited predictive value for cardiotoxicity assessment	• Human cells not readily available • Cells rapidly dedifferentiate • Representative of the adult heart morphologically and functionally • Low throughput • High predictive value	• Amenable to long-term culture • Terminally differentiated • Phenotype often immature • Respond to electrical stimulation • Replicate pharmacological effects	• Amenable to long-term culture; multi-cell micro-tissues viable and functional for weeks • Predominantly human iPSC-CMs • Phenotype often immature • Replicate pharmacological effects	• Human iPSC-based and perfusion systems • Non-cardiomyocytes can be integrated • Maturing phenotype • Can measure mechanical and electrical loading • Low throughput • Predictive value largely to be determined
Liver	• HepG2 used for identification of cytotoxic compounds • Limited functionality; for example, low metabolic and transporter competence • HepaRG cell line offers greater metabolic transporter competence and phenotypic stability	• Hepatocyte phenotype with some metabolic capability • Rapidly lost in culture • Strong donor-to donor CYP variation • End points mainly assess cytotoxicity or specific organelle function	• Stem-cell-derived hepatocyte-like cells commercially available • No robust differentiation protocols yet available • Lack maturity of adult hepatocytes	• Microtissue and co-culture models available (cell line and primary cells) • May offer a model for detection of chronic and inflammation-mediated toxicity • Increased sensitivity to liver toxins • Lower throughput for screening and size limitations to avoid producing necrotic spheroid cores	• Flow removes drug metabolites and bile acids, which could affect toxicity of static models • Flow and oxygen-mediated zonation phenotypes more closely recapitulate centrilobular, midzonal and periportal functionality • CYP zonation reproduces in vivo toxicity regionalization • Low throughput
Lung	• A549 cells exhibit many features of alveolar type II cells • Immortalized BEAS-2B cells used as model for upper airway • Rat NR8383 cell used for phospholipidosis	• Normal human trachea–bronchial epithelial cells or disease biopsy material • Used in cytotoxicity, function and barrier resistance assays	• Protocols for producing lung epithelial cells available • Further optimization and testing required	• 3D models of human trachea–bronchial epithelial cells • Differentiate to form mucociliary structures • Airborne drug/particle assays mimicking inhalation in vivo	• Biomimetic microdevice with mechanical breathing • Can model lymphocyte recruitment from the circulation • Movements for mechanistic toxicity and disease testing passed proof-of-principle • Low throughput
Kidney	• Kidney cell lines rarely used for safety assessment • Drugs mostly affect tubules • Cellular models of cortex include TERT-immortalized proximal tubular cells (RPTEC-TERT1)	• Primary cells from kidney (proximal tubule epithelia, podocytes, mesangial cells), obtained from various sources • Used for mechanistic understanding of specific cases	• iPSC-derived kidney cells show promising predictivity • Validation ongoing	• Engineered 3D proximal tubules exhibit enhanced epithelial morphology and functional properties compared with same cells grown in 2D • Validation ongoing	• Bioprinting used to create 3D human renal proximal tubules in vitro within an extracellular matrix and in perfusable chips • Glomerular structure in MPS has been reported • Long-term culture • Low throughput • Validation needed
CNS	• Various cell types, such as mouse P19 neurons, human neuroblastoma SH-SY5Y cells • Used for CNS toxicity assessment	• Microelectrode array technology • Determination of spontaneous electrical activity from networks of primary rat cortical neurones • Validation ongoing	• Different types of human iPSC-derived cells and MEA technology • iPSC-derived peripheral neurons show burst-like activity on MEA chips, indicating functional neuronal network	• Extracellular matrix scaffolds with primary human cortical neurons assessed • iPSCs differentiated into neural progenitor cells and neuronal astroglia lineages and cultured in 3D • Biological characterization promising	• iPSC-derived human 3D models comprised of differentiated mature neurons and glial cells • Appear to reproduce neuron–glia interactions and connectivity after 8 weeks in culture • Exhibit key neuronal function • Low throughput • Highly promising
GI	• Caco-2 (colorectal adenocarcinoma) as standard for GI function and drug absorption • Increased functionality for safety end points in models with flow	• Tissue-derived intestinal epithelial cell cultures • Cytotoxicity, absorption and metabolism	• Primary stem cells are used for organoid cultures • iPSCs differentiated into intestinal-like tissue in vitro	• Self-organizing 3D organoids and multicellular microtissue derived from intestinal stem cells • Enable functional comparison across species, GI regions and healthy versus diseased cells	• Gut-on-chip with potential for pulsatile flow, peristaltic stretch • Co-culture with inflammatory cell and/or commensal microbes • Activation of sensory neurons • Low throughput • Validation needed • Significant potential

Level of confidence: ☐ Low ☐ Medium ☐ High ☐ Very high

Fig. 3 | In vitro models of target-organ toxicity. The figure summarizes the status of in vitro test systems that are amenable across key target organs. Key characteristics of each organ and its model options are summarized. Boxes are coloured according to the currently perceived confidence in safety translation among the survey participants. This empirical assessment is based on published data, considering the ability of the model to emulate key organ phenotypes (and stability for extended periods of culture) and toxicology validation data. More details on in vitro cell culture assays for each organ system with associated references are available in Supplementary Table 1. CNS, central nervous system; CYP, cytochrome P450; GI, gastrointestinal; iPSC, induced pluripotent stem cell; iPSC-CM, iPSC-derived cardiomyocytes; MEA, microelectrode array; MPS, microphysiological systems.

organoids or MPS, or even with organ explants and slices to provide in-depth mechanistic understanding. Indeed, connected organ models in single MPS chips are emerging and are starting to be applied for drug discovery risk assessment. A 'heart-on-a-chip' connected to a 'liver-on-a-chip' via a microfluidics system was used to derive in vitro temporal pharmacokinetic/pharmacodynamic (PK/PD) relationships for the

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histamine receptor antagonist terfenadine: the terfenadine-induced increase in QT interval of the 'heart-on-a-chip' was reduced when coupled to a metabolically competent liver compartment. Importantly, when the model was applied to a small-molecule drug discovery programme, a representative molecule was identified as having a hERG liability, due to the formation of a hERG active metabolite[21]. Thus, such 'heart-on-a-chip' coupled with a 'liver-on-a-chip' systems have the potential to reveal complex metabolic and toxicological relationships earlier in the drug discovery process.

