Archives of Toxicology
https://doi.org/10.1007/s00204-019-02613-4

REVIEW

Toxicity testing in the 21st century: progress in the past decade and future perspectives

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Received: 25 April 2019 / Accepted: 5 November 2019

Abstract

Advances in the biological sciences have led to an ongoing paradigm shift in toxicity testing based on expanded application of high-throughput in vitro screening and in silico methods to assess potential health risks of environmental agents. This review examines progress on the vision for toxicity testing elaborated by the US National Research Council (NRC) during the decade that has passed since the 2007 NRC report on *Toxicity Testing in the 21st Century* (TT21C). Concomitant advances in exposure assessment, including computational approaches and high-throughput exposomics, are also documented. A vision for the next generation of risk science, incorporating risk assessment methodologies suitable for the analysis of new toxicological and exposure data, resulting in human exposure guidelines is described. Case study prototypes indicating how these new approaches to toxicity testing, exposure measurement, and risk assessment are beginning to be applied in practice are presented. Overall, progress on the 20-year transition plan laid out by the US NRC in 2007 has been substantial. Importantly, government agencies within the United States and internationally are beginning to incorporate the new approach methodologies envisaged in the original TT21C vision into regulatory practice. Future perspectives on the continued evolution of toxicity testing to strengthen regulatory risk assessment are provided.

Keywords Toxicity testing · New approach methodologies · Computational toxicology · High-throughput in vitro testing · High-throughput exposomics · High-throughput pharmacokinetics · In vitro to in vivo extrapolation

Introduction

In 2007, the US National Research Council (NRC) published a landmark report on *Toxicity Testing in the 21st Century*, which put forward a long-term strategy designed to take

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00204-019-02613-4) contains supplementary material, which is available to authorized users.

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Published online: 17 December 2019

advantage of new tools and technologies to increase the efficiency of toxicity testing in order to permit the assessment of the large numbers of environmental agents to which human populations may be exposed (US NRC 2007). Key elements of this strategy included increased use of high-throughput in vitro test systems and methods in computational toxicology, with less reliance on more time-consuming and costly toxicological studies using experimental animals (see Fig. 1). The NRC vision has received widespread support internationally and has provided a blueprint for change in toxicological science. A subsequent NRC report on *Exposure Science in the 21st Century* provided a parallel vision for the advance of exposure science (US NRC 2012b) emphasizing high-throughput exposomics and computational methods for exposure assessment. The US Environmental Protection Agency's *NexGen* initiative subsequently integrated new developments in toxicological risk assessment within an overarching framework for the *Next Generation of Risk Science* (US EPA 2014b; Krewski et al. 2014) (see Fig. 2). This paper provides a review and update on the NRC vision, including concomitant advances in risk science, and applications in assessing the risks of environmental agents.

TT21C: the need for change

TT21C was born out of the need to improve the safety assessment of environmental and industrial chemicals (Hartung 2009a), both existing and new ones coming to the market (Hartung 2009b, c). The number of substances to be tested is impressive: more than 85 million chemicals have been synthesized; about 140,000 of these have been commercialized on a larger scale, but many more are found in natural products.

In comparison, the number of well-studied substances such as drugs and pesticides is only in the realm of a few thousand, while the number of those which were tested at all—typically only done for acute and topical endpoints—is likely between 10 and 20 thousand. Furthermore, the apparent bias toward acute and topical effects is owed to feasibility rather than what investigators would ideally like to know about the safety of products.

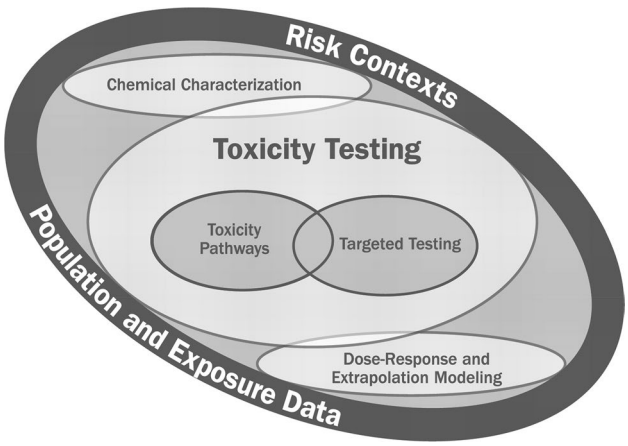

Fig. 1 US NRC framework for toxicity testing in the 21st Century

The reason for not testing all substances or important chronic endpoints is primarily economical (Bottini and Hartung 2009). For example, a full assessment of a pesticide can amount to $20 million USD, which is not affordable for many chemicals given typical production volumes and profit margins of these substances. In addition, the volume of substance required (about 20 kg) and the time required to process the results of these assessments (4 + years) can be prohibitive. There is a need to strategically develop adequate and feasible approaches here (Busquet and Hartung 2017).

Even if such assessments are available, such as those primarily in rodents, rabbits and guinea pigs, with very few available in other species such as dogs and monkeys, the relevance to humans must still be considered (Hartung et al. 2013). It is difficult to determine the extent to which these species resemble humans in their toxic effects; however, it may be easier to determine how representative such species are of each other. Toxicology is one of few fields where testing is standardized across species: for more complex endpoints such as cancer and reproductive toxicology, the correlation is only about 60% (Basketter et al. 2012); for severe eye irritation, approximately 70%; and for skin sensitization, 77% (Luechtefeld et al. 2016a, b). For most hazards, we can only assess their reproducibility: these results are not very impressive, despite the fact that toxicology is highly standardized, studies are often conducted using GLP quality assurance, and high-dose effects are assessed. Compared to pharmacology, where human-relevant doses are tested in a disease model with very few parameters being measured, toxicology is more likely to generate reproducible results.

Testing very high doses in order not to miss effects impairs relevance for human exposure situations. What does the high-dose exposure to a single substance in a short-lived animal of a few hundred grams for a short period of time tell us about human hazards? It definitively does not reflect human diversity (such as age, gender, weight, race, or comorbidities), special susceptibilities and exposure scenarios. Most importantly, we are not exposed to single chemicals but to their mixtures in very different quantities and patterns.

In consequence, there is a requirement for more human models, which are more reproducible, faster and more cost-effective. All these are design criteria identified for TT21C. Over the last ten years, the evidence for these problems has strongly increased (Hartung 2017). The most recent discussion of a "reproducibility crisis" (Baker

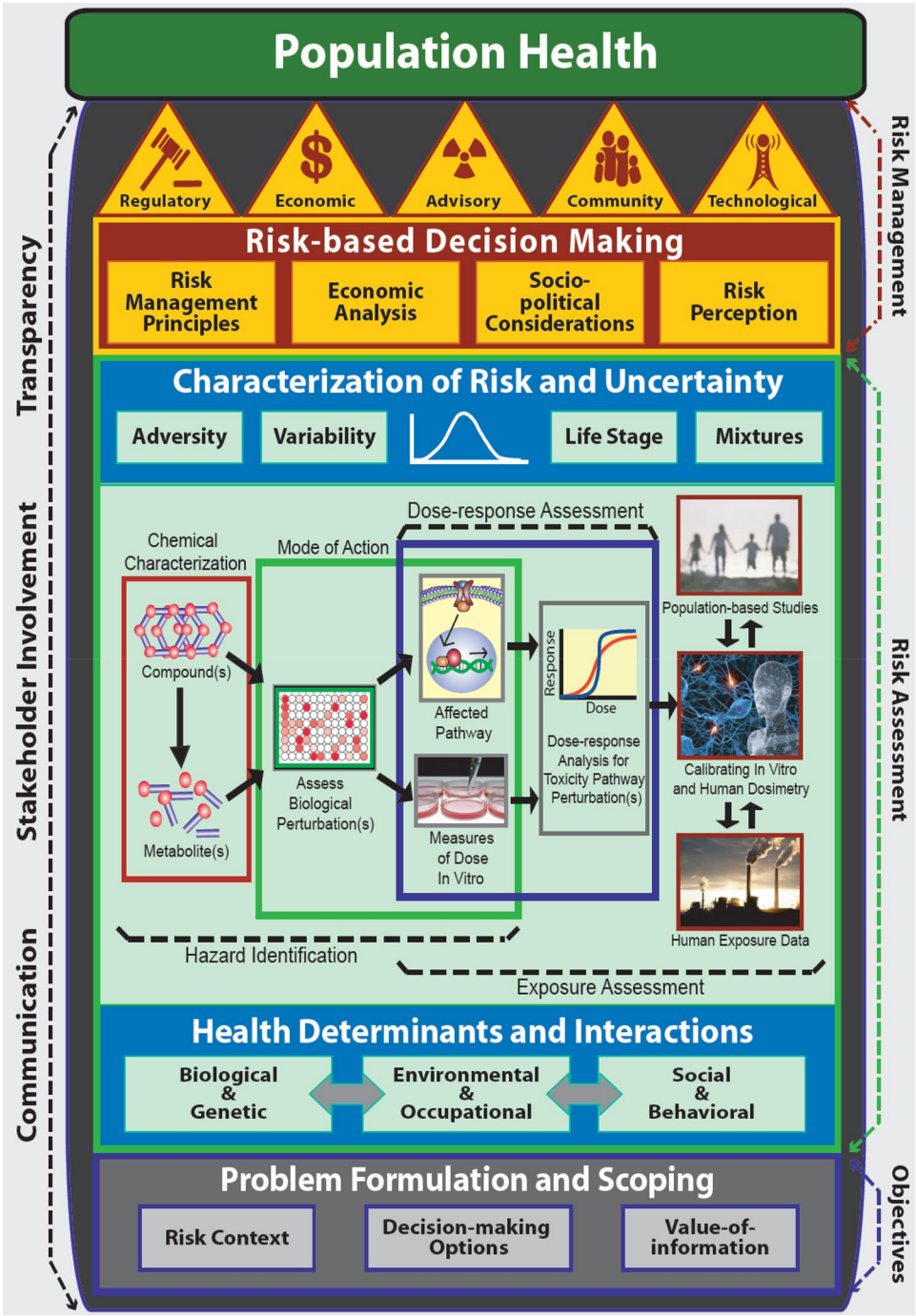

Fig. 2 A framework for the next generation of risk science

2016) in science is an example, but also large-scale toxicological databases such as the one accumulating through the European REACH process (Luechtefeld et al. 2016a, b) allow such assessments, e.g., acute fish toxicity (Hrovat et al. 2009), for eye irritation (Luechtefeld et al. 2016a, b) or skin sensitization (Luechtefeld et al. 2016a, b). This emphasizes the foresight of TT21C.

Toxicity testing in the 21st Century

Toxicity pathways: taking the first steps by bringing toxicity pathways to safety assessment

The TT21C vision and strategy arose largely from considerations of advances in understanding human biology

and in the explosion of tools for assessing perturbations of test systems by chemicals and other stressors. This new knowledge formed the basis for proposing a novel, higher throughput system for toxicity testing and chemical safety assessment. Obviously, there are many changes that can be measured using cell and tissue platforms. For toxicity testing purposes, it is necessary to ascertain which of these changes would be of sufficient concern to serve as the basis of risk or safety assessment. The concept of a toxicity pathway was the nexus on which to build a strategy for collecting and utilizing information on perturbations in biological systems and assessing adversity. Toxicity pathways were defined as normal cellular response pathways and cellular signaling networks whose disruption would likely lead to adverse consequences. The examples provided were related to oxidative stress pathways and estrogenic signaling through a nuclear receptor-mediated pathway. Other examples might include G-protein coupled receptors (GPCRs) that are frequent targets for pharmaceutical intervention, but less frequently affected by environmental chemicals.

Systems biology approaches for assessing pathway perturbations and analysis of their dose–responses behaviors had already been discussed, including the manner in which in vitro and ex vivo assays could provide information on pathway components and assess dose–responses characteristics for pathway perturbations (Andersen et al. 2005a, b). Cellular perturbations would be expected to show dose-dependent transitions—from sub-threshold regions, through responses leading to adaptation and on to those that would be sufficiently intense and long-lasting to cause adverse responses in the test system. While the comparison to previous efforts was not highlighted, toxicity pathways clearly represent an extension of concepts of mode-of-action (MOA) that had been developed to aid the use of mechanistic studies in chemical risk assessment and especially in assessing the human relevance of animal toxicity studies (Seed et al. 2005; Boobis et al. 2008). The initial interactions of chemical with biological targets was similar to concepts of molecular initiating events (MIEs) and dose-dependencies had been discussed for many compounds and a broad range of toxicological responses in intact animals (Slikker et al. 2004).

If toxicity pathway assays are to form the basis for developing new test procedures and dose–response assessment, some inventory of likely pathways would be valuable to ensure coverage during toxicity test procedures. Papers appeared categorizing canonical stress pathways (Simmons et al. 2009) and pathways associated with nuclear receptors (Jennings 2013; Jennings et al. 2013). While initiatives to implement the recommendations related to higher throughput testing were jointly proposed by US Environmental Protection Agency (US EPA) and National Institutes of Health (NIH) (Collins et al. 2008), other groups moved forward to either define toxicity pathways from cellular responses to compounds or to focus on specific pathways, creating assays based on known characteristics of pathway biology. General principles for developing assays and dose–response models for DNA-damage pathways were first described in a more prospective fashion (Bhattacharya et al. 2011) leading on to more detailed work on assay development, data collection, safety assessment applications (Clewell et al. 2014; Adeleye et al. 2015) and systems biology approaches to pathway-based safety assessment (Zhang and Andersen 2007; Li et al. 2014; Zhang et al. 2014). Another pathway selected for study was estrogenic signaling in uterus (Miller et al. 2016, 2017). These examples represent developing a clear rationale for measuring cellular endpoints related to the pathway responses and components—developing so-called "fit-for-purpose" assays that are based on the biology of the pathway and the tools available for querying pathway function (Clewell et al. 2016).

Biological pathways post-2007: In assessing overall progress on the specific recommendations from the TT21C report, we need to reflect on the evolution of pathway-based concepts and look to instances where the idea of toxicity pathways is now guiding new directions. The term 'pathway' is ubiquitous in life-science research and is used to convey mechanistic understanding about a process. A pathway is a description of a process within a larger system that involves the interaction of components of the system leading to a particular outcome (Kleensang et al. 2014). Pathways capture knowledge in either a narrative (textual), graphical or mathematical form, with varying degrees of formality and compliance with different established conventions utilized by various research communities. In the last 10 years, significant resources have been invested in the elucidation of pathways and there are now many related databases available: Pathguide.org, for example, has a catalogue of well over 500 pathway databases. Unfortunately, there remains a considerable lack of formalization, harmonization and standardization for describing and reporting pathways (O'Hara et al. 2016), which hinders sharing, collaboration and, ultimately, the practical exploitation of pathway information by end-users.

Typically, biological pathways describe sets of molecular interactions that lead to a change in the phenotypic state of a cell. Thus, according to this definition, toxicity pathways and biological or cellular-response pathways are intrinsically linked (i.e., no biological pathway, no toxicity pathway). Owing to the nature of typical biological pathways, a region of adaptation is expected to exist that precedes injury, dysfunction and adversity. Jennings (2013) points out that doses causing adaptation compared to those with overt toxicity may not differ substantially. For a proper description of a toxicity pathway, the dose/concentration–response

relationship (dynamics) needs to be fully characterized (experimentally) and described (ideally, mathematically) (Jennings 2013).

Regarding application of results from toxicity pathways, in some risk contexts a dose–response model based on in vitro results (e.g., from which a point-of-departure or benchmark dose can be derived) might provide adequate data to support a risk-management decision. Essentially such an approach is about identifying regions (exposures) of safety, as opposed to risk per se (Andersen and Krewski 2009). For most risk contexts (i.e., chemical-specific assessments) the TT21C vision and strategy would entail extrapolation modeling to link in vitro with in vivo dosimetry and low-dose modeling either with safety factors or systems biology modeling of the pathway (Zhang et al. 2010).

Toxicogenomics

Toxicogenomics was identified in TT21C as a transformative approach that was expected to play a pivotal role in identifying the toxicity pathways and cellular responses associated with exposure to environmental agents. Although it was noted that toxicogenomics would not be "the staple technology", expectations were high that in combination with advances in bioinformatics, systems biology, and computational toxicology, toxicogenomic approaches could support the evolution of toxicity testing toward the TT21C paradigm.

State of the science circa 2007

TT21C defined toxicogenomics as "a broad field combining expertise in toxicology, genetics, molecular biology, and environmental health and includes genomics, proteomics, and metabonomics", although the predominant focus and prevailing application since 2007 has been transcriptional profiling. Toxicogenomics was viewed as a key platform on which to expand knowledge of toxicity pathways and implement higher throughput methods for querying cellular effects.

In 2007, the field of toxicogenomics was dominated by the application of DNA microarrays for gene expression analysis, with a variety of well-established commercial technologies available (e.g., Affymetrix, Illumina, Agilent Technologies Inc., and others). However, early studies in this field were often poorly designed and executed, applied faulty analytical pipelines, failed to apply sufficient statistical rigor and data filters, or suffered from errors and incomplete bioinformatics annotation of probes and pathways. This led to a lack of reproducibility across studies, a decline in confidence in the technologies in the first decade of application, and a plateau in the number of papers applying toxicogenomics in research after 2007 (Chen et al. 2012). Concerns as to the possibility to validate such approaches prevailed (Corvi et al. 2005).