Crucially, there is an opportunity for the large amounts of multi-factorial data generated to be used to feed algorithms for the mathematical modelling of drug liabilities[22]. However, full integration of multi-factorial data and further refinement of test systems are still required. This necessitates extensive work on qualitative and quantitative understanding of complex metabolic and toxicological relationship[23,24] models to ensure that in vitro data are sufficiently predictive of in vivo non-clinical and clinical findings. This in turn will help to identify a fit-for-purpose battery of target-organ assays and test systems for use in early selection of the safest compounds through a deeper understanding of the mechanisms of drug toxicity.

The general approach and challenges for understanding the potential for target-organ toxicities can be illustrated for the liver, for which a wide range of in vitro assays has been and is being developed for toxicological assessment. Hepatic cell lines constitute an integral part of most pharmaceutical companies' safety screening strategies and can provide an initial hazard warning system[13,25,26]. However, several of these transformed cells can poorly reflect native primary human hepatocytes, as revealed by transcriptomic and proteomic phenotype differences[27–29]. Consequently, their toxicological utility without a priori knowledge of the physiological and pharmacological characteristics and mechanistic end point under investigation can be relatively low[30–32]. Primary human hepatocytes in 2D cultures also have limitations to their use for toxicological studies, because cells de-differentiate[27]. Culturing hepatocytes in 3D 'spheroid' configurations appears to overcome this issue to some extent[33,34]. Other examples are discussed in the section on advanced cellular models below.

In addition to understanding potential target-organ toxicities through in vitro assays, another key investigative toxicology activity at this stage of the drug discovery process is pharmacological profiling to identify undesirable off-target activity that could hinder or halt the development of drug candidates. At the molecular level, secondary pharmacology studies evaluate drug effects against a broad range of molecular targets that are related to or distinct from the intended therapeutic target[35–37]. Numerous targets — including G protein-coupled receptors, transporters, ion channels, nuclear receptors and kinases — have been associated with significant side effects in non-clinical or clinical settings[35–37], and the list is continually expanding. It has been reported that compounds with a target hit rate of ≥20% (where the target hit rate is the percentage of a panel of at least 50 targets for which more than 50% binding is noted at a compound concentration of 10 μM) are associated with a higher attrition rate[37–39]. Higher hit rates are often, but not exclusively, related to physicochemical properties of compounds, particularly lipophilicity[40]. These properties may also lead to artefactual potent target inhibition due to colloidal aggregation, and efforts are needed to avoid such false positives[41,42]. Various strategies have been used to address off-target binding[43,44], as have empirical guidelines to avoid unfavourable compound profiles and/or minimize off-target effects. For example, compounds beyond the boundaries of Pfizer's 3/75 rule ($\log P > 3$ and tPSA < 75 Å^2)[40] or GSK's 4/400 rule ($\log P > 4$ and M > 400 Da)[45] may be considered more likely to be problematic.

Various protocols for in vitro secondary pharmacology screening approaches are available, and these generally consist of binding assays, functional assays and enzymatic assays, all of which provide important information regarding the pharmacological activity of a drug (such as potency, efficacy and type of interaction), including possible undesirable side effects that may be anticipated in humans[37,39,46,47]. This is a cost-effective and efficient approach commonly used as a safety screen for hazard identification and elimination in the early phases of drug discovery.

During hit identification and lead series selection, the main objective is to identify toxicological hazards and understand the potential for promiscuity (that is, the incidence of off-target hits) in the initial lead chemical series. The approach consists of screening chemicals against a panel of fewer than 60 targets strongly linked to safety liabilities, thus enabling prioritization of lead molecules and chemical series for further development[36,37,39,48]. Data from this initial profiling can be used during the lead optimization phase to enable medicinal chemists to establish structure–activity relationships, allowing mitigation of undesirable off-target activity from the molecules by design. The profiling can also be used to select drug candidates to progress into preclinical development, as well as to trigger and influence the design of investigative in vivo toxicology studies. In later phases of drug discovery, before carrying out first-in-human trials, screening against a broader panel of targets can be performed to aid in mechanistic understanding of in vivo effects[35,39].

The impact of such investigations is exemplified by our next case study: the off-target profiling of a novel antimalarial chemical series belonging to the triaminopyrimidine (TAP) class. The TAP lead series, with potent activity against a panel of clinical strains of *Plasmodium falciparum* that harbour resistance to known antimalarial drugs, were found to have relatively potent hERG channel activity (associated with adverse cardiovascular risk) and acetylcholine esterase activity (associated with adverse neurological risk). Extensive and focused sub-panel off-target screening was employed to develop structure–activity relationships and optimize the in vitro selectivity. The activity of the final drug candidate at the primary pharmacological target and potencies against the affected off-targets was improved (>100-fold) and translated into an acceptable in vivo toxicology and in vivo safety pharmacology profile, providing confidence in the candidate drug therapeutic index[49].

In contrast, a simple routine cellular off-target assay such as in vitro phospholipidosis (PLD)[50], which is commonly used either prospectively to screen out potential PLD inducers, or retrospectively to explain the nature of vacuoles seen in vivo, may be used to help understand in vivo lack of efficacy. In a recent example, a PLD assay based on high-content imaging (HCI) was used to investigate the high hit rate in coronavirus disease 2019 (COVID-19) drug-repurposing screens and why these 'hits' were not efficacious in vivo[51]. This study showed that, independently of their putative mode of antiviral activity, many of these hits were inducing PLD, which can itself inhibit severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replication. So, in this case, an assay that originated from investigative toxicology activities could help early deselection of artefactual hits and prevent wastage of further resources on their study[51].

Safety profiling can also be approached by combining different technologies to gain insight or to confirm findings. For example, the identification of off-targets of drug candidates with biophysical and

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proteomic techniques can provide the basis for developing hypotheses for observed adverse effects. Side effects associated with panobinostat, a histone deacetylase (HDAC) inhibitor, include hypothyroidism, seizures and tremors[52]. Thermal proteome profiling (TPP) and affinity enrichment-based chemoproteomics identified four specific off-target proteins of panobinostat, including phenylalanine hydroxylase (PAH)[52]. PAH has not previously been identified as a panobinostat target and was not stabilized in TPP experiments with vorinostat, a structurally distinct HDAC inhibitor. Inhibition was confirmed through enzymatic assays with rat liver PAH. Binding of panobinostat to PAH resulted in an increase in cellular phenylalanine and a decrease in tyrosine levels: a potential molecular initiating event related to panobinostat side effects[52]. Thus, optimizing the PAH selectivity of panobinostat analogues may improve HDAC inhibitor tolerability. More generally, earlier deployment of TPP and chemoproteomic approaches could enable a fuller assessment of off-target liability before candidates are selected for toxicology evaluation in animals.