Nonetheless, by 2007 a variety of studies had demonstrated strong correlations across microarray technologies from high-quality studies (Yauk and Berndt 2007), and the US Food and Drug Administration (FDA) Microarray Quality Consortium (MAQC) published its first series of papers defining the reproducibility of DNA microarray technologies and outlined some best practices (Consortium et al. 2006). This, in parallel with more judicious implementation of the Minimal Information About a Microarray Experiment (MIAME) standards (Brazma et al. 2001), including requirements to make expression data publicly available through a variety of public repositories (e.g., ArrayExpress, Chemicals Effects in Biological Systems, and Gene Expression Omnibus) by scientific journals, led to increases in the quality and availability of the data and analyses applied in toxicogenomic experiments.

Prior to the release of TT21C, numerous experiments had been conducted to explore the utility of toxicogenomics both in vivo and in vitro for a variety of applications in regulatory toxicology, and large-scale efforts to develop databases of expression profiles were underway. For example, Iconix Biosciences, Inc. produced expression profiles for over 600 drugs in vivo and in vitro in male Sprague–Dawley rats and commercially released their DrugMatrix database for the development of predictive toxicogenomic signatures and discovery of mode of action of prototype and new chemicals (Ganter et al. 2005; Fielden and Kolaja 2006; Fielden et al. 2007). In parallel, there was increasing regulatory interest in the technology (e.g., US Environmental Protection Agency (EPA) produced a variety of reports including '*A framework for the use of genomics data at the EPA*' (Dix et al. 2006). Indeed (US NRC 2012a), there was sufficient information, experience and momentum by 2007 that the NRC released its report outlining the potential use of toxicogenomics for hazard identification, analysis of mechanism of action, chemical classification, exposure assessment, genetic susceptibility, and toward reductions in animal testing (US NRC 2007; Ganter et al. 2005; Fielden and Kolaja 2006; Fielden et al. 2007).

Overall, despite acknowledged limitations, at the time of publication of TT21C, toxicogenomics appeared to be in a position to relatively efficiently derive mechanistic information associated with toxicity pathway perturbation from in vivo and invitro experiments. However, significant challenges included the high cost of microarray experiments and a lack of: (a) automation for higher throughput applications; (b) quality control standards and international guidelines for analysis/application; (c) understanding of the associations between pathway perturbations and apical effects; and (d) efficient bioinformatics tools/pipelines for meaningful interpretation of toxicogenomics data in a reasonable timeframe.

Although significant progress has been made, a number of these challenges still exist one decade later.

State of the science circa 2017

Toxicogenomics continued to evolve rapidly post-TT21C. Major technological advances since 2007 include the development of RNA-sequencing (RNA-seq) approaches and higher throughput platforms to query gene expression changes, and improvements in bioinformatics platforms, databases and analytical tools. In parallel, the increasing availability of open sources of data and bioinformatic tools has broadened the community's ability to mine toxicogenomic data to derive biomarkers of toxicity and mode of action, and refine pathway analyses. Experiments and efforts aimed at defining standards and best practices have moved the field forward. Importantly, over this time period, a variety of case studies demonstrated practical examples of how toxicogenomic data may be used in a regulatory context, and a framework for tiered testing that integrates toxicogenomics was published (Thomas et al. 2013a).

Technological advances Development and refinement of microarray platforms continued in this decade, bringing about higher quality output, declining costs, and increased automation. Analytical approaches for microarrays were increasingly standardized, leading to more reproducible results across studies and improved confidence in the technology. Widely available quantitative real-time PCR (qPCR) arrays in micro-well plates (96- and 384-well formats) also now provide high-quality tools for more specific query of the genome, enabling smaller laboratories without specialized genomics infrastructure to apply toxicogenomics approaches. However, although microarrays and qPCR continue to be standards, a pivotal technological advance in toxicogenomics has been in the area of whole genome sequencing, which has continued to progress at an astounding rate in the last decade.

Next generation sequencing has emerged as a powerful, more sensitive and precise method to quantify transcriptional changes. Because RNA-seq does not rely on pre-defined probes, it can, in principle, be used to study the entire transcriptome. Moreover, the count-based approach has a much greater dynamic range than microarray technologies, allowing more precise measurement of low- and high-abundance transcripts. With multiplexing and increasing automation, large numbers of samples can be analyzed simultaneously. Within the field of toxicology a variety of studies have demonstrated reproducibility of RNA-seq relative to microarrays at the pathway level, but increased sensitivity to identify differentially expressed transcripts (Consortium 2014; Wang et al. 2014; Webster et al. 2015a, b; Zhang et al. 2015a, b). Moreover, RNA-seq is more effective for analysis of highly degraded archival samples (such as those preserved in formalin), which, in moving forward, provides the unprecedented opportunity to derive molecular signatures from well-characterized (i.e., phenotypically anchored) tissues that are in archives (e.g., the National Toxicology Program's archive) (Auerbach et al. 2015; Webster et al. 2015a, b; Hester et al. 2016). Leveraging this opportunity to query expression changes in archived tissues would be very useful for defining toxicity pathways linked to adverse effects and developing transcriptional signatures that can serve as biomarkers of toxicity.

In parallel with RNA-seq, a number of approaches have emerged that enable high-throughput screening (HTS) for gene expression changes. These technologies have been developed to permit an analysis of transcripts in cells in culture that work on crude cell lysates, rather than requiring the extraction of RNA from cells, which makes them amenable to automation. For example, the L1000 assay queries RNA from cell lysates using ligation-mediated amplification (Peck et al. 2006). The L1000 technology measures 1000 'landmark genes' that are used to capture information contained within the entire transcriptome (Lamb et al. 2006). Similar approaches include RNA-mediated oligonucleotide Annealing, Selection, and Ligation with Next-Gen sequencing (RASL-seq) (Li et al. 2012) and the TempO-seq™ technology (BioSpyder Technologies, Inc., Carlsbad, CA), which is integrated with a bioinformatics pipeline for the rapid identification of differentially expressed genes (Bushel et al. 2018). These and other ground-breaking techniques are changing the field of HTS to enable the assessment of a more complete and complex biological space than has been possible using standard HTS approaches.

Analytical/bioinformatics advances A major area of development in the past decade has been in analytical tools and knowledge bases for genomic data. A variety of both commercial and public applications are now available for rapid assignment of changes in gene expression to molecular pathways and processes. For example, commercial software packages for functional analysis of genomic data, such as Ingenuity Pathway Analysis (Qiagen) and MetaCore (Thomson Reuters) now provide straightforward analytical tools to identify perturbations in curated pathways, biological processes and molecular functions; identify regulatory agents and signaling networks that are driving responses; and have specifically tailored applications for toxicology. Publicly available sites also continue to be refined to provide high-quality approaches to mine genomics datasets and identify perturbed pathways, processes and networks (e.g., Bioconductor http://www.bioconductor.org, and The Database for Visualization and Integrated Discovery (Huang da et al. 2009). This progress has meant that identifying the molecular alterations induced by exposures to toxicants

is more straightforward; however, linking these perturbations to associated apical effects or mode of action remains a time-consuming challenge. To circumvent this problem, increasing focus has been placed on the development of transcriptional signatures that can be used to predict mode of action/toxicological effect. Of substantial benefit to research and development in this area has been the public release of a number of large databases of toxicogenomics profiles in vivo and vitro. Predictive toxicogenomic signatures and transcriptomic databases are described in more detail below.

Another critical advance has been in the area of quantitative toxicogenomics, with the release of the BMDExpress software (Yang et al. 2007; Phillips et al. 2019). BMDExpress enables high-throughput benchmark dose (BMD) modeling of global gene expression data. The tool has been used to demonstrate that the lowest pathway BMDs derived from toxicogenomic data in short-term rodent studies (e.g., 5, 14, 28 or 90 days studies) are consistent with BMDs derived from conventional endpoints (e.g., histopathological changes and cancer) (Thomas et al. 2013a, b). In addition, BMDs from specific perturbed pathways associated with different modes of action are similar to BMDs for later apical effects (e.g., Bhat et al. 2013; Jackson et al. 2014; Moffat et al. 2015; Labib et al. 2016). Toxicogenomic BMDs derived from different gene expression platforms are largely concordant (Black et al. 2014; Webster et al. 2015a, b), indicating that as platforms evolve BMD values derived using toxicogenomics approaches should remain relatively consistent. The US National Toxicology Program recently convened an expert panel to establish an acceptable approach for genomic dose–response modeling, which will ensure greater consistency in this application to facilitate the use of transcriptomic dose–response data in risk assessment (US NTP 2018). Overall, transcriptomic BMD analysis provides insight into the doses at which molecular changes occur, and is poised to become a key tool in next generation risk assessment (Thomas et al. 2013a, b; Moffat et al. 2015; Cote et al. 2016).

Significant resources to advance the development of predictive signatures of mode of action/toxicity, and for other types of data mining, include the public release of two large toxicogenomics databases: DrugMatrix and The Japanese Toxicogenomics Project (TG-GATES). As described above, DrugMatrix contains in vivo and in vitro global toxicogenomics profiles of hundreds of drugs across two doses [a fully effective dose (defined as the dose used for treating disease, converted from human), and the maximum tolerated dose (defined as 50% reduction in weight gain)], in 13 tissues and across multiple early time points (hours to 5 days). This database was purchased by the US National Institute of Environmental Health Science in 2011 and made publicly available. TG-GATES was produced by the Japanese National Institute of Health Science, the National Institute Biomedical Innovation, and 15 pharmaceutical companies (http://toxico.nibio.go.jp/open-tggates/search.html), and was also released publicly in 2011. TG-GATES contains in vitro and in vivo expression profiles with liver as the primary target organ (Uehara et al. 2010). Data for approximately 170 compounds (predominantly drugs) are available for single- and repeat-dose study designs. Experiments spanned three dose groups (1:3:10 ratio) up to the maximum tolerated dose alongside concurrent controls, which provides an opportunity to explore both dose- and temporal-response. The strengths of these databases lie in both the size and scope of the projects, and the controlled nature of the experiments that enables direct comparison across chemicals. Moreover, the availability of toxicogenomics profiles from both in vivo and in vitro studies for the same chemicals facilitates assessment of the relevance of pathway perturbations across models.

Databases of HTS transcriptomic data are also being produced and used. For example, the Library of Integrated Network-based Cellular Signatures (LINCS) L1000 dataset contains gene expression profiles from human cells exposed to over 20,000 small-molecule compounds, including most of the FDA-approved drugs, with measures taken before and after the exposures. A compound signature discovery pipeline that spans raw L1000 data processing to drug screening and mechanistic analysis has now been developed to expedite signature-based toxicological analyses (Liu et al. 2015).

Although there are other excellent databases that are available in this area, the above provides key examples of resources available for the discovery of toxicity pathways and the development of signatures of mode of action and toxicological effects. Moreover, these databases can be leveraged immediately to assess similarities of transcriptional changes induced by novel chemicals to prototypes within the database for hazard identification and mode of action analyses.

In addition to mode of action analysis, a promising area of research has been in the development of gene expression signatures that can be used to predict whether a chemical agent produces a specific toxicological effect, or operates through a specific mode of action. Such signatures can facilitate rapid analysis of transcriptomic datasets through simple pattern recognition approaches integrated with probability assessment. For example, the TGxDDI biomarker comprises 64 genes that were derived from analysis of 28 training compounds in human TK6 cells (Li et al. 2015, 2017; Yauk et al. 2016; Cho et al. 2019). Transcriptional changes in these genes following toxicant treatment predict whether an agent induces DNA damage or not. Similar methods have been applied in vivo to identify interaction of chemicals with key transcription factors such as constitutive activated receptor (CAR) (Oshida et al. 2015a) and peroxisome proliferator-activated receptor alpha (PPARalpha) (Oshida et al. 2015b).

Overall, many published gene expression signatures have been generated to predict diverse toxicological endpoints including genotoxicity, carcinogenicity (Gusenleitner et al. 2014; Saito et al. 2016), hepatotoxicity (Hrach et al. 2011; Van den Hof et al. 2014), nephrotoxicity (Fielden et al. 2005; Minowa et al. 2012), developmental toxicity (Pennings et al. 2011), and specific exposures (Hochstenbach et al. 2010, 2012; Chauhan et al. 2014). A key gap is the development of bioinformatic tools to rapidly apply these signatures to the analysis of new datasets, although promising approaches have been recently proposed (Rooney et al. 2018; Corton et al. 2018).

Although toxicogenomics has not been widely applied to develop toxicity or adverse outcome pathways (AOPs), there has been some progress in this area. Overall, it has been challenging to precisely define the associations because of redundancies across pathways, and lack of tools to mine the networks produced. A recent publication leveraged the TG-GATES and ToxCast datasets to develop computationally predicted AOPs (Bell et al. 2016). The authors found that computationally derived AOPs approximated manually curated AOPs and suggested that their approach could be used to accelerate expert-curated AOP development. Applications of such machine-based approaches followed by manual validation through text and data mining will greatly advance the TT21C agenda. Moreover, these efforts will define the sequence of transcriptional perturbations that are linked to adverse effects to support effective use of transcriptomic data in toxicological testing. In the context of the Human Toxome project (see below), transcription factor analysis including weighted gene network analysis proved to be especially helpful (Andersen et al. 2015; Maertens et al. 2015; Rahnenfuhrer and Leist 2015; Pendse et al. 2016a, b).

Development of standards and best practices A critical gap in the field of toxicogenomics is the absence of guidance describing the specific details of experimental approaches and analyses as they should be conducted for application in regulatory toxicology (e.g., an OECD test guideline). This is generally viewed as an impediment to the use of toxicogenomics data in human health risk assessment. The US FDA's Microarray Quality Control consortium (MAQC) conducted a variety of validation studies to demonstrate that toxicogenomics technologies are robust and reproducible, and suggested best practices for the identification of differentially expressed genes for both microarrays and RNA-seq (Consortium et al. 2006; Guo et al. 2006; Shi et al. 2010; Consortium 2014; Wang et al. 2014). Additional work from this group demonstrated the consistency of predictive signature genes and classifiers across transcriptomic technologies (Fan et al. 2010), which is critical given the rapidly evolving technologies in genomics. However, although individual international regulatory agencies have produced policies, guidance documents and reports relating to the use of toxicogenomics and transcriptional profiling in human health risk assessment (e.g., the US EPA's 'A Framework for the Use of Genomics Data at the EPA' and the FDA's 'Guidance for Industry: Pharmacogenomic Data Submissions' in 2003, and their 'Voluntary Exploratory Data Submissions (VXDS)' program), the lack of international guidelines for toxicogenomics tests has hampered regulatory uptake. A variety of case studies have provided specific examples of applications of toxicogenomics in human health risk assessment, which have helped to advance the field (described in more detail below). Moreover, recent efforts to more specifically define the experimental design and study quality criteria in this field have been published (e.g., McConnell et al. 2014; Bourdon-Lacombe et al. 2015). These efforts will facilitate the development of formal, harmonized, international guidance to advance this field in the future. Finally, the OECD has initiated projects to follow on important recommendations from working groups of the European Centre for Ecotoxicology and Toxicology of Chemicals to develop reporting standards for transriptomics, metabolomics and proteomics (Gant et al. 2017; Buesen et al. 2017).

Summary

The field of toxicogenomics has matured to a state where automatedprocedures facilitate rapid and cost-effective production of these data, and analytical tools are available for relatively efficient data analysis and interpretation. BMD analyses have demonstrated that transcriptional perturbations occur at doses that are highly predictive of adverse effects and tools are available for rapid BMD modeling on global transcriptomic datasets. Databases and tools are available to develop and implement the use of signatures of specific toxicities and modes of action for chemical assessment. However, significant efforts are required to improve data mining to define the specific transcriptional perturbations that are causally linked to adverse effects, and to produce the guidelines required for routine uptake of these types of data by the regulatory community.

High-content imaging

While the 2007 NRC report provided an overall roadmap of the knowledge and thought processes that conceptually frame the new toxicity testing paradigm, the actual in vitro toxicity testing platforms and interpretative tools needed to acquire the data for safety assessments were acknowledged to be evolving works in progress. The ideal in vitro toxicity testing platform will provide broad coverage of the biological response landscape, be simple enough to be medium- to high-throughput, and produce an inherent indication of adversity with toxicant exposure. To achieve these goals,

three-dimensional (3D) predictive biology platforms composed of various microtissues are likely needed, as argued below, and require experimental, technical, and computational innovation in three areas:

- demonstration that 3D microtissue cultures manifest important biologically relevant behavior;
- design and testing of simple medium- to high-throughput array platforms in a simple and inexpensive format suitable for living 3D microtissue culture and high-content confocal imaging; and
- evidence that the morphogenetic and cytopathological signals produced by the 3D microtissues identify adverse responses and points of departure for safety assessments.

Each of these identified areas of required innovation is discussed in the context of the problem it solves, and how it contributes to the development of a fit-for-purpose predictive biology platform for in vitro toxicity testing.

3D microtissue cultures manifest important biologically relevant behaviors. Two examples of in vitro toxicity testing approaches developed by the USEPA (ToxCast) and the Hamner Institute use protein and cell-based reporter systems and 2D cell culture plus omics detection techniques, respectively. While important and ground-breaking in many ways, these testing approaches have significant limitations. Often, the reporter assays are designed as protein–protein interaction indicators or as artificial constructs inserted into cells without the usual co-factors that modulate responses, raising concern about their relevance to in vivo biology. The 2D cell culture plus omics detection techniques are dependent upon the biology of 2D cells in culture which may be distinctly different from in vivo biology (Petersen et al. 1992). In addition, the omics detection techniques require a large expenditure of resources (time and money) for data generation. While these approaches have been necessary and highly informative in developing the tools and strategies for the new toxicity testing paradigm, it is now important to devote resources to optimizing the test platforms themselves.

A key feature of an optimal test platform is that it be able to manifest the broad spectrum of responses inherent to living humans, a property we are calling broad coverage of the biological response landscape. To achieve this broad coverage of biological responses requires the incorporation into the test system of as many of the properties of in vivo cells as possible. In vivo cells interact in 3 dimensions to form tissues, and such 3D interactions markedly change the behavior of cells, resulting in cell sorting, differentiation, and polarization (Bissell et al. 2003). Therefore, in vitro test systems that incorporate 3D cellular models are likely required to fully represent the biological response landscape. Cells grown in 3D more closely mimic the differentiated function, phenotype, and biology of tissues than the same cells grown in conventional 2D mono-layers (Cukierman et al. 2001; Griffith and Swartz 2006) because of increased cell–cell contact, enhanced cell–ECM signaling, and the absence of an unnaturally stiff and highly adherent plastic substrate (Baker 2012).

A critical need in the development of fit-for-purpose 3D predictive biology platforms for in vitro toxicity testing is a way to rapidly assess living 3D cultures repeatedly over time. To meet this challenge requires scale-up of the existing 3D culture platforms for screening, which implies a design based on simplicity, a key feature of any medium- to high-throughput test platform. Numerous 3D technologies have been developed, including cells on membranes, cells in gels/scaffolds, cells in micro-fluidic devices and cells aggregated into spheroids or microtissues. From a biology perspective, each has its advantages and disadvantages as it tries to replicate the complex in vivo environment in a simple in vitro system. Likewise, from a technology perspective, each has its niche.

Microtissue systems based on attaching cells to scaffolds (natural and synthetic) are non-permissive for many complex three-dimensional morphological events because their cell density is low and cell-to-scaffold interactions predominate (Lee et al. 2008). Human-on-a-chip technologies are complicated devices difficult to adapt for rapid testing (Marx et al. 2012). For example, a human biomimetic lung-on-a-chip device has been developed to model the effect of cyclic mechanical strain on uptake of nanoparticles by alveolar epithelial cells (Huh et al. 2010). However, this complex microfluidic system is not easily translated to traditional biomedical laboratories due to the need for specialized equipment and training, nor does it accurately model subchronic pathological endpoints such as pulmonary fibrosis. Predictive in vitro models for adverse chronic pulmonary outcomes, including rodent lung slices, human lung airway tissue constructs, airway epithelial cell cultures at an air–liquid interface, and open porous lung scaffolds are promising but are not yet sufficiently reliable or reproducible as alternatives to in vivo animal toxicity testing (Patel et al. 2012; Nichols et al. 2013; Sauer et al. 2014; Jeannet et al. 2015).

Therefore, the most advantageous 3D microtissue designs are likely scaffold-free, allowing the cells to make their own extracellular matrix rather than relying on externally supplied materials that might vary batch-to-batch and dominate the biological behavior of the cells. This approach also creates 3D microtissues of high cellular density, mimicking the conditions of normal in vivo tissues. Examples of scaffold-free 3D microtissues culture approaches include the hanging drop system and the use of non-adhesive hydrogels. The hanging drop system, commercialized by In Sphero®, collects cells at the bottom of a small drop where they self-assemble a spheroid. However, the small drops are prone to spillage and evaporation, media changes are difficult,

spheroids are difficult to image in the drop (beyond working distance of objective) and the culture environment is relatively unstable during the longer time frames needed for morphological assays (Mehta et al. 2012). Another scaffold-free system in use is non-adhesive hydrogels, as commercialized by MicroTissues, Inc® (Napolitano et al. 2007). This technology creates a stable, long-term, reproducible culture environment for microtissues to form at normal organotypic density and architecture, with maximal cell-to-cell communication and movement, allowing mixtures of different cell types to interact while undergoing complex 3D morphological changes and differentiation (Achilli et al. 2012, 2014).

Morphogenetic and cytopathological signals identify adverse responses. Animal toxicity tests are phenotypic "apical" tests—complex experiments that measure integrated biological endpoints, each of which is an integrated measure of multiple facets of the machinery necessary for in vivo function. As such, these apical tests may provide little insight into the cellular and molecular events, mechanisms, and targets responsible for toxicant action. The traditional definition of an adverse effect is "A biochemical, morphological or physiological change…that….adversely affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge" (Lewis et al. 2002). By this definition, adverse effects are limited in scope to apical events manifested by the whole organism. On the otherhand, the new toxicity testing paradigm is non-apical, focused on human cells, in vitro approaches, and high-throughput techniques. There is an obvious disconnect between the accepted definition of adversity used in risk assessments and the requirements for interpreting adversity signals from the new system of toxicity testing.

Rudolf Ludwig Karl Virchow (1821–1902), the famous German physician, said, "… the cell is really the ultimate morphological element in which there is any manifestation of life …" in articulating his vision of the cellular basis of disease. The implementation of the microscope as a diagnostic tool, and the development of an explanation of disease organized around cellular dysfunction contributed greatly to the advances in medicine that took place during the first half of the twentieth century. The determination of the double helix structure of DNA in 1953 by Watson and Crick heralded the modern era of molecular pathogenesis, enhancing our understanding of the cellular basis of disease based on altered gene and protein expression. The tools now at our disposal are phenomenal, rapidly evolving, and supported by remarkable computational power.

Aided by advances in technology, the morphological study of cells (cytopathology) and of cells in tissues, including their organization and alterations (histopathology), continues to be a powerful diagnostic indicator of adverse effects. The 3D predictive biology platforms being developed inherently manifest morphogenetic and cellular alterations over time that depend upon 3D interactions that can be altered by toxicant exposure. The in vitro morphologic manifestations of adversity include alterations in the appearance, organization, and number of cells, and alterations in subcellular organelles. An advantage of relying on these morphological manifestations as indicators of adversity is the long track record of success using this approach in diagnostic pathology as well as chronic toxicity assays in animal models.

The distinction between an adaptive response and an adverse response plays a fundamental role in toxicology, defining the level of exposure associated with a significant effect that can lead to disease. In risk assessment, the boundary between the no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) has guided regulatory practice. Cytopathology and histopathology have played key roles in defining this boundary and identifying adversity. An example of the power of morphological assessment as an adversity indicator using high content images is the measurement of changes in neurite outgrowth (Crofton et al. 2012). The morphological tools used to identify the effects of toxicant exposures include high content image analysis of the patterns of neurite outgrowth and differential cytoplasmic distributions of neurite biomarkers (Harrill et al. 2013). Live cell imaging of in vitro cultures of human neuronal cells has further demonstrated the power and relevance of this approach (Stiegler et al. 2011).

Therefore, technical advances in imaging of 3D microtissues (van Vliet et al. 2014) allow a rejuvenated "in vitro pathology" to integrate morphological indicators of adversity identified in the 3D microtissues with functional and molecular endpoints of effect, such as action potentials and transcriptomics. This combination of tried-and-true morphological signals of adversity plus mechanistic molecular validation will lead to the development of a new adversity standard suitable for 3D predictive biology platforms. This effort will be guided by the following re-definition of adversity focused on an understanding of toxicant-induced perturbations at the molecular and cellular level: "Adversity is defined as a combination of molecular and cellular events that are dose and treatment related by statistical test and evolve over time across biological pathways to lead to a pathophysiological alteration."

Exposure science in the 21st Century

Exposure science includes quantitative estimation of the external dose received by a human or ecological species. Prior to the introduction of high-throughput screening technologies that have enabled the implementation of toxicity

testing in the 21st century, exposure science was, perhaps, in a better place than toxicology. There were ongoing efforts monitoring the biomarkers of dozens to hundreds of chemicals in human tissues (Calafat et al. 2006; US CDC 2012; Dodson et al. 2014) and large, sustained efforts for environmental monitoring of hundreds of chemicals in media such as water and air (Alexander et al. 1998). Environmental fate and transport models could provide estimates of human and ecological exposure for thousands of other chemicals. The developers of these "high throughput" exposure models for thousands of chemicals specifically noted the lack of "consistent" toxicology information, often with no human data available (Arnot et al. 2006; Rosenbaum et al. 2008). The relative standing of exposure and hazard for thousands of chemicals was abruptly inverted by high-throughput screening for bioactivity and bioinformatic approaches to organize existing chemical toxicity information. As noted by Egeghy et al. (2012): "Of the roughly 100,000 chemicals that have at least limited toxicity information available, less than one-fifth also have exposure information—and for most of these the information is of limited utility (e.g., production volume)."

Risk is a combination of hazard and exposure—if the exposure level is sufficiently low, even if uncertain there is little or no risk. Similarly, if the hazard level is sufficiently low, exposure is less relevant (Wetmore et al. 2015). High-throughput risk prioritization efforts that tried to combine rapid estimates of chemical hazard and exposure illustrated how for some chemical classes (i.e., pesticides) there were hundreds of example chemicals with exposure estimates while for other classes (e.g., industrial process chemicals and ingredients in consumer products) there were almost no traditional exposure estimates (Wetmore et al. 2012). This "knowledge gap" was compounded by the continued addition of thousands of new chemicals and/or new chemical uses every year (US NRC 2012a). Thus, in 2012 the National Research Council (US NRC) released a new report entitled "Exposure Science in the 21st Century" (US NRC 2012a).

The NRC report identified significant data needs to inform the estimation of chemical risk (US NRC 2012a). Since for many chemicals only the total production volume or volume released to the environment was known, the development of methods for determining the fraction of that total chemical to which populations are exposed (i.e., the "intake fraction") was a primary goal (Nazaroff et al. 2012). Efforts to relate basic exposure information to markers of exposure in biomonitoring data had limited success (Gangwal et al. 2012). The existing fate and transport models were designed for making predictions for exposure due to chemical migration from industrial releases (i.e., "far-field" sources) (Arnot et al. 2006) through the environment (including air, water, and the food web) for tens of thousands of chemicals (Arnot et al. 2012). However, there was a growing realization that the primary source for many organic chemicals that were present in biomonitoring data was due to "near-field" sources in the home, such as consumer products and articles of commerce (e.g., furniture and flooring) (Wallace et al. 1987; Wambaugh et al. 2013). Unfortunately, there were no high-throughput models for describing this near-field exposure to chemicals (Arnot et al. 2012; Wambaugh et al. 2013). In particular, there was a great need to determine the presence and weight fraction of chemical ingredients in products that were commonly in homes but for which there were few public sources of consistent information (Goldsmith et al. 2014; Dionisio et al. 2015; Isaacs et al. 2016).

To address the need to provide exposure context for twenty-first century toxicity testing, programs such as the EPA's Exposure Forecasting (ExpoCast) project (Hubal 2009) and the Emory Health and Exposome Research Center: Understanding Lifetime Exposures (HERCULES) were initiated. Through a combination of model development and new experimentation, in particular the advent of new, non-targeted screening mode high-resolution mass spectrometry (e.g., metabolomics and exposomics), exposure science has begun to implement the NRC vision as well as develop similar capacity to high-throughput toxicity testing (Park et al. 2012; Goldsmith et al. 2014; Wambaugh et al. 2014; Auerbach et al. 2015; Dionisio et al. 2015; Egeghy et al. 2016; Rager et al. 2016).

Advances in exposure science

Within "Exposure Science and the 21st Century" the NRC specifically mentions the need for advances in the technology and analyses of exposure monitoring data (US NRC 2012a). This is perhaps epitomized by the exposome, i.e., "every exposure to which an individual is subjected from conception to death" (Wild 2012). The NRC recognized that the use of tools such as high-resolution mass spectrometry to identify factors including diet, behavior, and disease might fundamentally alter the way in which exposure science could be conducted (US NRC 2012a).

Advances in raw computational power have also allowed the implementation of new algorithms and approaches (e.g., computational tools) to further transform the way in which exposure science was conducted (Egeghy et al. 2016). Computational exposure science has been enabled by algorithms such as Markov Chain Monte Carlo (Metropolis et al. 1953) for Bayesian analysis and the Random Forest algorithm (Breiman 2001) for machine learning. For example, more systematic analyses of data such as exposure biomarkers revealed trends, enabling simple models largely based on whether or not chemicals are used in the home to explain half of the chemical-to-chemical variance in exposures that can be inferred from exposure biomonitoring aspects US Centers for Disease Control and Prevention National Health and

Nutrition Examination Survey (NHANES) (Wambaugh et al. 2014). The need for describing near-field sources to chemical exposure (e.g., consumer products) has been addressed both on a whole home level (Goldsmith et al. 2014) and with greater precision for specific product classes (e.g., personal care products) (Csiszar et al. 2016). Computational exposure science has been enabled by algorithms such as Markov Chain Monte Carlo (Metropolis et al. 1953) for Bayesian analysis and the Random Forest algorithm (Breiman 2001) for machine learning. For example, more systematic analyses of data such as exposure biomarkers revealed trends, enabling simple models largely based on whether or not chemicals are used in the home to explain half of the chemical-to-chemical variance in exposures that can be inferred from exposure biomonitoring aspects of the US Centers for Disease Control and Prevention National Health and Nutrition Examination Survey (NHANES) (Wambaugh et al. 2014). The need for describing near-field sources to chemical exposure (e.g., consumer products) has been addressed both on a whole home level (Goldsmith et al. 2014) and with greater precision for specific product classes (e.g., personal care products) (Csiszar et al. 2016).

The ability to algorithmically process large data sets to reveal sometimes remarkable trends and correlations is described as "Big Data" analytics and has been applied with success to exposure science (Egeghy et al. 2016). In order to feed these algorithms, large amounts of data are needed in a format that can be processed by a computer (i.e., "machine readable"). Efforts have been made to capture data that was previously only human readable in computer databases (Goldsmith et al. 2014), and to harmonize databases allowing broader analyses to be conducted (Dionisio et al. 2015). These analyses have identified as many or more gaps as they have answered. For example, while chemicals within consumer products are now well characterized, chemicals within articles of commerce are not.

Big Data analytic techniques show promise for addressing long outstanding problems in the field of toxicology, in particular with respect to mixtures (Simmons 1995; Sturla et al. 2014; Hartung 2017). For example, although HTS is, by definition, high-throughput, testing all permutations of even 100 chemicals (2^{100} combinations, or roughly a 1 with thirty zeros after it) would be literally impossible. However, it has been recognized that the occurrence of chemicals in environmental media (such as dust in a child care center) are structured (Ryker and Small 2008; Tornero-Velez et al. 2012). Similar combinations of the same chemicals often recur, likely due to patterns in consumer purchasing and other human activities. Kapraun et al. (2017) recently used the technique of Frequent Itemset Mining (FIM) (Borgelt 2012) to identify combinations of chemicals that frequently occurred in the US blood samples obtained by NHANES. FIM is an example of a Big Data analytics technique that is more commonly used by retailers to identify products that are frequently purchased together (for example, bread is purchased in conjunction with peanut butter and jelly). What Kapraun et al. (2017) found were a few dozens of combinations of chemicals that co-occurred in significant fractions of the US population (> 30%). By reducing the mixtures problem from all possible mixtures to dozens of the most relevant mixtures, FIM allows HTS to examine relevant mixtures for potential synergistic effects (Ryker and Small 2008; Kapraun et al. 2017). Further, recognition that there are frequent combinations of chemicals may increase the statistical power of environment-wide association studies (EWAS, discussed below) that have typically focused on univariate associations between single analytes and health effects (Patel et al. 2013) and can now focus on more multifactorial environmental causes of adverse outcomes (Patel and Ioannidis 2014; Bell and Edwards 2015).

Though human activity can and does give rise to structure in chemical exposure, it often remains difficult to determine when and why a chemical is used (Dionisio et al. 2015; Isaacs et al. 2016; Phillips et al. 2017). Chemical use in turn dictates the exposure pathway (e.g., direct application or accumulation through the food web) (Auerbach et al. 2015). Chemical exposure information problems can be addressed on a per chemical basis, but are important to overcome more rapidly and systematically (Wambaugh et al. 2013; Auerbach et al. 2015). New tools and databases allowing greater capacity for systematic analysis should be expected to continue to answer some questions while identifying new research areas capable of transforming exposure science.

For example, describing thousands of chemicals with sufficient accuracy to allow high-throughput exposure models to make predictions remains an open and difficult challenge (Arnot et al. 2012; Goldsmith et al. 2014). For example, the High-Throughput Stochastic Human Exposure Dose Simulator (SHEDS-HT) can simulate aggregate exposure to a chemical from multiple pathways in the home, including chemical emission from and use of diverse consumer products (Goldsmith et al. 2014). However, in order for SHEDS-HT to run, estimates of the chemical presence, weight fraction, and emissivity must be available to "parameterize" the model. Although public databases exist to describe consumer products in this manner for many thousands of chemicals and products (Goldsmith et al. 2014; Dionisio et al. 2015; Rager et al. 2016), there are many more chemicals and products for which this information is complete. One recent example of applying machine learning is the development of "functional use" models which can predict the role served by a chemical within a formulation (e.g., dye or surfactant) from physico-chemical properties that can be predicted from structure. The outputs of functional use models allow exposure models like SHEDS-HT to make predictions for chemicals and chemical-containing products where data are

not available, albeit with greater uncertainty (Rager et al. 2016). One tantalizing prospect offered by predicting the use of chemicals from structure alone is that libraries of chemicals that have been screened for bioactivity, as in the Tox21 project, can be further screened to identify novel chemical uses among chemicals with lesser bioactivity (Phillips et al. 2017). Though the current models fall short of commercial needs (e.g., the potential use of a fragrance can be predicted but the pleasantness of that fragrance is more challenging (Juberg et al. 2017)), there is enormous potential for "green chemistry" (Anastas and Warner 2000) using high-throughput exposure tools and HTS (Phillips et al. 2017).