Classical histopathology may also gain power when combined with analytical methods. Mass spectrometry imaging (MSI) approaches encompass a range of direct ionization methods that sample endogenous and exogenous compounds directly from the surface of tissue sections. Coupled mass spectrometry enables multiplexed label-free spatial analysis of compounds directly from tissue sections and is now being used in drug discovery[53]. Such spatial resolution when coupled to histopathological images has the potential to eliminate bulk analysis artefacts of pathological tissue sampling and focus on adverse biological perturbations at the cellular level[54]. The potential of MSI to complement other methods has recently been demonstrated in a study of the metabolic changes induced by cisplatin nephrotoxicity. Kidney tubule injury biomarkers and morphological effects in rats exposed to cisplatin were complemented by MSI revealing molecular alterations on a spatial level[55]. Early metabolic changes in the corticomedullary boundary metabolism were coincident with pathological changes. Interestingly, effects that included changes in nucleotides, antioxidants and phospholipids detected by MSI persisted longer than changes in renal biomarkers and after morphological recovery, highlighting the potential of MSI for detecting long-term liabilities.

De-risk preclinical in vivo findings and address human relevance

In vitro assays may be valuable in understanding the molecular and cellular mechanisms of pathological effects observed in animal studies in various species and clarifying their potential relevance to humans. The following two examples illustrate the impact of mechanistic understanding of toxic effects in the progress of a drug candidate either into clinical trials or onto the market.

Drug-induced neoplastic changes are a frequent phenomenon in in vivo safety studies, particularly in rodents exposed up to life time. For example, foci of altered hepatocytes are considered putative preneoplastic lesions that can occur spontaneously or be induced by chemicals or drugs[56]. Progression of these foci to hepatocellular neoplasms has been reported, but increases in foci in rodents do not necessarily lead to tumours in carcinogenicity studies[57]. A non-genotoxic nicotinic α7 receptor partial agonist drug candidate, RG3487, was found to induce foci of altered hepatocytes and subsequently tumours in rats[58], potentially precluding human clinical studies. However, the hepatocyte alterations observed in rats were not seen in the liver of mice or dogs. To assess human relevance, primary rodent, canine and human stem cell and patient-derived 3D cell model phenotypic assays

were used to explore potential mechanisms of neoplasia. Rodent phenotypic models were found to recapitulate the in vivo effect whereas human and canine models clearly showed an absence of effect, and the study revealed drug-induced effects that were not relevant in humans, such as nuclear receptor-driven liver proliferation in rodents but not in human models[58]. These data supported the progression of RG3487 into clinical trials.

Another example in which a high incidence of haemangiosarcoma induced by siponimod, a $S1P_{1/5}$ receptor modulator, in mice but not in rat 2-year carcinogenicity studies was addressed by a mixed in vivo and in vitro approach. A 9-month investigative mouse study with multiple time-point euthanasia explored molecular mechanisms that underlie the emergence of haemangiosarcoma, in parallel with a similar rat 3-month study[59]. These studies showed equal activation of vascular endothelial cells (VECs) by gene expression profiling in the two species, but mitotic gene expression was only transiently induced in rats whereas it remained constantly activated in mice. Additionally, out of many cytokines assessed, placental growth factor 2 (PlGF2) was induced in blood from day 1 of treatment and sustained in mice, whereas it was weakly and transiently increased in rats. In vitro experiments using primary VECs of both species showed a similar PlGF2 induction and mitotic activation in mice but not in rats. Human primary VECs behaved like rat VECs, showing no induction of mitosis or PlGF2 over a very large concentration range[59]. After review by regulatory agencies, this set of studies supported the approval of siponimod for the treatment of secondary progressive multiple sclerosis[60]. The data from these two cases highlight the importance of humanizing toxicology research and illustrate the use of cross-species in vitro comparison at the molecular and phenotypic levels to address the human relevance of preclinical findings.

Support identification of mechanisms underlying clinical events

Investigative toxicology may also be crucial in assessing safety issues that arise during clinical development or post-approval. This support is not limited to parent drugs, but may encompass drug impurities, degradants and metabolites.

For example, the development of antibody-mediated pure red cell aplasia (PRCA) in patients with anaemia treated with aggregated forms of recombinant human erythropoietin (rhEPO) is a rare and severe example of the consequences of protein immunogenicity[61,62], and so understanding the potential causes of protein aggregation is crucial for the safe use of rhEPO. Tungsten-induced rhEPO aggregates arising from the manufacturing process were hypothesized to be the root cause, and dendritic cell–T cell co-culture assays were used to differentiate non-aggregated from aggregated rhEPO and the immunogenicity of tungsten-induced rhEPO aggregates. T cell assays confirmed the immunogenicity of tungsten-induced aggregates of rhEPO in a clinical batch associated with one case of antibody-mediated PRCA, a finding that contributed to regulatory approval as part of a root-cause analysis[63]. Consequently, the syringe mode of production was modified to avoid traces of tungsten contamination.

Post-hoc investigations of clinical trial adverse events do not always lead to salvation of the product, but may provide solutions for follow-up drug candidates. For example, following the termination of the clinical development of an anti-CD40L antibody for the treatment of autoimmune disorders owing to unexpected thrombotic complications, studies using a blood vessel-on-a-chip model unveiled the mechanism of the prothrombotic effects[64]. Modifying a follow-up

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antibody with an Fc domain that did not bind to the FcγRIIa receptor on human platelets alleviated the prothrombotic effects in the vessel-on-a-chip model.

High-impact innovations in toxicology

On the basis of our survey of pharmaceutical companies (Box 1), which was first performed in 2015 and repeated in 2020, we were able to identify clear changes in the value of certain technologies and also prioritize those technologies that could improve the impact of investigative toxicology on drug discovery and development, according to their perceived impact at the time and over the next 2–5 years (Fig. 2). The perceived game-changing potential increased substantially over the 5 years between the two surveys for HCI, modelling and simulation, mRNA profiling, iPSCs and organs-on-chips. Decreases for game-changing potential were seen for in vivo imaging and MSI, gene editing and microRNA (miRNA). A more mixed picture developed for systems toxicology and metabolomics. In general, these changes can mainly be attributed to experience accrued by the participating partners over the 5-year period, as well as the perceived value of a technology for internal decision-making or the accessibility and deployment of some of the technologies (for example, MSI and metabolomics). Interestingly, although there has been an explosion in application of CRISPR to gain biological insights, its use is more nuanced in the investigational toxicology space because adverse pharmacology and/or toxicity will not necessarily be phenocopied by a gene deletion.