Advances in exposure science have most importantly provided critical "real world" context for data obtained from HTS for chemical hazards. Risk prioritization on the basis of toxicity predicted from in vitro HTS and estimates of exposure show great potential. Risk-based prioritization separates those chemicals where, for the general population, the margin between putative hazardous dose and exposure is small (i.e., greater risk) from those chemicals where the expected margin is quite large (i.e., lower risk) (Rotroff et al. 2010; Aylward et al. 2011; Wetmore et al. 2012; Thomas et al. 2013a, b). Unfortunately, it was quickly recognized that for non-pesticidal chemicals there are few estimates of exposure rates for the general public (Wetmore et al. 2015). Empirical, i.e., statistical (Gangwal et al. 2012) and mechanistic (Wambaugh et al. 2013) descriptions of human exposure were not initially found to be adequate for dealing with the large numbers of chemicals being screened for toxicity. Once large-scale information on chemical use (Dionisio et al. 2015) became available, however, statistical models that focused on exposure pathways (i.e., route of exposure) as identified by use became able to provide very approximate, but useful, estimates of human exposure (Wambaugh et al. 2013, 2014). The approximate estimates were very coarse, with between six and eight orders of magnitude of uncertainty (e.g., exposure is between a μg/kg body weight/day and a pg/kg body weight/day). Yet, if the hazard was estimated to occur at a higher rate (e.g., tens of mg/kg bodyweight/day) there still might be a sufficient margin to consider a chemical as a lower risk priority. In fact, when combining high-throughput exposure estimates with toxicity estimates from the ToxCast project, Wetmore et al. (2015) found that many chemicals have an expected margin of, at worst, a million fold. Thus, approximate but high-throughput exposure tools can be combined with HTS to sift among thousands of chemicals to identify the highest priority targets for additional research (Thomas et al. 2013a, b).

In an effort to advance exposure science, US EPA has initiated the Systematic Empirical Evaluation of Models (SEEM) framework, which is designed to calibrate exposure predictions based on empirical measurements. This framework implements a consensus approach for validation of exposure predictions, and is being applied in a wide range of occupational and environmental exposure circumstances (Wambaugh et al. 2018).

Notably, first attempts to combine the Exposome concept with the Adverse Outcome Pathways (AOPs) of TT21C are on the way (Escher et al. 2017). They promise to make sense of the exposure patterns by using mechanistic information.

High-throughput toxicokinetics

High-throughput in vitro screening has the potential to identify concentrations which cause biological perturbations To relate these perturbations to in vivo hazard methods for in vitro–in vivo extrapolation (IVIVE) methods are needed for toxicokinetics (Coecke et al. 2013; Bell et al. 2018). Toxicokinetics describes the absorption, distribution, metabolism, and excretion of a chemical and its metabolites by the body (O'Flaherty 1981). Toxicokinetics allows the relationship between an external exposure (e.g., chemical ingestion) and tissue concentrations caused by that exposure to be described. For most chemicals, however, chemical-specific toxicokinetic models are unavailable (Wetmore et al. 2012). Toxicokinetic data have been typically obtained using laboratory animals. These studies are expensive, but can be used to make human predictions via physiologically based toxicokinetic (PBTK) models if sufficient data are available (Tan et al. 2018; Cohen Hubal et al. 2019).

In vitro tools have been developed to anticipate pharmacokinetic behavior for the pharmaceutical industry using in vitro methods (Shibata 2002; Waters et al. 2008). These methods typically produce predictions within a factor of three of what is observed in human clinical trials (Wang 2010). In the last 10 years a series of studies have addressed the use of these pharmaceutical methods for environmental chemicals (Rotroff et al. 2010; Tonnelier et al. 2012; Wetmore et al. 2012, 2013, 2015). These so-called "high-throughput toxicokinetic (HTTK)" approaches provide chemical-specific predictions of toxicokinetics for chemicals where there are no other data.

Chemical-specific hazardous doses can be predicted from in vitro bioactive concentrations using IVIVE based upon HTTK. An approach known as "reverse dosimetry" (Tan et al. 2006) is used to predict the dose rate (mg/kg body weight/day) that would be needed to cause a steady-state plasma concentration equal to the bioactive in vitro concentration. A model (Wilkinson and Shand 1975) for predicting steady-state plasma concentrations can be used based on the measured plasma binding and metabolic clearance Comparisons between the bioactive doses predicted by reverse dosimetry and hazardous doses observed in toxicity studies found this method for IVIVE to be conservative (i.e., predicting lower hazardous doses than observed) (Wetmore et al. 2013).

HTTK currently relies on two medium throughput in vitro assays: The first assay characterizes the amount of chemical that is free in the presence of human plasma protein (Waters et al. 2008) and the second assay characterizes the rate of metabolism of the parent chemical by incubating a known initial concentration of the chemical with a suspension of human hepatocytes, and drawing aliquots over to time (Shibata 2002). Both assays require the development of chemical-specific methods for the determination of chemical concentration in samples, for example, to examine the disappearance of chemical due to metabolic activity. Development of chemical-specific analytical methods is time consuming, and not necessarily always successful (Tolonen and Pelkonen 2015). For this reason, attempts have been made to develop and apply quantitative-structure property relationships (QSPR) for both protein binding (Ingle et al. 2016) and clearance (Sipes et al. 2017). While HTTK data have so far been obtained for hundreds of chemicals, QSPR methods based on these data allow the application of HTTK to much larger sets of chemicals. While currently limited to two primary assays, additional in vitro measures, e.g., absorption (Artursson and Karlsson 1991; Wetmore et al. 2012), are under evaluation and may be adopted as predictive performance can be demonstrated for non-pharmaceutical compounds.

Lacking clinical data on environmental chemicals, the uncertainty in HTTK must be carefully quantified through comparisons with in vivo data (Yoon et al. 2014). The predictions of HTTK have been shown to be often within a factor of three, and generally biased toward overestimation of plasma concentration (Wambaugh et al. 2015). HTTK IVIVE has been evaluated by collecting matched in vitro and in vivo toxicokinetic data in rats for several dozen chemicals. Generally, peak, time-integrated (area under the curve, AUC), and steady-state plasma concentrations could be moderately well predicted, while properties like overall clearance rate were generally underestimated for non-pharmaceutical chemicals (Wambaugh et al. 2018). This difference is potentially due to the omission of processes like extra-hepatic metabolism and active secretion/resorption in the kidneys from current in vitro toxicokinetic assays (Rotroff et al. 2010). Additionally, the duration of the hepatic metabolism assay, which is conducted over four hours, may be too short to properly characterize chemicals with relatively slow clearance (Wambaugh et al. 2015). Supplemental Table 3 provides a summary of analysis methods and tools.

High-throughput exposomics

The NRC report "Exposure Science in the 21st Century: A Vision and A Strategy" summarized successes of exposure science and development of powerful new approaches to complement the strategies of "Toxicity Testing in the 21st Century. Examples are summarized in Figure S2 of the NRC report, and include remote sensing, use of geographic information systems, nanosensors, participatory assessments, biomonitoring and integration by source to dose models (US NRC 2012a). The report omitted critical review of the practical limitations of these approaches to address the exposome and personalized environmental health. Environment, defined broadly, and gene-environment interactions account for most human disease, while only a small fraction is attributable to genetics alone (Rappaport 2016). Humans have more than a million exposures over a lifespan (Idle and Gonzalez 2007), and this spectrum is amplified by the sequential nature of exposures, exposure interactions, cumulative biological responses to exposures and variable reparative/restorative functions in response to frequency and intensity of exposures (Miller and Jones 2014). Only a small fraction of the million or more human chemical exposures have been evaluated for health effects, and development of effective means to monitor lifelong exposures and health impacts poses a considerable challenge. At the same time, increased attention to personalized health brings awareness that each individual has a unique set of exposures. Additionally, stakeholders are increasingly demanding greater specificity and sensitivity to address individual health concerns. Some of these challenges are summarized in Fig. 3 as a foundation and motivation for new approaches to complement the NRC vision for toxicity testing in the 21st Century.

Perhaps the most critical challenge to toxicity testing and exposure science is the logistics of measuring and testing one chemical at a time. While fundamentally sound to address a relatively small number of hazardous agents causing severe toxicities, the approach becomes impractical for a million or more exposures having more moderate or delayed toxicities. For instance, the Human Metabolomics DataBase (HMDB) began cataloguing chemicals in human plasma about 10 years ago and has added about 6000 new chemicals per year; at the current rate, it will take more than a century to have a catalogue of one million chemicals in human plasma (Uppal et al. 2016). One can similarly project from the remarkable achievements of ToxCast/Tox21 screening of 8000 chemicals during the past decade; to screen one million chemicals at this rate will take a millennium. If this can be scaled to 10,000/y, it will only take 100 years. Unfortunately, the latter approach may not capture toxicities of products of environmental chemicals produced within the biosphere unless they are within the suspect list of chemicals subjected to screening. So without diminution of the important advances in toxicity testing and exposure science, motivation exists to complement these strategies with efforts to broadly measure the human exposome and support personalized environmental health assessments.

High-resolution metabolomics (HRM) was developed as a practical approach for personalized medicine (Johnson

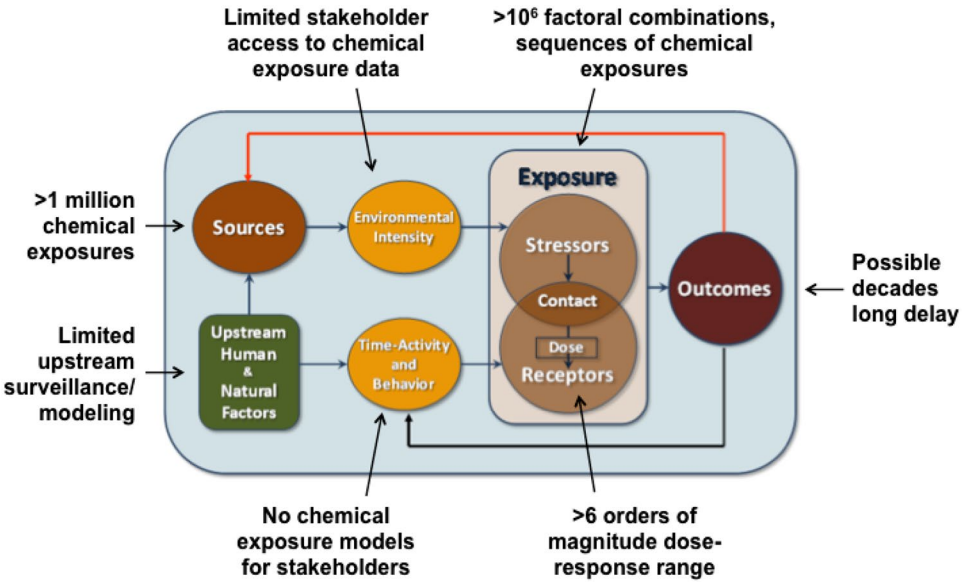

Fig. 3 Selected challenges to central framework for exposure science resulting from exposome research and personalized health initiatives. Challenges are listed on the periphery of the central framework (in the blue rectangle) (NRC 2012) (color figure online)

et al. 2010; Walker et al. 2016a). The approach uses ultra-high resolution mass spectrometry with liquid chromatography and electrospray ionization to measure metabolites in most human metabolic pathways (Jones et al. 2012). By measuring metabolites in most pathways, HMR provides a global approach to measure biological effects (see Fig. 4). Importantly, by pairing reverse phase liquid chromatography (C18) and hydrophilic interaction liquid chromatography (HILIC) together with switched negative and positive electrospray ionization in a dual chromatography protocol, the approach supports routine detection of more than 20,000 ions and can be performed for about $100 per sample, analyzed with three technical replicates (Jones 2016). Computational methods provide confidence scores for thousands of metabolites (Uppal et al. 2016), and absolute quantification can be obtained for known chemicals using reference

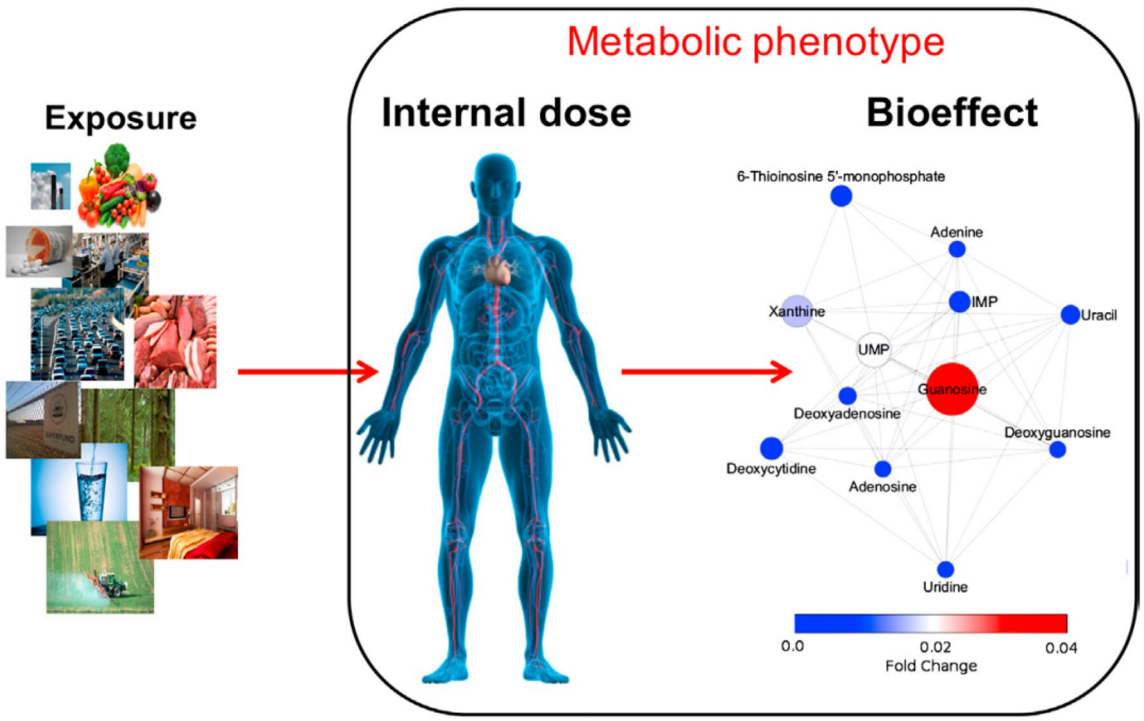

Fig. 4 Conceptual framework for metabolic phenotyping in exposome research. High-resolution metabolomics provides means to measure bioeffects of exposures and link these to internal dose of environmental chemicals obtained from biomonitoring (Walker et al. 2016a, b, c)

standardization (Go et al. 2015). If such an approach was applied to a large-scale study with a million or more individuals with documented exposures, the results would provide a large-scale reference for bioeffects. The feasibility of this has been established in smaller studies of occupational exposure (Walker et al. 2016a, b).

Application of HRM to evaluate metabolic differences between seven mammalian species provided the unexpected finding that environmental chemicals, including plasticizer, insecticide, flame retardant and fungicide contaminant, were present in all species (Park et al. 2012). Such environmental chemicals are often present at three to five orders of magnitude lower concentrations than intermediary metabolites (Rappaport et al. 2014). Quantification in human plasma using reference standardization showed that chlorobenzoic acid, chlorophenylacetic acid, chlorsulfuron dibutylphthalate, dipropylphthalate, octylphenol, pirimicarb, styrene, tetraethylene glycol, triethylphosphate, triphenylphosphate, tris(2-chloropropyl)phosphate and xylylcarb are present in nanomolar and sub-nanomolar concentrations (Go et al. 2015). The results raise the possibility that affordable analytical platforms can be developed for biomonitoring to broadly survey the occurrence and concentrations of environmental chemicals in populations. If such data were available, then a metabolome-wide association study (MWAS) could be performed to determine which metabolites vary in association with a chemical. Examples are available for benzo(a)pyrene (Walker et al. 2016a) and chlorophenylacetic acid (Walker et al. 2016b), trichloroethylene (Walker et al. 2016c) and cotinine (Jones et al. 2016). Applied to large populations, this approach could evaluate large numbers of environmental chemicals and chemical combinations for associated changes in metabolism. Those associations could then be experimentally verified using existing toxicity testing procedures. An example of the complete sequence from external exposure to internal dose to metabolic effect to disease marker outcome is available (Walker et al. 2016a).

HRM provides a foundation for universal exposure surveillance of environmental chemical exposures (Jones 2016); while chemical space is effectively infinite (Kirkpatrick and Ellis 2004), development of affordable, complementary analytical platforms to maximize coverageof chemical space is practical (Uppal et al. 2016). For instance, gas chromatography (GC) enables detection of non-polar aromatic chemicals that do not ionizewell by electrospray ionization so that coupling of GC to ultra-high resolution mass spectrometry complement HRM to detect hundreds to thousands of additional environmental chemicals. Thus, even though universal exposure surveillance cannot be construed to mean "comprehensive" exposure biomonitoring, feasibility studies indicate that measurement of 100,000 or more chemicals may be possible with current analytical methods if appropriate protocols and computational tools are developed. A computational framework to extend this to a million or more chemicals is available (Uppal et al. 2016).

Detailed cost–benefit analysis of universal surveillance methods to complement toxicity testing of known chemical entities is beyond the scope of the current discussion. The most critical argument for inclusion of such an approach is, however, important as a possible approach to improve toxicity testing. Currently, about half of the ions detected in mass spectral analyses of human samples are un-identified and about half of the ions associated with human disease do not match known chemicals in human metabolomics databases (Uppal et al. 2016). This suggests that up to half of the chemicals in blood that are associated with disease are chemicals that are not within suspect chemical lists. It is unclear whether these represent food or microbial metabolites, or possibly uncharacterized environmental products derived from the more than 80,000 agents registered with the US EPA for commercial use. HRM and associated universal surveillance approaches could provide an alternative means to prioritize chemical substances for toxicity testing. Specifically, if applied to large-scale population studies with health outcome data, mass spectral features associated with specific diseases can be subjected to purification, identification and prioritization for targeted toxicity testing. Such data would then provide reference data, which could be used to address many of the limitations identified in Fig. 3: for example, individual profiles could be compared to this reference to evaluate personalized exposures of concern, and personalized exposure models could be developed to facilitate remediation.

Making toxicity pathways useful in the regulatory process

There have been some efforts to elaborate the toxicity pathway concept itself and to make pathway knowledge more readily available for application in safety assessment (Whelan and Andersen 2013; Kleensang et al. 2014). Although progress has been primarily at the theoretical level, the concept itself has gained widespread appeal and has helped a great deal to encourage scientists and regulators to start thinking and talking on a mechanistic basis. The challenge of exploiting pathway knowledge to inform actionable decisions has been approached, at least in part, by the development of adverse outcome pathway (AOP) descriptions of toxicological processes (Ankley et al. 2010) that incorporate toxicity pathways, describe their linkages to apical responses and allow identification of specific assays needed to actually test a chemical for hazardous properties of regulatory concern. Thus AOPs provide a framework to more easily bring toxicity pathway assay results into the

risk assessment process and represent a formalization of the more ad hoc approaches attempted with specific case studies.

The epistemic properties of AOPs

An AOP is a practical analytical tool for collecting, synthesising, reviewing and disseminating knowledge about a toxicological process (Vinken et al. 2017). Thus one can consider an AOP as a way of 'managing' biological knowledge of a mechanistic nature. Moreover, although there are certainly gaps in our knowledge that should be filled through research, an extensive body of knowledge already exists primarily in the form of peer-reviewed papers that needs to be extracted and made available in a form that can be readily applied within a risk assessment context.

Analytically, an AOP is a discretization of a process occurring within a system into a chain of sequential causally related key events (KEs) linked by key event relationships (KERs). A molecular initiating event (MIE) triggers the process that, under the appropriate conditions (e.g., magnitude and duration of insult), leads to an adverse outcome of regulatory concern, for example an anticipated specific organ toxicity in a human or a population decline of an environmental species. Essentially, a KE reflects the state of the system at a particular stage or time, while a KER describes the reasons or conditions for the system transiting from one KE (upstream) to another (downstream). An important premise of AOP theory is that toxicological processes tend to share KEs and KERs, not only within an individual organism but also across species. Moreover, one MIE may be associated with different adverse outcomes, and vice versa. Thus the collective knowledge captured by AOPs is best represented as a causality network, with KEs being the nodes and the KERs being the edges. From a practical perspective, an AOP is a structured document that is the product of an AOP development process (OECD 2013, 2016a; Villeneuve et al. 2014a, b) which involves collaborative input from subject specialists, expert peer review, and in some cases endorsement by a regulatory body (OECD 2017a).

Another important element of the framework is the AOP Knowledgebase (AOP-KB) that provides public-domain access to AOP content (http://aopkb.org). The AOP-KB developed under the auspices of the Organisation for Economic Co-operation and Development (OECD) currently comprises a number of complementary on-line software modules, with the AOP-Wiki (https://aopwiki.org) currently being at the core. However, with the anticipated adoption of an OECD harmonized template for reporting AOPs, it is envisioned that interoperable AOP platforms maintained by different parties across the world will soon emerge thus transitioning the KB from a centralized to a distributed infrastructure. The framework is built with crowdsourcing and knowledge-sharing very much in mind, not only for the initial development of an AOP but also during ongoing refinement and review. Since an AOP covers many levels of biological organization, this naturally stimulates and relies upon extensive collaboration across numerous scientific disciplines, from molecular toxicologists to epidemiologists. Thus the AOP framework and the associated AOP development program at the OECD can be seen as very much addressing the priority of "knowledge development" as described in the TT21C Vision and Strategy and the proposed focus on "elucidating toxicity pathways and developing an associated data-storage, -access, and -management system". Although AOPs have gained widespread appeal both in the scientific and regulatory communities, there is clear impetus to continue to refine the framework (LaLone et al. 2017; Leist et al. 2017; Vinken et al. 2017) to ensure it can sufficiently capture key information relevant to many important aspects of toxicology including: sensitive life stages; species specificity; acute versus chronic and high versus low levels of activation (exposure); quantification of response dynamics; and simultaneous triggering of multiple pathways. In addition, the relationship between AOPs and computational models for predictive toxicology is also being explored (Wittwehr et al. 2017).

AOPs and risk assessment

An AOP is not a magic bullet. Neither is an AOP a test method, a computational model nor a risk assessment. In its primary form, today, an AOP is simply a peer-reviewed, highly structured textual description of collective knowledge of a toxicological process which is supported by scientific evidence. To be practically useful in risk assessment therefore, the knowledge conveyed by an AOP has to be applied through some practical means and for some purpose that is relevant to a decision-making context. Outside the context of AOPs, mechanistic knowledge has been used for many years by different agencies and companies in a variety of sectors to support chemical risk assessment. Thus the use of mechanistic knowledge is nothing new per se. However, mechanistic information has typically been a 'nice to have' rather than a 'need to have' since the assessment of the potential toxicity posed by a chemical still relies heavily on information derived from pathological observations manifest in animal studies. This continuing over-reliance on animal testing has had the result that for the most part, regulatory toxicology is driven more by observation rather than by mechanistic reasoning. In reality therefore, mechanistic information, if actually available, is typically used to trigger follow-up animal studies or to increase confidence in a risk assessment decision based on animal data.

Responding to the need to increase the efficiency and effectiveness of chemical risk assessment through the incorporation of modern toxicological tools and methodologies,

the OECD's Working Party on Hazard Assessment (WPHA) recently proposed a framework for Integrated Approaches to Testing and Assessment, or IATA (OECD 2017b). The output of an IATA is intended to be a conclusion concerning hazard identification, hazard characterization or safety assessment that can inform a decision in a certain regulatory context. Much like general provisions in risk assessment, an IATA integrates existing information and employs weight-of-evidence to decide if a conclusion can be drawn or if more information is required through, for example, targeted testing. An IATA draws data from any relevant source including: physicochemical properties, computational models, grouping and read-across, in vitro methods, in vivo animal studies and human data. However, an important goal of IATA is the use of new data-streams coming from non-animal approaches to not only address the ethical issues surrounding animal testing but also to further enhance levels of protection of human health and the environment in an economically beneficial and socially acceptable manner. Moreover, the core or blueprint of IATA is intended to be based on mechanistic information and reasoning, ideally presented as AOPs and the toxicity pathways therein (OECD 2016b).

An important consideration in the development of the IATA framework has been how additional toxicological data could be best generated when existing information is insufficient to draw a conclusion about a potential chemical hazard or risk. In the interests of efficiency both in the generation of additional data and the consideration of it within an assessment, it was considered important to be explicit as possible about means of filling typical information gaps. This led to the concept of a Defined Approach (DA) to testing and assessment which "consists of a fixed data interpretation procedure used to interpret data generated with a defined set of information sources, that can either be used on its own, or together with other information sources within an IATA, to satisfy a specific regulatory need" (OECD 2016c). According to DA principles, the following attributes should be clearly defined: the toxicological endpoint being addressed; the intended purpose in a regulatory context; the underlying rationale including mechanistic basis (e.g., AOP); the individual information sources (methods) used; how the individual information sources are combined and processed to arrive at an outcome (prediction); and the known uncertainties including limitations of the approach. These principles and associated attributes are captured in a recommended reporting template (OECD 2016d) that facilitates understanding and acceptance of a DA used within an IATA by end-users and decision-makers. This addresses the reality that irrespective of the scientific validity of a DA, trust in the information it delivers depends a great deal on how it is described and presented. To complement and exploit the development of IATA/DA guidance, in 2015 the OECD launched the IATA Case Studies Project within its Cooperative Chemicals Assessment Programme (CoCAP) to facilitate a collective learning-by-doing process. The project is cyclic in that annually member countries and organizations submit case studies to the WPHA that typically reflect particular assessment priorities or interests they may have. These are then subject to analysis and review (OECD 2017c) by WPHA members with the aim of identifying and describing aspects such as the overall strengths and weakness of the IATA, principle sources of uncertainty, needs for guidance development, and any potential regulatory applications of the IATA in addition to that foreseen by the developer. The case studies considered so far have been quite varied and cover a number of human health and ecotoxicological endpoints; different chemical classes including nanomaterials; predictive approaches such as grouping, read-across and ab initio; and assessment aims including hazard identification, hazard characterization, and screening and prioritization. In addition to the OECD programs, there are many other IATA related activities being pursued internationally (Tollefsen et al. 2014; Berggren et al. 2015, 2017) which are providing a solid foundation for transitioning to pathway-informed approaches to achieve the aims of sound chemicals management worldwide.

There have been a number of notable successes regarding the impact of IATA using non-animal methods on chemicals regulation. In 2016 the European Union changed the REACH information requirements for skin corrosion/irritation, serious eye damage/eye irritation, acute dermal toxicity (Commission Regulation (EU) 2016) and skin sensitization (Commission Regulation (EU) 2016) to make data from in vitro methods the default. Concerning skin sensitization, the revised REACH legal text now makes specific reference to 'key events' and the combination of test data derived from associated in vitro methods. There were many elements and actors that had to come together to make this happen which not only represents a scientifically superior way to assess chemicals regarding skin sensitization potential, but also heralds a substantial shift in the formulation of information requirements within a legislative context, i.e., with a reference to toxicological pathways that can be captured within an in vitro assay rather than to conventional pathological observations in animal models (Casati et al. 2013, 2018). There were many important elements that had to be put in place to ultimately put forward an alternative approach to skin sensitization assessment that was acceptable to regulators (JRC (Joint Research Centre) 2017) These included the development and endorsement of the skin sensitization AOP (OECD 2012), the adoption of the first 3 in vitro methods as OECD Test Guidelines (442C, 442D and 442E) and the publication of OECD guidance on the reporting of DAs and individual information sources to be used within IATA for skin sensitization (OECD 2017d). The latter includes (in annex) descriptions of 12 different case studies which clearly

demonstrate that the same mechanistic knowledge base (i.e., the AOP in this case) and tool box (i.e., in vitro and in silico methods) can rationally inform the design of different DAs that address the same problem demonstrating similar levels of predictive performance. In 2015, the US EPA announced its plans to incorporate a pathway-informed in vitro approach (Browne et al. 2015) into the Tier 1 battery of the Endocrine Disruptor Screening Program (EDSP) to test chemicals for estrogen receptor bioactivity (US EPA 2014a, b). This has paved the way for the anticipated introduction of similar in vitro high-throughput screening solutions to address endocrine disruption via androgen and thyroid toxicity pathways. The EPA's Office of Pesticide Programs (OPP) has put forward its "Strategic Vision for Adopting 21st Century Science Methodologies" (US EPA 2017) and has committed itself to the development and evaluation of new technologies that can be combined with a hypothesis-driven approach to underpin IATA that can supplement or replace more traditional methods of toxicity testing and risk assessment. One near-term objective is to propose a set of IATAs based on non-animal methods that satisfy the information requirements for acute toxicity (the "6 pack") requested for pesticides. Additional case studies involving endocrine active compounds are currently underway within the OECD IATA initiative (OECD 2019).

Looking to the future, AOPs and IATA will likely continue to serve as a bridge. A bridge between different scientific communities, from computational biochemists and molecular toxicologists to epidemiologists and clinicians, who can contribute to the development of AOPs; between method developers providing the tools and risk assessors who need solutions; and between regulatory scientists and decision-makers whose have a collective responsibility to translate new approaches into the regulatory arena. But AOPs also provide another important bridge, that is between toxicity pathways described at the molecular and cellular level and the apical endpoints that current regulation is based on, including topical toxicities, skin sensitization, acute and chronic systemic toxicities, cancer, and reproductive toxicity. There are many who highly value this aspect of AOPs since it may offer the possibility of retaining the current regulatory basis for characterizing the toxicological hazard of chemicals while at the same time exploiting new types of data derived from in vitro and computational methods. This of course implies that it will be eventually possible, and desirable, to use AOPs as a means to construct models or DAs that predict conventional endpoints. This "marrying the old with the new" strategy may indeed accelerate the uptake of new science in risk assessment to some extent. However, it is debatable if this would eventually constitute the paradigm shift actually proposed by TT21C. An alternative strategy to fully realize that vision would be to use the AOP framework to facilitate a pivotal, paradigm-shifting transition from defining toxicological hazard not in terms of conventional endpoints associated with animal models but instead in terms of intermediate key events specific to the species of concern. Only then will there be a sound scientific basis to adapt regulatory information requirements and risk assessment methodology to exploit twenty-first century tools and thinking to its fullest.

The next generation of risk science

In the last 25 years, a transformation in biological understanding has happened, but the practical implication for our daily work as risk assessors and risk managers often goes unrecognized. The exploration of the genome and epigenome, advent of high-throughput biological assays, and the explosion of new scientific information pertaining to disease causation alone dictate that we must embrace new ways of thinking about risk assessment science. New science is being used to inform risk assessment, but probably has not been as widely applied as warranted. It must be recognized that environmental regulations, risk-management decisions, and the scientific support of these actions are based on legal precedent in successful court cases. This is a criterion not generally required of science in general. Consequently, there is a tendency to rely on "tried and true" approaches that have been utilized successfully in the past. New types of science will also have to withstand challenge in courts of law. The initial uses of new types of science in support of regulation likely will be accompanied by traditional, confirmatory science (Chiu et al. 2013). Such side-by-side evaluations will help build legal precedent for new methods, as well as stakeholder confidence in new methods. As precedent develops, new types of science to inform risk assessment will begin to stand on their own. In a variety of non-regulatory applications, such as prioritizing chemicals for testing and additional assessment, emergency response, and clean-up activities, new science is playing a significantly larger role, particularly when traditional data are not available or are limited. In this paper, we focus on integrating information across an array of data types to evaluate risks in a variety of contexts. Almost all new data types can help us understanding public health risks in some way. The challenge is how to correctly interpret data and fit the puzzle pieces together.

Here, we discuss risk assessment approaches that have broad applications: (1) informatic methods to find, organize, and integrate risk assessment information; (2) data confidence evaluation in different risk assessment applications; (3) data mining and bioinformatic analyses; (4) novel ways to build and use mode of action networks; and (5) new data types in dose–response assessment.

Informatic methods to find, organize and integrate risk assessment information

One change that is occurring in risk assessment is the identification, organization and synthesis of the relevant literature itself. Often, we can no longer rely exclusively on human talent alone for this central task of risk assessment. The amount of literature that must be reviewed for risk assessments is growing exponentially. A disease of interest, such as cancer, will have more than 100,000 new papers published a year, adding to the > 30 million pre-existing studies. For well-researched chemicals, the potentially relevant number of publications identified in traditional key word searches often includes tens of thousands of papers. Upon reflection, it becomes obvious that individual scientists or even teams of scientists are no longer able to do what has been done in the past, i.e., to read the relevant literature, organize and integrate information into new knowledge for risk assessment, without computational help. Therefore, scientists are turning to natural language processing to facilitate the systematic review of the literature.

Natural language processing is a discipline at the intersection of linguistics, computer science and artificial intelligence. EPA and other regulatory agencies are beginning to apply this approach to help identify the likely most relevant information for further human review (Painter et al. 2014; Gabb and Blake 2016; Gonzalez et al. 2016; Howard et al. 2016). Computer scientists and a new breed of librarians use computer algorithms to help identify and sort papers into topics of interest, and exclude off-topic papers, thus, playing a substantial role in the development of risk assessments. Essentially, computer algorithms search the text of published papers for similarities in language and group papers accordingly; a "seed" set of highly relevant papers, selected by humans, informs this process. Computers make the process of identifying information substantially faster and less resource intensive, but human curation is still required to refine and ensure accuracy. Computer algorithms, which "read", summarize textual descriptions, and write draft documents are being developed but are not yet sufficiently refined for routine deployment in risk assessment (Blake and Lucic 2015). Also in development are programs that can extract tabular and quantitative data (e.g., dose–response/concentration–response data, disease incidence) from the primary literature and manage it within databases. This latter category is important as it allows for re-analysis and meta-analysis of a chemical's data on specific effects (Druwe and Burgoon 2016).

Criteria for data confidence evaluation in different risk assessment applications

Common to all risk assessments is an evaluation of uncertainty in the underlying data and, hence, the conclusions based on these data. Some criteria for reducing uncertainty are the same as for traditional data, such as reproducibility and adequate reporting of methods. Other criteria are specific to new methodologies. The first step in this process is to determine which studies meet minimum technical quality. This is particularly important in a rapidly developing field like omics where the best practices and our scientific understanding is rapidly changing. A study that was considered as 'state of the art' 2 years ago may now have obvious flaws. The Systematic Omics Analysis Review (SOAR) protocol was used for evaluation of transcriptomic data (McConnell et al. 2014). This approach is currently being expanded to other data types. Others have published systematic review schemes that can be very useful, although SOAR appears to be the most parsimonious (Dearfield et al. 2016; Vandenberg et al. 2016).