In this section, we highlight the current applicability of technologies for investigative toxicology based on experience in the companies participating in the survey, using published examples where possible. We also discuss the associated challenges and steps needed to achieve their potential, as well as the reasons for the perceptional changes described above.

Imaging technologies

Spatial resolution of drug effects on cells and tissues provides invaluable insight into drug toxicity; moreover, multi-parametric imaging biomarkers provide a basis for mechanistic understanding. There have been significant advances and application of imaging technologies, both in vitro and in vivo, including HCI, fluorescence and bioluminescence imaging, MRI, CT and high-dimensional molecular profiling of cell and tissue samples using MSI[65,66].

HCI technology combining automated microscopy and quantitative image analysis can be used to investigate mechanisms of compound and organ-specific toxicity[67–69], and in genotoxicity screening[70]. Consequently, HCI has emerged as an important technique for monitoring the spatial and biological changes induced by small molecules, biologics or other modalities, and integrating them to provide toxicological insights. It can discriminate subsets of cellular phenotypic features from a larger set, enabling the construction of machine-learning models for phenotypic profiling of drug candidates according to their mechanism of action in an unbiased way. For example, microtubule inhibitors and mitochondrial toxicants showed distinct clustering when 1,008 annotated reference compounds were tested in a panel of 15 reporter cell lines[71]. Hussain and coworkers[72] predicted direct hepatocyte toxicity in humans by using a subset of 30 phenotypic features, with a test sensitivity, specificity and balanced accuracy of 73%, 92% and 83%, respectively. Data from a single high-throughput imaging assay can be repurposed to predict the biological activity of compounds in other assays, even those that target alternative pathways or biological processes[73].

Cell painting is a high-throughput phenotypic profiling assay that uses fluorescent cytochemistry to visualize various organelles and HCI to derive many morphological features at the single-cell level[74]. Recently, this approach was used to test a set of reference chemicals in six biologically diverse human-derived cell lines. Similar biological activity profiles were obtained in the various cell lines without the requirement for cell type-specific optimization of cytochemistry protocols[74,75]. The availability of high-throughput systems, together with robust protocols and rapid data processing, are the main reasons why the game-changing potential of HCI significantly increased between the two surveys.

In vivo imaging using techniques such as MRI, positron emission tomography (PET), single photon emission tomography and CT offer the possibility of investigating biological events in any region of the body in real time and can provide the basis for minimally invasive biomarkers for safety profiling. Nonspecific biomarkers, such as those based on MRI relaxometry, CT density or PET with [^{18}F]fluoro-2-deoxy-D-glucose, are better suited for preclinical general toxicology and clinical monitoring, whereas specific biomarkers, such as those based on most PET and single photon emission tomography ligands, are better suited to clarify mechanisms of toxicity or unwanted target engagement[76,77].

The potential of MSI to simultaneously determine the discrete tissue distribution of the parent compound and/or its metabolites has paved the way for in situ studies on drug development, metabolism and toxicology[66]. MSI also has high molecular specificity and allows comprehensive, multiplexed detection and localization of hundreds of proteins, peptides and lipids directly in tissues[78]. In addition, single-cell imaging has advanced to the point where it is possible with nanoscale secondary ion mass spectrometry (NanoSIMS) to directly measure the absolute concentration of an organelle-associated, isotopically labelled pro-drug directly from a mass spectrometry image. A nano-electrochemistry method for single organelles has recently been developed[79] that allows subcellular quantification of drugs and metabolites. Nevertheless, the game-changing potential of in vivo imaging and MSI was downgraded in the 2020 survey relative to 2015, owing to the complexity and the high costs of these technologies, which still prevent their routine use in early phases of drug development. In the future, this potential could be boosted by the development of artificial intelligence (AI) tools for digital pathology[80]; for example, the recently launched 6-year public–private partnership BigPicture[81] aims to develop a repository of more than 3 million preclinical and clinical digitized pathology slides and AI tools[82] to analyse them and recognize abnormalities within tissue sections.

'Omics' technologies

Toxicogenomics (TGx) — the molecular assessment of toxicological effects via transcriptomics or the cellular output of gene expression (that is, proteomics and metabolomics) — generated considerable hype in the early 2000s, but its initial impact was disappointing. In 2010, a multi-sector survey showed that despite the application of TGx data for more than 10 years, the broad implementation of TGx to improve decision-making had not been achieved[83]. However, it is now considered that the use of such technologies can greatly strengthen preclinical toxicology studies. Approaches such as the L1000 methodology used within the NIH Library of Integrated Network-based Cellular Signatures (LINCS) programme allow high-throughput transcriptional profiling and signature analysis[84]. It is hypothesized that for most biochemical networks, mRNA expression levels of key representative

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genes (landmark genes) can be used as surrogates for all members of that network. If correct, measurement of the expression levels of an appropriate subset of landmark genes at a relatively low cost could be used to represent the whole transcriptome information, as has been demonstrated for EPA's ToxCast and the Tox21 data[85].

Transcriptional profiling for the prioritization of compounds during early drug discovery is a common approach within the pharmaceutical industry: 42% in our survey considered this a game-changer for investigative toxicology already in 2015, and 65% in 2020, illustrating the increasing maturity of this technology (Fig. 2). Gene expression signatures of compounds can be used to capture a range of poly-pharmacological effects and can be used to monitor the impact of medicinal chemistry optimization of a lead series. In addition, it may be used to generate hypotheses as to what compound substructures are responsible for a given effect and ultimately support the medicinal chemist in designing new structures to synthesize. More recently, non-signature-based transcriptome and gene network-based approaches for the analysis of drug-induced genomic effects have been demonstrated to explain mechanisms of hepatotoxicity and predict distinct toxicity phenotypes using gene network module associations. Network responses were found to complement traditional histology-based assessment in predicting outcomes of longer-term in vivo toxicology studies and to identify a novel mechanism of hepatotoxicity involving endoplasmic reticulum stress and NRF2 activation[86].