Subsequent to identification of the minimum technical criteria, additional restrictions may be placed on data use and these can vary by type of assessment. For screening and prioritization, the types of data used are more flexible and can be derived from various species, tissues, cell types and levels of mechanistic understanding. For example, data from invertebrate species like yeast and daphnia have proven very informative for comparative toxicity testing and investigation of basic biology (Garcia-Reyero et al. 2012; Hartman et al. 2015; Goldstein and King 2016; Gust et al. 2016; North et al. 2016). For regulatory assessment, we currently prefer data from either human cells or from vertebrate cells focusing on highly conserved processes in the tissue of interest, because of the importance on cell-type and tissue identity in disease (Greene et al. 2015; Gross and Ideker 2015). An exception to the tissue-specific data criterion is observed disruption of critical mechanisms common to most cell and tissue types (e.g., stem cell population modification, epigenomic remodeling, cell cycle alterations, DNA repair impairment, endocrine disruption). Such disruptions can be linked to either the same disease in different tissues (e.g., cancer) or different diseases resulting from a common mechanism (e.g., chronic inflammatory diseases). Additionally, the closer to the intact organism the experimental protocol, the greater the confidence in the data. This is because of the important roles that metabolism and cell-to-cell and tissue-to-tissue interaction play in disease. To the extent feasible, information on humans is also sought, as the absence of such data introduces significant uncertainty (Zeise et al. 2013; Krewski et al. 2014; Wetmore et al. 2014; Abdo et al. 2015; Eduati et al. 2015; Schulte et al. 2015; Iavicoli et al. 2016; Kenyon et al. 2016; McCullough et al. 2016; Bowers and McCullough 2017). Overall, the understanding of the mechanistic connections between exposure and response and the impact of various risk modifiers increases our confidence in the risk assessment.

Although tempting, it is simply not practical to create an ordered list of preferred data types in terms of determining causation. For instance, a blanket statement that animal studies are better predictors of human effects than in vitro human cell studies is not appropriate or correct. Each data type, be they from epidemiological studies, in vivo animal studies, in vitro human cells, has specific strengths and weaknesses. It is the combination of the available data that synergize to lead to a specific level of causal certainty and overall confidence in the assessment. In silico models, including models based on machine learning, also have varying confidence and certainty. There may be times when they outperform the individual assays they use as input data, and others where they underperform (Knudsen et al. 2015; Kleinstreuer et al. 2016a, b). Again, this is a place where predictive performance becomes important.

Data mining and bioinformatic analyses

Data mining and bioinformatic analyses are areas where significant progress in being made. The National Institutes of Health (NIH), the European Community and others have established enormous data warehouses that archive published biological data. As a consequence, data can be acquired and analyzed as never before (Luo et al. 2015; Snider et al. 2015; Zhang et al. 2015a, b; Gonzalez et al. 2016; Juberg et al. 2017). It should be noted that integration of information from various data types (genomics, transcriptomics, metabolomics, proteomics, systems biology, and network biology) is what is truly needed, but is difficult. A few common databases and their content include: NIH/NCBI BioSystems which annotates integrated molecular pathways by source, species, biological function/process, disease/toxicity relevance and availability of probing assays; NIH/NCBI Gene Expression Omnibus and European Community ArrayExpress with MIAME-compliant functional genomics data; the NIH Roadmap Epigenomics Project that provides epigenomic maps for stem cells and primary ex vivo tissues representing tissues and organ systems frequently involved in human disease; NIH/NCBI Genotypes and Phenotypes (dbGaP) that catalogues interactions of genotype and phenotype in humans; Tox21 Consortium's high-throughput assays results; and the US EPA's Safer Chemicals Program that has a computational toxicology database. Additional database resources are shown in Supplemental Tables 2 and 3; see also Zhang et al. (2015a, b). Currently, EPA is using these types of databases to help inform the understanding of mode of action networks, identify and characterize sensitive populations and risk modifiers, and to help characterize dose–response. Development of mechanistic knowledge has been fueled by the explosion in high-throughput omics data and development of various computational methods that facilitate integration of these data into biological networks (Greene et al. 2015; Gross and Ideker 2015).

Understanding environmental disease causation and risk modification involves understanding interactions within biologic networks (Geer et al. 2010; Bouhifd et al. 2015a, b; Greene et al. 2015; Gross and Ideker 2015; Zhang et al. 2015a, b; Oki and Edwards 2016). Due to functional interdependencies among molecular pathways, a disease is rarely a consequence of an abnormality in a single gene or even pathway, but reflects disruption of complex intracellular networks. Many genes contribute to each phenotype and each gene contributes to multiple phenotypes (Goh et al. 2007; Hartman et al. 2015; Darabos et al. 2016). Importantly, individuals or subpopulations with the same disease can have different perturbations (Schadt 2009; Barabasi et al. 2011; Ideker and Krogan 2012). Additionally, the critical role of epigenetic modifications has begun to be elucidated (Kuppusamy et al. 2015; Schulte et al. 2015; Bowers and McCullough 2017). Nuances in network functions are the basis for varying susceptibilities in the human population, and can define the underpinnings of mixture interactions (Yang et al. 2012). Overly simplistic modes of action (MOA) are often insufficient to capture our current level of biologic understanding or explain public health risks. In this section, we will explore innovative ways to develop MOA networks and how these more rapidly developed and more robust models can improve risk assessment. These approaches will be discussed in more detail below.

Innovative ways to build mode of action networks

Currently, the US EPA and others are making extensive use of the broader literature on pathogenesis combined with chemical-specific information to help develop mode of action (MOA) networks (US NRC 2017). The medical research community has worked extensively to develop robust disease models, such as those housed in the National Biosystems Institutes of Health's National Center for Biotechnology Information (NIH/NCBI) database. BioSystems (https://www.ncbi.nlm.nih.gov/biosystems/) is perhaps the most comprehensive biological pathways knowledgebase currently with distinct advantages: robust models exist for many major diseases; models are periodically updated and linked to the underlying publications; species and tissue specific, human models and "bioconserved" models that facilitate cross-species extrapolation are available; importantly, models include various data types measured with various methods (e.g., omic events, miRNA regulation, transcription, cell signaling, traditional upstream events and prototypic outcomes) at different levels of biologic organization, models are also linked to the broad array of other NIH databases, e.g., Gene, PubMed, BioAssay. Additionally, a variety of

existing database can provide useful data (Bunyavanich and Schadt 2015). These mechanistic models help: "fill in the blanks" in existing MOA networks; increase confidence in the causal connections between chemical exposures and effects; identify potential risk modifiers that are important for understanding population responses to chemical exposures (e.g., genetic polymorphisms or mechanistic connection among other environmental factors such as lifestyle, preexisting health status, and co-exposures); and inform dose–response. This approach is consistent with the adverse outcome pathway (AOP) philosophy of utilizing other-than-chemical-specific-data to understand mechanisms, but more oriented toward network and systems biology.

The National Research Council raises a number of questions in regards to use of mechanistic information to inform chemical risk assessments that our research, and that of others, is beginning to address (US NRC 2017). For several important chemicals (ozone, arsenic, polycyclic aromatic hydrocarbons, benzene) the data reflect the following elements.

Identified pathways, alone or in combination with other pathways, when sufficiently perturbed, increase the risk of an adverse outcome or disease in humans (Dangleben et al. 2013; US EPA 2014a, b; McCullough et al. 2014; US NRC 2014; Thomas et al. 2014; Bunyavanich and Schadt 2015; Cote et al. 2016). Multiple pathways generally underlie causation. Altered expression of various pathways within a network also can alter the severity of the disease, as well as incidence (Netto 2012; Hatzimichael and Crook 2013; McCullough et al. 2014; Bunyavanich and Schadt 2015).

Chemically induced pathways, leading to adverse outcomes, overlap with the pathways involved in "naturally" occurring disease. Thus, it appears that chemical exposures can exacerbate ongoing pathogenesis in some individuals, and initiate pathogenesis alone or in combination with other risk factors (Dangleben et al. 2013; US EPA 2014a, b; McCullough et al. 2014; Thomas et al. 2014; Cote et al. 2016).

The number of pathways and extent of activation varies with exposure–dose, as well as the time post exposure (McCullough et al. 2014; Thomas et al. 2014; Mirowsky et al. 2016).

Understanding the exposure–dose relationship can be critical for characterizing the dose–response and this can be critical to extrapolating across various experimental paradigms (e.g., across species) (Hatch et al. 2014).

The underpinnings of increased susceptibility due to co-exposures, pre-existing disease, genetic profile or life stage can be characterized mechanistically (Netto 2012; Hatzimichael and Crook 2013; Bailey and Fry 2014; McCullough et al. 2014; US NRC 2014; Bunyavanich and Schadt 2015; Davis and Burgoon 2015; French et al. 2015; McCullough et al. 2016).

While quantitative changes in the epigenome, gene expression, or patterns of gene expression can be quantified, it is currently difficult to use these changes to predict public health risks due to the complexity of network interactions, in the absence of in vivo data to anchor upstream data to public health risks. In the absence of such data, our current preference is to focus on quantification of events as downstream as feasible, as these downstream events already integrate much network activity. Additionally, quantitative changes in pathways are being used to explore comparative potencies of chemicals to disrupt important biological processes (Tice et al. 2013) and see "High-content imaging" and "The epistemic properties of AOPs" in this paper.

Mechanistic models can be useful in varying degrees of completeness (Perkins et al. 2015). Chemicals with some MOA data and limited or no traditional can be compared against diseases-specific networks for insights into potential phenotypic outcomes. In this manner, new data types can inform hazard identification, as well as dose–response in the absence of traditional toxicological data (see "New data types in dose–response assessment" below for more discussion).

This EPA effort has yielded more coherent and robust MOA networks by filling in missing pieces of information using existing nonchemical-associated disease models. We have not encountered chemical-associated-processes not captured in "normal" disease models. In other words, "normal" pathogenic processes appear shared with chemical-specific pathogenic processes. Because much of the disease-based research is fueled by a desire to develop therapeutics, experimental data are sometimes available where relevant pathways are blocked or altered by pharmacologic agents or chemicals with concomitant evaluation of the impact on the downstream phenotypic event(s) (Hatzimichael and Crook 2013; US EPA 2014a, b; McCullough et al. 2014). This provides powerful experimental evidence that informs several important issues: clarifying causal events and pathways, including sorting causal events from events related to having the disease; exposing pathway nuances (e.g., does blocking a pathway prevent the disease or ameliorate a quality of the disease such as invasiveness); and providing insights into quantitative relationships between specific events, pathways and outcomes.

For some chemicals and situations, simple MOAs and pathway analyses appear adequate (e.g., carbon monoxide and hemoglobin binding, organophosphate pesticides and cholinesterase inhibition), but for many metals and chemicals (e.g., arsenic, benzene, ozone, polycyclic aromatic hydrocarbons, lead) the salient pathogenic processes are more informatively described by MOA networks (McCullough et al. 2014; Thomas et al. 2014; Cote et al. 2016; Darabos et al.

2016). We have on several occasions attempted to collapse the network models into simpler MOAs. Unfortunately, in doing so enough information was lost that the simpler MOAs were not useful for risk assessment purposes. In particular, important events or potential risk modifiers that operate in via different parts of the network were lost. Looking across network analyses, the recurrent roles of various events and pathways in multiple diseases are evident, moving us away from the one chemical, one MOA, one disease model to a viewpoint more consistent with the latest science.

Key characteristics of human carcinogens

An notable advance in the understanding the biological mechanisms of human cancer is the elaboration of 10 key characteristics of human carcinogens (Smith et al. 2016). The specific attributes of human carcinogens comprising the key characteristics are that the agent: is electrophilic or can be metabolically activated to electrophiles; is genotoxic; alters DNA repair or causes genomic instability; induces epigenetic alterations; induces oxidative stress; induces chronic inflammation; is immunosuppressive; modulates receptor-mediated effects; causes immortalization; or alters cell proliferation, cell death, or nutrient supply (see also Smith 2019).

Krewski et al. (2019) conducted an exploratory analysis of the key characteristics of 86 Group-1 agents identified by the IARC as causes of human cancer, including: pharmaceuticals (20 agents); biological agents (10 agents); (c) arsenic, metals, fibers, and dusts (10 agents); (d) radiation (5 agents); (e) personal habits and indoor combustions (8 agents); and (f) chemical agents and related occupations (33 agents). As indicated in Fig. 5, these 86 agents demonstrated a range of key characteristics, consistent with the notion that human carcinogenesis involves multiple pathways. These agents demonstrated an average of four characteristics, ranging from 1 to 9 characteristics across the individual agents.

These key characteristics are now being used by the International Agency for Research on Cancer (IARC) in its evaluation of the carcinogenic potential of agents evaluated within the *IARC Monographs Programme*, in accordance with guidance provide in the recently updated *Preamble to the IARC Monographs* (Samet et al. 2019), using systematic review to assemble all relevant information from human/animal/in vivo/in vitro sources. Examples of the use of the key characteristics in mechanistic evaluations conducted by the IARC in Volumes 112–119 of the *IARC Monographs* are described by Guyton et al. (2018).

Characterizing population variability and co-exposures to various environmental factors using MOA networks

The goal for environmental risk assessment is to estimate the population exposure–response relationship. Consequently, risk modifiers (environmental co-exposures, lifestyle, genetic and pre-existing morbidities) that affect the underlying population response distribution are important factors in understanding risks. In turn, considerations of underlying networks can be key to identifying risk modifiers and the sizes of the affect populations. While the task seems daunting, progress has made in recent years (Zeise et al. 2013; Abdo et al. 2015; Davis and Burgoon 2015; Eduati et al. 2015; McCullough et al. 2016).

As an example, a number of human polymorphisms likely to modify disease risks resulting from inorganic arsenic exposure have been identified. These polymorphisms appear

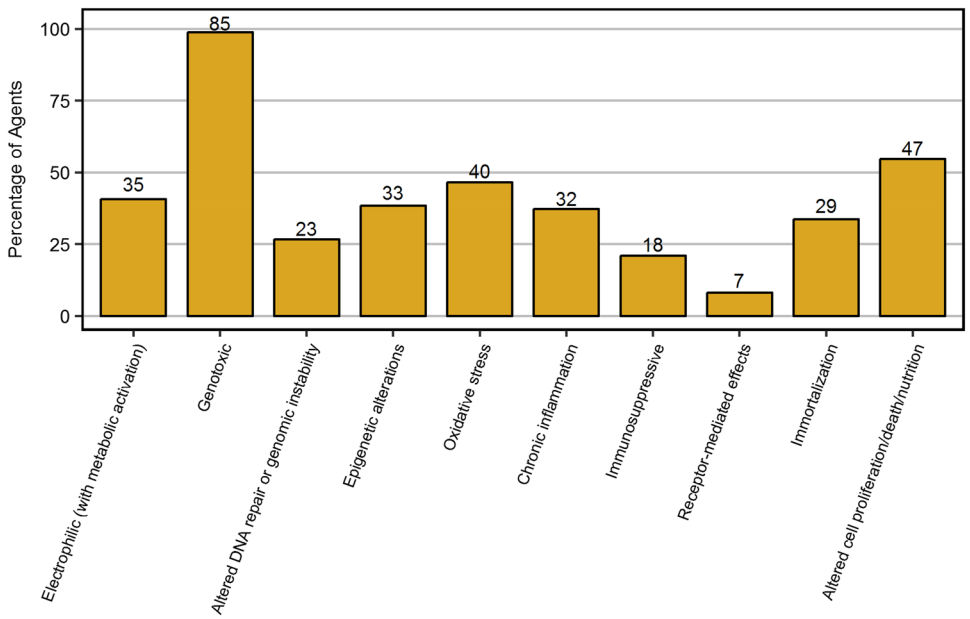

Fig. 5 Key characteristics of 86 agents known to cause cancer in humans

in genes involved in metabolism, oxidative stress responses, DNA repair, and tumor suppression. The network analysis results are consistent with epidemiological observations, thus adding to the weight of evidence that certain populations may be at greater (or lesser) risk based on their genetic profiles (Faita et al. 2013; Antonelli et al. 2014). Risk modifications by polymorphisms, as well as epigenetic alterations, have been reported for other chemicals as well (US EPA 2014a, b; Ravegnini et al. 2015; Carbonari et al. 2016; McCullough et al. 2016; Bowers and McCullough 2017). This work can be extended to identify populations potential susceptible to the effects of other chemical exposures, based on common underlying mechanisms or key events. The interactive potential of various polymorphisms can be visualized in MOA network analyses (see Fig. 6), a technique employed in the development of Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa 2019; Kanehisa et al. 2017, 2019).

Additionally, via network analyses, the interactions of co-exposure can be evaluated. For example, we have characterized some potential interactions between arsenic and tobacco smoke exposures relative to bladder and lung cancer risks (not shown). While there are some common events between arsenic and tobacco smoke, other interactions for these two pollutants occur at the pathway or network level. Similar network level interactions among various environmental factors also have been observed for benzene, and ozone (US EPA 2014a, b; Vawda et al. 2014). A linear MOA would not reveal these interactions.

New data types in dose–response assessment

Three approaches to dose–response assessment are discussed: (1) efforts in network modeling; (2) use of selected molecular or cellular events as the basis for a point of departure and (3) use of structure activity and read-across approaches. This discussion focuses on pharmacodynamic aspects of dose–response assessment, although the critical role of pharmacokinetics is well recognized.

Network models Dose–response analyses can be facilitated by the network analysis approaches described in the MOA section (above). Currently, efforts are focusing on quantifying changes in individual key events and processes using analyses of pooled data from multiple, high-quality studies. The most attention is focused on the farthest downstream events, closest to phenotypic outcomes, as they are least affected by dynamic upstream feedback and feed forward loops. The effort provides a static snapshot in time for what is inherently a dynamic process. An analogy is that of a roadmap which provides limited information on the most likely route to the destination or traffic flow along the various routes to the destination. Dynamic network models are in their infancy and hold great promise (Knudsen et al. 2015). Rarely are the types of data collected to drive such models (Jaeger et al. 2012; Cohain et al. 2016). Such models, which are similar to computer information processing models, depend on understanding the kinetic relationships among event and pathways rather than static measures.

High- and medium-throughput data to estimate points of departure A point of departure (POD) is an estimate of the lowest or no observed effect level for an adverse effect and is used as a starting point for low-dose extrapolation. In traditional risk assessment, PODs range from a 1 to 10% response rate; uncertainties around low-dose extrapolation are generally addressed by dividing the POD by various factors or by linear extrapolation to zero. When using molecular or cellular events to estimate a POD, the lowest concentration where a chemically induced

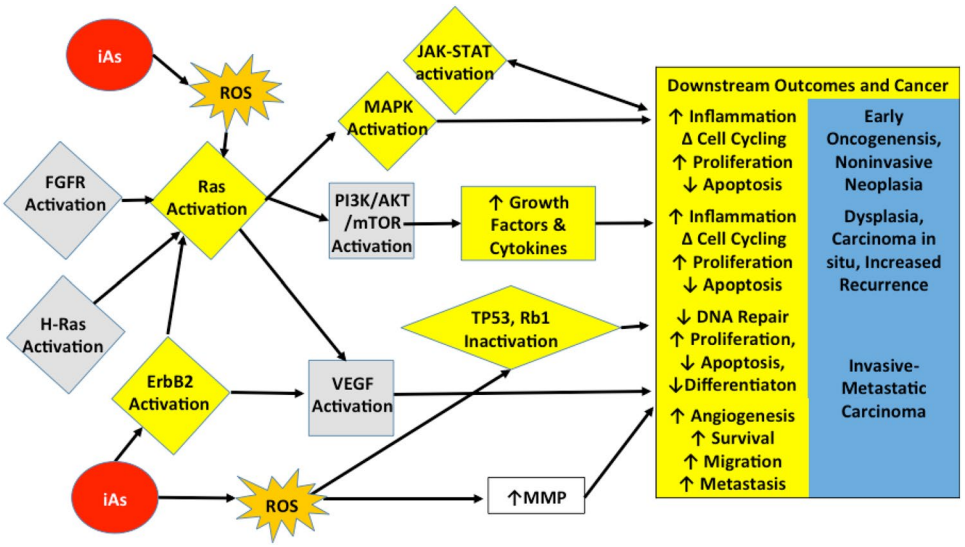

Fig. 6 Human bladder cancer network with common events associated with inorganic arsenic (shown in yellow). Adapted from NIH BioSystems #83115 (KEGG: hsa05219, 2015) (color figure online)

response exceeds background noise has been used. Given that no phenotypic change happens in the absence of some preceding molecular or cellular change, it has been proposed that measurements of such early changes could substitute for more traditional, in vivo data to model points of departure (Burgoon and Zacharewski 2008; Judson et al. 2011; Chiu et al. 2012; Tice et al. 2013; Sand et al. 2016; Burgoon et al. 2017). Correlation of in vitro-measured upstream events to phenotypic changes, however, has had mixed results (Thomas et al. 2012; Sand et al. 2016). The limited correlation to specific phenotypic outcomes is not surprising. Even in situations where the upstream change is known to be biologically significant, the impact of network interactions and common variables relative to extrapolation to humans (e.g., metabolism species, tissue, cell type, interactions among cell and tissue) makes prediction of a specific outcome based only on upstream data uncertain.

Specific knowledge of adverse outcomes, however, may not be necessary for some screening and risk assessment purposes, if there is confidence in the general importance and biologic context of the event being measured (Tice et al. 2013; Attene-Ramos et al. 2015; Chen et al. 2015; Huang et al. 2016). From such knowledge, effects in humans (although not specifically defined), could be anticipated. Additionally, specific chemical-associated network modification could serve as biomarkers of both exposure and effect (US NRC 2012a; Thomas et al. 2014; DeBord et al. 2015). One such example is the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) which used predictive computational models and high-throughput screening data to screen 32,464 chemicals identifying ~ 12% as active and ~ 21% as potential active estrogen disruptors for which further testing is needed (Mansouri et al. 2016a, b). Such an effort is possible because of the extensive body of knowledge about endocrine function in humans. The ability to sort chemicals, based on new approaches and data, into high, medium and lower concern is a tremendous achievement and begins to address the backlog of unaddressed chemicals in the environment, as well as makes additional research, testing and assessment more efficient. Proceeding to develop some risk assessment screening values for the chemicals of greatest concern seems warranted.

Sand et al. (2016) have recently explored, in detail, the feasibility of and methods for using high-throughput Tox21 data in dose–response assessment. While the authors note a number of science policy decisions necessary for proceeding with use in risk assessment, the technical approach is feasible. Several other examples, using various data types, are also available (Behl et al. 2015; DeBord et al. 2015; Adler et al. 2016; Kuo et al. 2016). Several approaches to improving consideration of population variability in such dose-response estimates also have been suggested (Zeise et al. 2013; Abdo et al. 2015; Eduati et al. 2015; Chiu et al. 2016; Iavicoli et al. 2016).

Combinations of high- and medium-throughput assays are being used to fill critical knowledge gaps between high-throughput-only and traditional type of information in a resource efficient manner (Thomas et al. 2013a, b). These approaches are associated with reasonable confidence because the evaluation relies on multiple data streams, and the phenotypic outcomes are usually suggested by the data. It is anticipated that this type of combined testing is the wave of the future for information supplied for regulatory risk assessment. As an example, considerable attention has been given recently to evaluation of a wide variety of brominate and organophosphate flame-retardants focusing on developmental, neurodevelopmental and neural activity impairment. One interesting approach has used a combination of short and medium throughput assays (high content assay screening using several in vitro cell-based assays, mouse embryonic stem cell differentiation, neuroprogenitor cell proliferation, and neurite outgrowth from differentiated neurons and firing activity in neural networks) and alternative short duration in vivo developmental models (*C. elegans* and zebrafish) (Behl et al. 2015; Ryan et al. 2016). The combination of information from these assays provides convincing evidence for chemical impairmentof important biologic process involved in development and neuronal function and the potential for neurologic impairment in exposed juveniles, although the exact phenotypic outcome is unknown. Additionally, assays were run using multiple concentrations, allowing for determination of a point of departure and the ability to compare the potencies of various chemicals in these assays. Uncertainties around species differences, metabolism, exposure–dose in humans and, in particular, inter-individual variability in human responses clearly exist. However, these flame retardant data, which was much more rapidly developed than would be possible with traditional bioassays, provides valuable insights into potential risks, as well as informing "green design" choices (Schulte et al. 2015). The combined assay results also were used to further refine strictly high-throughput assays, thus further increasing the efficiency of toxicity testing (Ryan et al. 2016).

Structure–activity relationships and read-across Research has focused on expanding the use of structure–activity relationship (SAR) approaches to identify appropriate surrogates and/or predict toxicological phenotype(s) and associated adverse effect levels. A tiered surrogate approach (i.e., decision tree) based on three main types of surrogates (structural, metabolic, and toxicity-like) was developed to inform selection of chemical analog(s) and the associated surrogate toxicity value(s), and a weight-of-evidence approach based on the proposed decision tree applied (Wang et al. 2012). This methodology is techni-

cally significant as it has been used to date to derive provisional screening reference values in Provisional Peer Review Toxicity Value (PPRTV) assessments for picramic acid, methylphosphonic acid, n-propylbenzene, tert-butylbenzene, sec-butylbenzene, 1,3-dibromobenzene, picric acid (2,4,6-trinitrophenol), o-aminophenol and n-heptane. (See the US EPA's Provisional Peer Review Toxicity Values PPRTV Assessments Electronic Library at: https://hhpprtv.ornl.gov/).

Summary of new bioinformatics data

Fascinating insights are being gained into how environmental factors alter risks of disease; including new understanding of how intrinsic and extrinsic factors modify risks. We are exploring how these new insights into disease can improve risk assessment. Analyses of data rich situations are providing insight and support of high and medium throughput risk assessments. Identification of hazards and risk modifiers facilitated by improved understanding of underlying mechanisms. Simultaneously, new approaches to dose–response appear ready to apply in selected situations. The greatest challenges that risk assessors and managers face, however may be to think in new ways about problems and solutions.

Prototype case studies

Implementation of the paradigm change presented in TT21C was anticipated to span approximately 20 years. One approach proposed to facilitate and accelerate implementation is the development of prototype approaches applied to well-characterized reference compounds to demonstrate application (Andersen et al. 2011). The prototype case studies published to date generally involve a comparison of the hazards, modes of action, and estimated points of departure or margins of exposure identified for reference compounds using TT21C approaches that are then compared back to conclusions or metrics from conventional toxicological testing. Alternatively, case studies have been conducted on larger batches of chemicals to demonstrate the significant gains in efficiencies provided by alternative testing approaches for applications such as prioritization. A variety of well executed case studies have been undertaken that include focused applications exploring the use of toxicogenomics, HTS and other TT21C approaches for regulatory applications. Below we provide key examples of case studies in this area that advance TT21C application. We also highlight the growing role of adverse outcome pathways (AOPs) in this area and consider case studies on integrated approaches to testing and assessment (IATA) that apply the AOP concept.

Case studies on individual chemicals or chemical groups: advancing qualitative and quantitative uses of toxicogenomics in risk assessment

Extensive work has been undertaken by the US EPA to explore how toxicogenomic data can be used in the assessment of the effects of dibutyl phthalate (DBP) on male reproductive system development. A series of companion papers was published to describe the results of this effort (Euling et al. 2013a, b; Makris et al. 2013; Ovacik et al. 2013). Initial scoping phases were conducted to review the available DPB genomic and conventional data to determine what aspects of risk assessment toxicogenomics might inform. This first phase of the project identified data gaps and research needs. Endpoints with unexplained modes of action in the toxicity data set were specifically identified for exploration by toxicogenomic analysis (in phase 2) to provide mechanistic insight. The overall analysis enabled the generation of hypothesized modes of action that were analyzed concurrently with apical phenotypes. In phase 2, high-quality toxicogenomic data from in vivo studies in male rats exposed to DBP during gestation were evaluated for use in risk assessment. A weight-of-evidence evaluation revealed strong evidence of DBP-induced downregulation of genes in the steroidogenesis and lipid/sterol/cholesterol transport pathways, as well as other signaling pathways in the testes of rats. The results supported existing hypotheses on the mode of action of DBP exposure leading to reductions in fetal testicular testosterone production, as well as revealing other potential molecular perturbations that might play a role in this and other adverse reproductive effects of DBP. This suggested that toxicogenomics can be used to identify mode of action and contribute to weight of evidence analysis. Overall, this early case study provided examples of how toxicogenomic data might be integrated in chemical assessment to advance the use of twenty-first century data in risk assessment.

The US EPA also generated an extensive database of toxicogenomic signatures on a variety of conazole fungicides that provide excellent case studies to demonstrate the potential use of toxicogenomics in regulatory toxicology. Initial studies in livers and thyroids of rodents exposed to various conazoles were used to define transcriptional signatures that differentiate tumorigenic from non-tumorigenic conazoles (Allen et al. 2006; Hester et al. 2006; Wolf et al. 2006; Hester and Nesnow 2008; Nesnow et al. 2009). These signatures are of value for future screening to predict the tumorigenic potential of other similar chemicals, and also provide significant insight into the mode of action. Alterations in the rodent transcriptional profiles led investigators to conduct biochemical analyses to support that hepatic retinoic acid metabolism plays a critical role in the hepatocarcinogenicity of conazoles (Chen et al. 2009). This is an example of how toxicogenomic pre-screening can provide insights into

key event perturbations that can be confirmed by follow-up testing. Dose–response studies were also conducted on mice (livers) exposed to five different conazoles. Transcriptional BMD analysis revealed concordance with apical benchmark doses (increased liver weight at 30 days) for the conazoles (Bhat et al. 2013). The median transcriptional BMD was within an order of magnitude of the BMD for hepatocellular tumors. Potency rankings based on the transcriptional BMDs were consistent with rankings based on apical effects. These results demonstrate the potential utility of gene expression changes measured in short term studies for potency assessment and for quantitative risk assessments of longer-term exposures.

In vivo dose–response experiments have been conducted by Health Canada scientists to explore the use of toxicogenomics in the risk assessment of furan, a rodent hepatocarcinogen found in heat-treated foods (Jackson et al. 2014; Webster et al. 2015a, b; Dong et al. 2016). The first study explored toxicogenomic response in the livers of male B6C3F1 mice exposed by oral gavage to both carcinogenic and non-carcinogenic doses of furan over 21 days (Jackson et al. 2014). Unsupervised clustering approaches of furan-induced transcriptomic profiles revealed strong similarities to mouse models of liver regeneration and inflammation, and rodent liver cancer, providing insight into key events in the mode of action of furan and suggesting these outcomes as potential hazards. In parallel, functional enrichment analysis indicated an important role for oxidative stress response leading to cytotoxicity and alterations in cell cycle and inflammatory pathways in furan-exposed mouse livers. The BMDs for key pathways were highly similar to the BMD for mouse liver cancer in the same model. A 90-day study on male and female F344 rats exposed to furan by oral gavage for 5 days/week over 90 days yielded very similar findings to the mouse work, supporting the major role of oxidative stress, cytotoxicity, cell cycle perturbations and inflammation in rat hepatocarcinogenicity (Dong et al. 2016). The magnitude of the transcriptional responses was much greater in male rats, which is consistent with male rats being more susceptible to furan-induced hepatocarcinogenicity. Moreover, the underlying functional analysis provided hypotheses to explain male-susceptibility. The toxicogenomic data showed that males express much higher levels of cytochrome p450 genes, which may lead to higher levels of oxidative stress, whereas females express higher levels of phase 2 transcripts and may have increased tolerance to the oxidative stress induced by furan exposure relative to males. The median pathway BMDs in male rats approximated those derived from traditional histopathology and the cancer BMD in rats. Overall, the gene expression data from these studies contribute significantly to the weight of evidence that furan causes rodent hepatocarcinogenicity through a cytotoxicity-regenerative proliferation mode of action and demonstrate that transcriptional BMDs can be used to approximate a point of departure that is consistent with the points of departure derived from assessment of adverse apical effects.

In 2015 Health Canada regulatory and research scientists collaborated in an extensive study to explore the use of published toxicogenomic datasets in an evaluation of risk posed by exposure to the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) in contaminated drinking water (Chepelev et al. 2015; Moffat et al. 2015). In this project, three risk assessments were conducted: (1) a traditional risk assessment as conducted by Health Canada's Water and Air Quality Bureau (WAQB); (2) a traditional risk assessment that considered available toxicogenomics data in parallel with conventional toxicological data; and (3) a risk assessment in which only toxicogenomics data were used to inform toxicological effects (no conventional data considered). The investigators identified high-quality DNA microarray dose–response experiments in rodent tissues and in human cells in culture. The toxicogenomic analysis provided clear evidence that BaP is an aryl hydrocarbon receptor agonist that induces a genotoxic stress response in both rodents in vivo and human cells in culture, in parallel with inducing multiple additional modulating factors (e.g., oxidative stress, inflammatory responses, immunosuppression) that could contribute to risk of carcinogenicity. Concordance in transcriptional response between rodent tissues and human cells in culture was used to support the human relevance of BaP health effects. Thus, the additional data provided by transcriptomics to inform mode of action analysis and human relevance enhanced the conventional risk assessment. The risk assessment that applied toxicogenomics in the absence of apical toxicology data demonstrated that this approach can provide useful information in data-poor situations. Transcriptional BMDs were within an order of magnitude of cancer BMDs in rodent tissues, and the lowest pathway BMD that was proposed for use as a point of departure was similar to the BMD for the apical endpoint (rodent forestomach cancer) used in the traditional assessment. Overall, the work shows that toxicogenomics can provide an effective tool for hazard identification, mode of action analysis, assessment of human relevance, and provides support for its use in the selection of the appropriate endpoints for points of departure. The data sets and analyses conducted within this case study were used within the formal risk assessment produced by WAQB, providing the first use of toxicogenomics in a risk assessment by this bureau.

Many studies have used acetaminophen as a model toxicant to demonstrate how toxicogenomics might inform risk of hepatotoxicity. These papers span examples of how gene expression can advance understanding of inter-individual variation in response to acetaminophen (Jetten et al. 2016), to exposure characterization, mode of action analysis and

development of signatures of hepatotoxicity (Kiyosawa et al. 2006; Beyer et al. 2007; Bushel et al. 2007; Zidek et al. 2007; Kerns and Bushel 2012). Indeed, a case study explored the extensive published analyses on the induction of toxicogenomic changes in cells and tissues following acetaminophen exposure as a basis by which to assess the application of toxicogenomics in hepatic systems toxicology for risk assessment (Kienhuis et al. 2011). An interesting area of investigation has been in the use of acetaminophen as a model agent to establish the relevance of in vitro tools to predict hepatotoxicity. For example et al. (Rodrigues et al. 2016) evaluated gene expression profiles from four human hepatic cell systems compared to profiles from patients suffering from acetaminophen-induced acute liver failure. The authors found comparable profiles in pathways associated with hepatoxicity in the patients and three of the cell lines, with HepaRG cells providing the best predictor of hepatotoxic response in human livers. Thus, the authors demonstrated that toxicogenomics can inform not only mode of action, but selection of relevant in vitro models for hazard assessment. A parallelogram approach (Kienhuis et al. 2009) has also been used to compare sandwich-cultured primary human and rat hepatocytes to rat in vivo liver transcriptional profiles. The analysis identified similarities in these models based on modulated biochemical pathways and biological processes. Overall, the authors demonstrated how a toxicogenomics-based parallelogram approach can be used to extrapolate in vitro to in vivo, and across species, to support the relevance of mechanisms in these liver models to humans in vivo for risk assessment.