Expression of some miRNAs − small evolutionarily conserved endogenous non-coding RNAs that regulate the translation of protein-coding genes − have been associated with adverse biological effects[87,88]. More than 1,000 human miRNAs have been identified so far (see the miRBase database and miRNA tissue Atlas[89,90]), and a growing number of miRNAs have been reported to be expressed in a specific tissue or to be highly tissue-enriched[90,91]; for example, miR-122 in the liver[92], miR-192 in the kidney cortex[93] and miR-208 in the heart[94]. Importantly, circulating miRNAs have recently been correlated with liver and heart toxicity or injury in preclinical models of hepatotoxicity and cardiotoxicity and in patients with drug-induced liver and cardiac injury[95,96]. Consequently, circulating miRNAs are increasingly being investigated as tissue-specific toxicity biomarkers in investigative toxicology. However, perception of the game-changing potential of miRNAs in the survey decreased between 2015 and 2020, most probably owing to the still-limited translational potential.

Advances in NMR and mass spectroscopy workflows as well as analytical techniques now mean that metabolomic changes in cells, supernatants and also bio-fluids from in vivo animal studies or clinical samples can now be readily quantified and used either for hypothesis-free profiling (often consisting of thousands of cellular metabolites) or for biased approaches that measure more focused metabolic subsets[97]. Metabolic changes after drug treatment can be monitored, and together with bioinformatics processing analysis to either identify perturbed biological process or make statistical comparisons with metabolic signatures of chemical/drug libraries, can aid in the identification of toxicological mechanisms or hazards[98,99]. In the two surveys, views of the technology were mixed, with an increase of "already a game-changer" from 0% to 22% between 2015 and 2020, but also a concomitant increase of "no potential game-changer" from 17% to 58%. On the basis of discussions within the surveyed companies, we concluded that this heterogeneous perception is due to project- and company-specific experiences with this technology, which may provide high value in specific, targeted cases, but may also too often fail to provide interpretable data, as well as presenting a set of challenges and requiring substantial investments of money and time[100].

Computational and systems-level modelling to predict drug toxicity

The integration of multiple testing platforms generates large data sets and can require the use of high-performance computing and machine-learning-based modelling to fully realize mechanistic insights or make effective predictions. Successive integration of chemical, protein target and HTS cytotoxicity data has been shown to improve predictive performance, and studies with Tox21 chemicals[101] show that models based on in vitro assay data may in some specific cases perform better in predicting human toxicities than animal toxicity studies[102]. In addition, the combination of structural and activity data results in better models than using structure or activity data alone. For example, a Bayesian machine-learning approach, integrating in vitro assay data (such as hepatic transporter inhibition, mitochondrial toxicity and cytotoxicity in spheroid liver cell lines) with compound physicochemical properties and in vivo animal exposure, allowed binary yes/no prediction of drug-induced liver injury (DILI) with a balanced accuracy of 86%[103]. More recently, 'big data compacting' and 'data fusion' approaches, coupled to machine-learning techniques, have been applied to public domain transcriptomic data and have yielded impressive results in explaining dose-dependent cytotoxicity effects, predicting in vivo chemically induced pathological states in the liver and predicting human DILI from hepatocyte experimental data[103,104]. Thus, predictive modelling of chemical hazards by integrating numerical descriptors of chemical structures and short-term toxicity assay data benefits from chemo-informatics approaches and hybrid modelling methodologies for in vitro−in vivo extrapolation[105]. In one example, deep learning out-performed many other computational approaches for computational toxicity prediction, such as naive Bayes, support vector machines and random forests[106].

The computational power to efficiently analyse a wealth of data using machine-learning techniques (chemogenomics) is now available, and together with enhancements in more human-relevant models (3D models, cellular co-cultures, MPS), improved transcriptomics work-flows and freely available public data sources, we have the opportunity to incorporate data from early screening sources and derive more quantitative risk assessments using systems toxicology approaches based on pharmacokinetics, pharmacodynamics and physiology-based models. Indeed, investments in translational science are proving fruitful in moving from descriptive toxicology to a more quantified understanding of human translation[107]. The company survey revealed that physiologically based pharmacokinetic (PBPK) modelling and simulation (M&S) approaches are being adopted to predict the pharmacokinetic and pharmacodynamic behaviour of drugs in humans[108]. One of the first areas in which this is occurring is in vivo safety pharmacology, where it has been crucial in providing more quantified cardiovascular risk assessment[108] (Box 3).

M&S approaches promise to greatly impact drug safety by providing a method for integrating in silico, in vitro and in vivo preclinical data. In particular, mechanism-based models can be used to integrate disparate data types and predict the effects of new drugs in humans[109,110]. Indeed, M&S approaches are now being adapted to toxicological end points that use compound-related tissue injury biomarkers. Such biomarkers can be used to bridge between toxicity in in vitro and in vivo models and enable reliable translation between animals and humans. A leading example of such approaches is the

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Box 3

Improving strategies for cardiac safety testing

In the late 1980s and early 1990s, drug-induced ventricular repolarization/QTc interval prolongation and the appearance of rare but potentially fatal cardiac arrythmias called torsade de pointes (TdP) was identified, and several drugs were removed from the market[193]. Subsequently, it was discovered that these drugs (for example, terfenadine[194,195]) were delaying cardiac repolarization by inhibiting the cardiac potassium current, I_{Kr}, through the hERG channel. In 1997, early guidance on how to evaluate ventricular repolarization was published by CPMP (Committee for Proprietary Medicinal Products)[196] and in 2005, the ICH S7B guidance document was issued[197], introducing the functional I_{Kr} assay as a key component of the preclinical testing strategy alongside a preclinical in vivo QTc assay. The guidance triggered substantial activity within the pharmaceutical industry and service and technology providers that led to the development and implementation[198] of a plethora of preclinical platforms and assays[194]. Preclinical I_{Kr}/QTc assessment has been crucial for clinical candidate selection in industry, and has been successful, as it led to a low prevalence of QTc-prolonging drugs in clinical trials, and no newer drugs have been removed from the market owing to unexpected QTc prolongation[199].

However, the focus on I_{Kr} blockade and the QTc interval as proarrhythmic biomarkers came with a challenge. Studies showed that I_{Kr} blockade alone is not highly specific for predicting either QTc prolongation or TdP[170]. With the exception of selective I_{Kr} blockers, the correlation between the level of I_{Kr} blockade and proarrhythmic liability is molecule-specific (for example, blockade of non-I_{Kr} currents may mitigate proarrhythmic effects of I_{Kr} current blockade) and the QTc interval can be prolonged without leading to an increased proarrhythmic risk[200]. As a result, some drugs with effects on hERG and QTc prolongation but little actual TdP risk might have been excluded from further development[201]. Identification of this challenge led to the proposal of a new paradigm — the Comprehensive In Vitro Proarrhythmia Assay (CiPA) — in 2013. Over the past several years, data from the CiPA project[202] and the Japanese iPS Cardiac Safety Assessment (JiCSA) initiative provided input to the ICH with the goal of updating the S7B and E14 guidance[203,204]. CiPA aimed to evaluate

proarrhythmic risk on the basis of mechanistic electrophysiological understanding of proarrhythmia[199,205]. In its current form, CiPA involves an assessment of drug effects on multiple human cardiac ion currents, in silico reconstruction of human ventricular electrophysiological activity[206,207], the use of human iPSC-derived cardiomyocytes (hiPSC-CMs) to confirm findings of the in vitro and in silico assays[205,208,209] and evaluation of drug effects in phase I studies for potential unanticipated electrophysiological ECG signals[210].

Although the CIPA initiative provided crucial knowledge through collaborations between the pharmaceutical industry, regulators and academic investigators[202,211], implementation in the industry varied[212], and the regulatory standpoint was uncertain. In 2015, a new revision to the ICH E14 Q&A document was published[213] that enabled the use of concentration-QTc modelling as an alternative clinical approach to assess QTc prolongation[214], leading to a steady decline in the number of dedicated TQT studies submitted[215]. The downside was that this ICH E14 update left little focus on the ICH S7B core assays and the mechanistic insights and approaches put forward by the CIPA and JiCSA initiatives. The CiPA and JiCSA data led to the view that regulatory guidance was needed regarding best practices for the design, conduct, analysis, interpretation and reporting of in vitro, in silico and in vivo non-clinical assays for these assays to provide reliable non-clinical data for use in subsequent clinical evaluation[203,204,216,217]. Consequently, an ICH S7B/E14 concept paper has been proposed and endorsed by the ICH General Assembly[218]. In the concept paper, a two-stage strategy is proposed. The objective of the first stage of the work is to provide clarity on how to standardize currently developing methods including multi-ion channel assays, in silico models, in vitro human primary and hiPSC-CM assays, as well as in vivo evaluation[200,219,220].

In summary, the history of safety testing for drug effects on ventricular repolarization is a rare example in which understanding of the molecular mechanisms that underpin a given toxicity has enabled the development, implementation and refinement of non-clinical and clinical strategies, as well as regulatory guidelines, to support the development of medicines with an improved benefit–risk profile.

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prediction of myelosuppression by oncology drugs, which has often been a dose-limiting toxicity in the past. To predict and better inform the time course of myelosuppression after drug administration, semi-physiological mathematical models have been constructed[111]. These models explicitly represent drug-sensitive cells that correspond to the proliferating cells in the bone marrow and drug-insensitive cells that correspond to cells that subsequently mature without proliferating, as well as the circulating white blood cells. One such model was able to translate the full time course of myelosuppression from rodent data to patients for six different drugs[112]. A key feature of the model was a correcting factor that accommodated species differences in protein binding and sensitivity differences determined in in vitro colony-forming unit granulocyte macrophage assays. Predictions can be made not only of the magnitude of myelosuppression caused by novel drugs, but also how they interact with myelosuppressive standard-of-care therapies.

This enables oncology drug candidates with the most favourable profile to be prioritized and can also guide clinical use by informing the exploration of combination dose scheduling[113].

As risk assessment begins to move from single end point measures of toxicological effects towards pathway- and system-based studies, multiple parametric data coupled with M&S will offer further insight into complex effects of small-molecule and biologic drugs. Integrating classic toxicology approaches with network models and quantitative measurements of molecular and functional changes can enable a holistic understanding of toxicological processes, permitting prediction and accurate simulation of drug toxicity[86]. Moreover, systems toxicology frameworks, based upon mechanistic understanding of the ways in which chemicals perturb biological systems and lead to adverse outcomes, have the potential to provide a quantitative analysis of large networks of molecular and functional changes occurring

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across multiple levels of biological organization[114]. Crucially, such frameworks provide a supportive basis for translation between in vitro and in vivo model systems and translation into improved predictions of the therapeutic index in humans. Although such approaches are at first daunting, it seems possible that links could be established mathematically and statistically by leveraging public domain toxicological and bioinformatics databases. The key to success will be in successfully integrating the toxicological network components of approved drugs and failed drug candidates and enabling the construction of more holistic or global networks[115]. The sum of the accumulated knowledge-based molecular events, from molecular initial events to the intermediate multiple linear and/or branched key events, to adverse outcomes can be advantageously captured as so-called adverse outcome pathways for general reuse[116]. The field of adverse outcome pathways, which can be considered as the systematic capture of knowledge generated by mechanistic toxicology in general, has attracted enough attention and interest to trigger an Organization for Economic Cooperation and Development (OECD) guideline[117].

In our surveys, there was a slight increase in the perception of the overall game-changing perception of M&S, whereas the perception of the potential of systems toxicology declined. However, as described above, the borders of the two technologies have become blurred in recent years. Toxicologists have become familiar with M&S approaches through the use in translational in vivo exposure prediction, whereas systems toxicology was primarily seen as modelling responses in in vitro systems. Recent use of in vitro data for modelling human DILI risk together with PBPK models provides a good example of how these technologies are merging[118], and indicates that the game-changing potential should be considered from the M&S perspective.

iPSCs and advanced cellular models

To generate more translationally relevant data, toxicologists are increasingly using complex human and animal MPS models (organs-on-chips or organoids) to provide insight into organ-specific and inter-organ toxicity profiles[119–121].

iPSC-derived cardiomyocytes (iPSC-CMs) are the most advanced iPSC-derived model to be implemented across the pharmaceutical industry for the assessment of cardiotoxicity and cardiac electrophysiology[122,123]. Nevertheless, even the newer, more complex iPSC-CM disease models still possess a clear embryonic phenotype[124], and their implementation in routine testing within the pharmaceutical industry has been generally sporadic and slow.

Progress with 3D hepatocyte culture systems (including co-culture with non-parenchymal cells and incorporation of biophysical constraints such as oxygen tension or extracellular matrix) have led to additional improvement in recapitulating physiological tissue and/or organ level functions[125]. In addition, liver MPS based on primary hepatocytes, as well as non-parenchymal and immune cells, have the potential to emulate the genetic, physiological and disease setting of donors and reproduce context-specific toxicology[126–128]. However, differentiation of iPSCs towards hepatocytes has been more challenging than differentiation towards cardiomyocytes, and so toxicity profiling has focused on primary cell systems[129,130]. Nevertheless, promising translational toxicology studies are emerging. For example, repetitive exposure of hepatocytes to bile acids sensitizes them to compounds with cholestatic liabilities[131,132], and elevation of glucose levels in the culture medium resulted in features reminiscent of hepatic metabolic syndrome[133]. Models for insulin resistance, steatosis and altered drug metabolism have also been reported[133–135]. Advances with MPS-based liver systems now allow recapitulation of complex human liver lobule morphology and coupling to plasma flow and tissue perfusion[136]. Further development of these models is required to fully emulate the in vivo hepatobiliary situation and include chemically induced biliary hyperplasia in early safety assessment.

Substantial progress is also being made on other MPS tissue models, including of the gastrointestinal tract (primary tissue and stem cell-derived organoids, including 'stretch' models to mimic peristalsis)[137,138], lung (air–liquid interface epithelia with stretch to mimic breathing dynamics coupled to endothelia)[139,140] and kidney (mini-kidneys under microphysiological flow)[141,142]. These models still need to be developed and validated as fit for purpose for use in drug discovery and safety testing.

MPS models of individual organs could be integrated to make multi-organ configurations for investigative toxicology, as illustrated by the example connecting a heart-on-a-chip with a liver-on-a-chip discussed above[21]. Ideally, these systems will be integrated with robust systemic modelling of ADME pathways and toxicity circuits[143]. The development of MPS systems that are representative of specific human subpopulations using iPSCs derived from them could also be

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valuable[144–146]. With further advances, MPS have the potential to be more reliable, specific and predictive than conventional drug testing on animals and cell cultures, in line with the 3Rs framework (replacement, reduction and refinement of animals in research)[147].

In our surveys, both iPSCs and organs-on-chips were perceived to have a marked increase in their game-changing potential between 2015 and 2020, which is reflected in key publications[148,149]. Nevertheless, substantial bio-engineering, cell sourcing and characterization (including reproducibility and scalability) challenges with these advanced systems need to be addressed before they become widely integrated in industry workflows[150]. In addition, the cost of MPS, either developed internally, through academic collaborations, commercially purchased or assays contracted out, is still perceived as too high for routine toxicological use, until its impact on drug development is proved[151].

Genome editing

The development of genome editing platforms such as CRISPR–Cas9 has enabled researchers to gain fundamental insights into the contribution of single gene products to diseases[152,153]. When focusing on understanding the specific biological mechanism of toxicity, or adverse outcome pathway, having the ability to accurately and specifically delete or modify a particular gene can also provide insights into the potential safety liabilities of a drug target[154,155]. For example, a genome-wide CRISPR–Cas9 screen identified novel genes that are protective against, or cause susceptibility to, acetaminophen-induced liver injury[156]. However, although the technology is being used widely in fundamental life science research, published examples of direct application in investigative toxicology in the pharmaceutical industry are limited so far[154,155]. This may explain why there was a slight decline in the perception of the game-changing potential of this technology between the two surveys.

Challenges and outlook

The technologies discussed in this article are illustrative examples of some recent approaches considered most likely in our survey to address gaps in mechanistic understanding of drug safety and facilitate drug candidate selection, but the discussion is not exhaustive, as the purpose of this article is to clarify the current status of the field, rather than overview each approach in depth. There is much work remaining to advance technologies that are delivering promising results, such as organs-on-chips. Furthermore, as newer technologies emerge and are developed, there may be further potential for integration. For example, the recent integration of technologies such as single-cell genomics with in situ imaging approaches into the broader domain of spatial transcriptomics may help with mapping biological mechanisms relevant to toxicology from the cellular level to the tissue level and ultimately to the in vivo level. Single-cell genomics has been broadly adopted owing to the granularity of data it provides for heterogeneous primary cell cultures and native tissues, but unfortunately the necessary cellular dissociation from tissues leads to the loss of key information about local cellular interactions[157]. The potential to localize hundreds or even thousands of transcripts in situ with emerging spatial transcriptomics approaches could hugely improve understanding of biological processes, particularly for complex tissues such as the brain[157,158]. In addition, the growing volume of 'big data' generated by spatial transcriptomics is coinciding with the upsurge of AI capabilities applied to biology[159–161]. Digital pathology[162], AI-assisted protein folding and function prediction[163], synthetic biology[164], microbiome–drug interactions[165] and

biosensors for instant biological parameter monitoring[166] are other emerging areas with potential impact on investigative toxicology that could merit deeper evaluation.

The limited understanding of the true potential of an emerging technology and the extent of the work needed to establish it are fundamental issues that face investigational toxicologists when assessing which approaches to invest in, given that the balance between proven costs and hypothetical impacts will remain the driver for expenditure decisions within the pharmaceutical industry. Importantly, the assays and technologies used for investigative toxicology in industry are neither defined nor required by regulatory guidelines, in contrast to certain in vitro assays in genetic toxicology and phototoxicity testing. This differentiating factor carries both opportunities and challenges. Although the freedom to select, establish and verify test systems remains mainly at the discretion of individual companies, this liberty results in highly fragmented approaches to assays and systems, as evidenced by the findings of our survey. The selection of approaches is mainly driven by the history of failures in individual companies, as can be seen for off-target screens that are applied in most companies, but with a high degree of heterogeneity[37]. In many cases this heterogeneity, together with poor mechanistic understanding, precludes a statistical evaluation of assays or strategies to confidently predict risk. The added value of a new assay can then only be shown on a cursory basis and lacks evidence for a solid translational prediction.

Furthermore, it should be highlighted that the adoption and integration of any promising new technology into preclinical safety testing needs to be fully evaluated in the light of its translational value for human subjects and patients in clinical trials. Unlike fundamental research or ADME prediction approaches, which can afford trial and error in proving or disproving a hypothesis, technologies used to ascertain whether a candidate is sufficiently safe to enter clinical trials need proven robustness. Therefore, an important strategy for investigative toxicology is to gather assay performance data and impact metrics to justify the selection of specific assays and test systems and demonstrate the added value to drug design and compound selection. This can be illustrated by the adoption of in vitro hERG assessment (Box 3) or screening assays for genetic toxicity, where the concordance with the apical (torsade de pointes) or the corresponding regulatory end points (test battery according to ICH S2(R1)), respectively, with acceptable levels of mechanistic insight are well documented[167–171].

Given the outlined needs for harmonization and concomitant value analysis, and that the extent of the work to address these needs is very likely to be beyond any one organization, pre-competitive consortia will continue to have a crucial role in the investigative toxicology field. It is beyond the scope of this article to provide a comprehensive list of global consortia activities, and owing to the European affiliation of the authors, we focus on those in the umbrella of the European Innovative Medicines Initiative (IMI, now the Innovative Health Initiative (IHI)), a public–private partnership between the European Federation of Pharmaceutical Industries and Associations (EFPIA) and the European Commission.

A central aspect for harmonization and qualification of new technologies is the ability to share company internal data in a safe and trusted manner. The eTOX consortium is one example of a successful large-scale preclinical data-sharing project that has set the stage in terms of data clearance and sharing for further such consortia (Box 4), and is currently being pursued in the IMI eTRANSAFE project, which extends the scope to clinical safety data sharing for concordance analysis[172]. More pragmatic curated data-sharing approaches across industry

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partners can also be useful, as illustrated by a study from the Innovation and Quality in Pharmaceutical Development (IQ) consortium based on a blinded database composed of animal and phase I clinical data for 182 molecules that indicated that an absence of toxicity in animal studies strongly predicts a similar outcome in the clinic[173].

Consortia approaches are sometimes also needed to distil technological hype into a realistic assessment of an application, as has been done with gene expression profiling in vitro. Numerous international consortia investigated gene expression correlations for specific pharmaco-toxicological end points, but essentially refuted the holistic predictive potential of the technology that was anticipated when it first emerged. As a consequence, the focus of technology has moved to the mechanistic elucidation of toxicities[174,175], which was also confirmed in an EFPIA survey in which multiple respondents of the sampled companies indicated an initial intensive investment in toxicogenomics as a predictive tool[174–177], with a shift over time to a more targeted approach that emphasizes mechanism[178]. In line with this changed view on toxicogenomics, partner organizations have joined forces in the IMI-funded TransQST initiative to share expertise, data and workload to develop novel systems toxicology models of drug-induced toxicity in the liver, kidney, gastrointestinal and cardiovascular systems[24]. This project is expected to have a major impact with respect to systems toxicology and computational approaches. A further example is the IMI project TransBioLine, which aims to develop qualified biomarkers for the identification of injury of the liver, kidneys, pancreas, blood vessels and central nervous system. Finally, beyond the exploration of new technologies, the advantage of consortia is also the joint training of both academic and industry staff, which was particularly fruitfully implemented in the IMI project SafeSciMet, in which specific courses were offered to train future toxicologists in the understanding of molecular toxicology and its application in the context of drug development and regulatory submission.

Although timely mechanism-based hazard identification is a key goal of investigative toxicology, additional data are needed to exert maximum influence on lead design and candidate drug selection. Risk assessments based on two crucial components are required: robust translational principles that accommodate human relevance in both healthy and disease states; and provision of quantitative translational data that place the assay in the context of human (patho)physiology and, importantly, drug exposure. Understanding how toxicity relates to drug efficacy given the required dose and exposure is crucial in determining whether a drug has an appropriate therapeutic index[1,179]. Unfortunately, our survey (Supplementary information) revealed that for many observed drug toxicities, there is a major gap in understanding of the molecular, cellular, physiological and histopathological processes, and how observed effects in cell and animal models used in classical toxicology studies relate to potential adverse effects in healthy volunteers and specific patient populations[180–182]. Areas in which the development of emerging technologies could deliver greatest impact in addressing these limitations are highlighted above. Importantly, these limitations mirror a similar situation with regard to efficacy testing, where the target relevance to disease progression and management is only confirmed with proof-of-concept clinical trials, normally phase IIb or even sometimes phase III. Thus, the intention of investigational toxicology in drug discovery is to identify key liabilities by building a platform of evidence early enough using in silico and in vitro models. In addition, it should be ensured that there is chemical scope to design out risks instead of only profiling the most interesting compounds at candidate selection, since at this point the scope (and generally the time) to correct deficiencies will be inherently more limited.

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To efficiently exploit the available safety data for early target assessment, three hurdles have to be overcome.

- Access to scattered information sources: tools that allow versatile and easy access to the heterogeneous data sources need to be developed. For example, for the early assessment of a target, automated processes need to be developed that allow text and data mining of the scientific literature, in parallel with searches in pharmaceutical, toxicological and clinical databases.
- Management of divergent definitions of similarity: when querying databases for compound effect predictions, sophisticated computational tools to assess similarity beyond just chemical structure (for example, the Tanimoto similarity index) need to be available to relate chemistry to biological effect.
- Knowledge management databases: integrating the results from complex searches requires automated processing to be able to handle the big data volumes. New approaches to data visualization, machine learning and AI will help to develop hypotheses for early candidates.

Summarizing our survey and our observations, we propose the following action points to help address these hurdles and further advance investigative toxicology.

- Establish collaborations to evaluate existing and new assays to allow the development of standardized protocols that improve hazard identification and risk assessment (see MIP-DILI as an example[31]).
- Establish baseline data sets to provide a reference for progress and enable evaluation of the impact of various investigative toxicology approaches.
- Engage in open innovation and pre-competitive collaboration; share data within defined boundaries to fully leverage knowledge from the vast industry archive to build predictive safety models and more effectively evaluate the most promising emerging technologies.
- Develop and share system toxicology and adverse outcome pathway frameworks for drug candidate toxicity to facilitate mechanistic insight and quantitative translational utility.
- Advance 'humanized' models that reflect off-target effects, genetic background and disease.

Responding to this call to action will need broad scientific skills and experience. Investigative toxicology requires expertise in physiology, cell biology, organic chemistry, toxicology, ADME, mechanistic and molecular pharmacology and a good understanding of toxicology and safety regulations, and increasingly, computational toxicology, bioinformatics, systems pharmacology/toxicology, data analysis, genetic skills and pharmacometric skills are also needed. Translation of academically developed expertise in these latter areas into pharmaceutical toxicology will require intensive training, an exercise that the pharmaceutical industry has already successfully accomplished two decades ago with the development of the professional profile of medicinal chemists. To repeat this success, companies, professional societies and academia need to develop a common understanding of the professional profile of an investigative toxicologist and to shape the necessary training. International conferences can be instrumental on this path, as can be seen by the inclusion of specific symposia on investigative toxicology in the pharmaceutical industry during the annual conferences of the European Society of Toxicology (Eurotox)[183,184].

In conclusion, we emphasize the need for continued support of existing initiatives and the establishment of programmes that foster a closer and more open collaboration between pharmaceutical investigational toxicologists, academia and technology developers and providers. These alliances and coalition networks would have the goal of increasing the impact of investigational toxicology on the discovery and development of safer medicines.

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Competing interests

All the authors are employed by pharmaceutical companies, as listed in their addresses. In addition, some or all may hold shares or stock options in their respective companies.

Additional information

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