These case studies and others have been very informative in demonstrating a variety of toxicogenomic approaches that are useful to human health risk assessment. A significant hurdle that exists is the time-consuming nature of toxicogenomic data analysis, and the lack of international guidance on technical, analytical and reporting aspects of toxicogenomic studies. However, we note that many toxicogenomic studies are published that fail to fully describe the study designs/methodologies/analytical pipelines that were applied, or apply inadequate study designs and insufficient quality control. This severely restricts the use of toxicogenomic data in risk assessment. This should be alleviated by efforts to produce best practices and reporting standards for omics analyses (Gant et al. 2017).

BMD modeling of toxicogenomic datasets for quantitative risk assessment

A significant amount of effort has been invested in developing approaches for the use of toxicogenomics data in quantitative risk assessment. These efforts have focused primarily on BMD modeling of gene expression data. In addition to the single-chemical case studies described above, well-designed toxicogenomic dose–response studies (encompassing doses that span the NOAEL) have been conducted in rodents across a diverse array of time points and tissues for many model chemicals. An important study by Thomas et al. revealed that the lowest pathway BMD from short-term rodent studies (rats and mice, and including different tissues) closely approximates BMDs from both cancer and non-cancer apical effects (Thomas et al. 2013a, b). An additional study on a subset of these data showed that a variety of approaches (not just the lowest pathway BMDL) can be used to derive points of departure from gene expression data that approximate apical endpoints, indicating that regulatory agencies applying different approaches will not result in highly divergent points of departure (Farmahin et al. 2017). A report was also published by Hester et al. (2015) to describe a compilation of case studies exploring the use of transcriptional BMDs following short-term rodent exposures. The case studies included liver gene expression for conazole pesticides and prototype nuclear receptor-mediated (non-genotoxic) rodent hepatocarcinogens (CAR and PPARα agonists), and urinary bladder gene expression for a substituted urea pesticide associated with urinary bladder cytotoxicity and tumorigenesis in rats. Overall, comparisons with BMDs derived from apical effects across all of these studies revealed that short-term toxicogenomic BMDs are generally within an order of magnitude of BMDs derived from conventional endpoints. Two important points have emerged from these studies thus far: (1) toxicogenomic BMDs from short-term studies in rodents are highly consistent with BMDs derived from conventional endpoints assessed at much later time points (e.g., cancer and other adverse outcomes); and (2) toxicogenomic BMDs are not orders of magnitude lower than BMDs from conventional endpoints. The latter has been a serious concern within the regulatory community, and dispels the notion that the toxicogenomic BMD values would lead to regulatory safety threshold that would be too low to be achievable.

A variety of challenges remain in this field. For example, the analyses described above span a limited number of tissues and toxicological endpoints. Additional work is necessary to demonstrate that the toxicogenomic BMDs are predictive of other toxicities beyond those assessed (e.g., neurotoxicity, reproductive toxicity, etc.). However, important progress has been made toward the establishment of consensus best practices for BMD modeling of omic data (US NTP 2018).

Biological read-across: use of toxicogenomic profiles for hazard identification/potency analysis

Traditional read-across approaches, i.e., data-gap-filling from test results of similar tested compounds (Patlewicz et al. 2014a, b), in hazard identification have been based on

chemical structural analysis and assessment using quantitative structure–activity relationship (QSAR) models derived from prototype agents. Traditional read-across is fueled by the availability of Big Data in toxicology (Hartung 2016; Luechtefeld et al. 2016a, b; Luechtefeld and Hartung 2017). Notably, good read-across practices for this very pragmatic approach are currently being developed (Ball et al. 2016). More recent approaches have explored the integration of biological read-across (Zhu et al. 2016) using data derived from short-term and HTS assays. This is a promising and very active area of research.

Various studies have demonstrated the ability to discern hazardous from non-hazardous chemicals within chemical groupings and assess potency using toxicogenomics. For example, mathematical models derived from rodent liver expression profiles were developed to discern a panel of prototype hepatocarcinogens and non-carcinogens that were then applied to assess the hepatocarcinogenicity of alkenylbenzene flavoring agents (Auerbach et al. 2010). The models effectively differentiated between hepatocarcinogenic (estragole and safrole) and non-hepatocarcinogenic (anethole, eugenol and isoeugenol) alkenylbenzenes, in addition to differentiating carcinogenic and non-carcinogenic doses of safrole. The models were then used to analyze two alkenylbenzenes that have not been subject to rodent cancer bioassays (myristicin and isosafrole). The analysis predicted that these chemicals are weakly hepatocarcinogenic in male F344 rats. The authors used this evidence to suggest that these chemicals should be higher priorities for cancer testing. Nikota et al. (2016) used rodent in vivo pulmonary gene expression profiles for hazard identification of engineered nanomaterials. A total of 22 toxicogenomic studies were used to demonstrate increased probability of carbon-based nanomaterials (carbon nanotubes and carbon black) to induce pulmonary pathogenesis (e.g., fibrosis) relative to titanium-dioxide nanoparticles. The authors used the findings to propose that a tiered approach be used for nanomaterial screening to prioritize those with biological profiles consistent with nanoparticles that cause pulmonary pathogenesis for further testing. Another case study applied connectivity mapping to 34 different chemicals across a broad spectrum of modes of action at multiple time-points and concentrations, in four cell lines (De Abrew et al. 2016). The authors demonstrated how connectivity mapping can be used to group chemicals by mode of action, and also identify potentially undefined toxicological hazards.

An elegant example of the use of toxicogenomic data in potency assessment was published by Hannas et al. (2012). Male rat fetuses were exposed to increasing doses of five phthalate esters during sexual differentiation; these phthalates vary in their ability to induce reproductive tract malformations as a result of reductions in testosterone production and the expression of genes involved in steroidogenesis. The authors explored the use of real-time quantitative PCR analysis of a panel of genes involved in sexual determination and differentiation, steroidogenesis, gubernaculum development, and androgen signaling pathways to assess the potency of the phthalates to cause reproductive toxicity. Potency ranking based on gene expression changes in fetal testes were directly compared to measures of testosterone in fetal testes. Certain gene expression endpoints were more sensitive predictors of overall potency (based on ED_{50}) than testosterone production. Moreover, potency rankings based on testicular gene expression were nearly identical to those based on testosterone production.

The above studies provide important examples of analytical processes for high-content data that are very useful for predictive toxicological purposes. Overall, these studies demonstrate a very promising approach with the strength of leveraging alternative high-dimensional data sources in toxicity predictions.

Case studies summary Two recent publications synthesize the results of larger numbers of case studies, and describe several specific examples of the use of toxicogenomics in human health risk assessment to draw more broad conclusions surrounding application. These reports serve as important summaries of advanced applications in regulatory toxicology areas.

The US EPA-led Next Generation (NexGen) of Risk Assessment report was a multiyear collaboration that assessed new molecular, computational and systems biology approaches to risk assessment, with the objective of informing whether these new data sources provide increased understanding of public health risks posed by environmental exposures (Cote et al. 2016). The report summarizes the results of over 40 publications to describe the potential application of these new data sources to risk assessment and discusses strategic research directions (US EPA 2014a, b). The report includes the use of genomic data to assess population variability (genetic susceptibility), an important area that not covered within the overview of case studies herein. The collaboration involved eight prototypes that were evaluated in three risk assessment contexts: (1) major scope decisions (generally regulatory decision-making aimed at nationwide exposures and associated risks); (2) limited scope decisions (generally non-regulatory decision-making for limited exposure, hazard, or data situations); and (3) chemical screening and prioritization for further testing, research, or assessment, or for emergency response. In each decision context category, the analysis shed light on new methods and data types that could be used to inform assessment efforts. AOPs and networks emerged as important tools for use of these new data streams across all of the decision contexts. The lack of best practices for data reporting and study design were identified as a limiting factor in the state of the field

today. Limited availability of data integration and analytical tools/approaches was also noted as critical obstacles. Overall, although the authors reported a variety of obstacles and challenges, the study advanced understanding of how to apply new science to more rapidly identify chemicals and exposures of potential concern, clarify mechanisms of action and exposure–response relationships, and identify potential susceptibility and cumulative risk. The authors also noted that the report also led to increased dialogue between risk scientists and managers to improve confidence in interpreting and applying these alternative data streams.

A Health Canada-led collaborative effort took a different approach to advance the application of toxicogenomics in the regulatory domain. Based on the premise that uptake in the regulatory community, when chemical specific data is available, is limited by lack of published guidance and experience, Health Canada collaborated with international experts to produce a technical guide on the use of different transcriptional profiling technologies and analytical approaches in regulatory settings (Bourdon-Lacombe et al. 2015). The guide was specifically tailored to regulatory scientists with limited experience in toxicogenomics, providing basic information about how and why gene expression analysis is conducted. The guide presents tables to assist regulators in defining minimal quality and reporting criteria from different study designs (e.g., in vitro, animal, and human studies), technologies (e.g., real-time quantitative PCR, DNA microarrays, and RNA-sequencing), and analytical approaches (e.g., identify differentially expressed genes, pathway enrichment, hierarchical cluster analyses, benchmark dose modeling, etc.) to differentiate high from low quality studies for inclusion in risk assessment. In addition to providing checklists to identify studies that pass or do not pass specific criteria, this synthesis of quality criteria could be mined to produce reporting frameworks for regulatory submissions of toxicogenomic data, which will hopefully have the additional benefit of improving reporting in the peer-reviewed literature. The guide also takes users through a variety of specific cases to demonstrate how the approaches described were used to provide insight that could be leveraged fortoxicological risk assessment. The report concludes with a discussion of where genomics is currently adding value to risk assessment, and where it is envisioned to significantly add value in the future (Yauk et al. 2019).

HTS case studies

Case studies on the use of HTS data in regulatory applications have taken many forms and it is not possible to present an exhaustive review of these publications. Below we provide illustrative case studies that have advanced the application of HTS models in areas in regulatory toxicology.

Development of predictive toxicology models Studies have been conducted to apply HTS to develop predictive models for specific hazards. These studies typically compare the results of a panel of HTS assays (e.g., assays from ToxCast™ targeting a particular pathway) to the results of high quality in vivo studies to test the accuracy of the panel of HTS assays in predicting the apical outcome, or apply models developed from an HTS training set to data from reference compounds to test accuracy in identifying specific hazard categories. These experiments provide scientific confidence for the appropriate use of HTS data in various regulatory contexts. This type of work is also critical for the ultimate replacement of specific animal tests with HTS assays (e.g., the use of 18 HTS assays to identify estrogen receptor bioactivity without the need for the uterotrophic assay (US EPA 2014a, b)).

A large amount work in this area has focused in the area of endocrine disruption. For example, Cox et al. (2014) used endocrine screening prediction models published previously (Rotroff et al. 2013) as a case study for predicting in vivo androgen-, estrogen- and thyroid hormone-mediated effects, and perturbation of the steroidogenesis pathways using prototype chemicals. The authors noted that this was of particular interest because of the EPA's use of HTS assays as part of its Endocrine Disruption Screening Program Tier 1 screens (EDSP Tier 1). They first identified in vivo guideline study data from endocrine screening assays and non-guideline endocrine-related studies from the literature. They then compared the outcomes of these studies to ToxCast™ and EDSP Tier 1 assay results for the same compounds. The HTS results were used to develop a set of models to predict results for in vivo estrogen receptor- and androgen receptor-mediated responses in the guideline in vivo assays. The HTS cross-validation models had high balanced accuracies for androgen and estrogen effects (79% and 85%, respectively. However, very low balanced accuracies were obtained for thyroid and steroidogenesis pathways. The authors concluded that the HTS assay models are promising for priority setting for endocrine screening (in particular for androgen and estrogen effects), and proposed a framework for documenting scientific confidence in both the HTS assays and the models.

Browne et al. (2015) evaluated the performance of 18 HTS assays for estrogen receptor (ER) activity (measuring ER binding, dimerization, chromatin binding, transcriptional activation and ER-dependent cell proliferation) for compounds in which data from the uterotrophic assay were available. The HTS assays were highly predictive and the results provided strong support for inclusion of the HTS assays in the EDSP. Judson et al. (2015) developed a network model to use bioactivity patterns of these 18 HTS assays to predict ER activity (i.e., agonist or antagonist of ER). They applied the model to a library of 1812 commercial

and environmental chemicals, including 45 ER positive and negative reference chemicals. The model correctly identified the agonists and antagonists within the reference chemicals with the exception of very weak compounds, and the agonist score was correlated with the expected potency of the active reference chemicals. They then used the panel to assess the remaining compounds and predicted that 111 (6.1%) were strongly ER active in agonist or antagonist modes. The work demonstrates how the HTS assays can be used for prioritization of large number environmental chemicals for follow-up in vivo endocrine testing, with both studies serving as a basis for a proposal to accept data from these tests in lieu of the uterotrophic assay. Correspondingly, Kleinstreuer et al. (2016a, b) curated an uterotrophic database from available research studies as a way to evaluate the performance of in vitro assays that measure estrogenic activity.

Chemical and chemical family-specific case studies In addition to the use of the existing HTS data to develop and refine predictive models, case studies have examined a variety of regulatory applications. These studies have generally compared HTS results to conventional testing in vivo to assess the suitability of the HTS models for predicting hazard and/or risk for the case example chemicals. For instance, in keeping with the noted emphasis on the EDSP program where a significant amount of progress has been made, HTS and the EDSP Tier 1 screening assays for three triazole fungicides (triadimefon, propiconazole and myclobutanil) were evaluated against EPA guideline mammalian toxicology study data (Paul Friedman et al. 2016). A high degree of qualitative concordance across the assays was found. The authors noted that inclusion of guideline studies mitigated limitations of the HTS assays for thyroid and steroidogenesis pathways. Activity-exposure assessments revealed that HTS-predicted human bioactivity and in vivo mammalian bioactivity (against chronic human exposure estimates) yielded margins that were within 3–5 orders of magnitude. The authors noted that the HTS prioritization would have been protective of potential in vivo effects. This combined analysis was used to support that these agents would be low priority for subsequent higher-tiered endocrine testing, and provides an example of how HTS and guideline toxicology data can be integrated for EDSP tier 1 evaluation of pesticide active ingredients.

Silva et al. (2015) qualitatively compared ToxCast™ results (including both in vitro and zebrafish models) from endosulfan and methidathion experiments to in vivo and in vitro endpoints associated with neuro- and developmental toxicity, and endocrine effects. The authors also used in vitro–in vivo extrapolation to derive rat oral equivalent doses for the half-maximal activities of the ToxCast™ assays to compare with the lowest observable effects levels (LOELs) from in vivo studies. The results were mixed, with both concordance and non-concordance across the panels for both chemicals between HTS and conventional assays. Concordance was strong for several endpoints, including oral equivalent doses for estrogen and androgen receptor pathways, and zebrafish assays, for both compounds with in vivo LOELs. Non-concordant results were primarily false inactives, which the authors concluded may have been due to insufficient metabolic activation in some assays and limitations in assay design.

Other studies have also shown a balance of concordance/non-concordance between HTS and in vivo assays. A study to examine the activity and potency of ortho-phthalates in ToxCast™ assays revealed various commonalities between key molecular events identified in vitro and chemical-specific hazards from in vivo tests (Pham et al. 2016). The results were consistent in identifying parent ortho-phthalates as more active than their monoester metabolites, and in demonstrating concordance of toxicity with chain length. However, there was some discordance, including lack of effects on HTS assays associated with male reproductive toxicity. The authors recommended that HTS results be interpreted in the context of in vivo assays until more broad biological coverage or refined models for these pathways are available.

A variety of reports have discussed how HTS data can be used for hazard identification across various applications (e.g., Damoiseaux et al. 2011; Sipes et al. 2013; Wetmore et al. 2015)), with increased use of Big Data approaches emphasized as an important path forward (Zhu et al. 2014). These approaches are particularly powerful when combined with reverse dosimetry and human exposure estimates to derive bioactivity-to-exposure ratios (Wetmore et al. 2015).

Overall, because an exhaustive synthesis of HTS prototype studies is beyond the scope of this report, we have described select examples of how HTS assays are currently being considered to refine testing strategies for chemicals and for prioritization. Although the focus above is primarily on endocrine response, the results are generalizable to other molecular pathways, in particular when multiple upstream and downstream assays are available within a pathway. There has been good progress in this area and several studies have shown that exposure limits based on HTS assays (in combination with in vitro–in vivo extrapolation) are generally protective. However, lack of concordance for some endpoints has been a cause for concern and may arise due to insufficient pathway coverage and/or model relevance. More work is needed to increase biological space and model relevance (e.g., use of metabolically competent cell lines and 3D-organoid models), and refine the systems-biology models used to predict hazards and key events in a mode of action. Significant uptake in the regulatory community beyond the application in screening and priority setting will require continued work to define linkages between the molecular perturbations and adverse effects.

Integrated approaches

Increasing consideration is being given to the use of multi-dimensional datasets that integrate various data streams to develop systems-biology levels of understanding of the chemical effects (Ankley et al. 2016). Indeed, approaches to assess broader biological space spanning a variety of model systems are increasingly prevalent in the literature. A variety of strategies have been conceptualized for regulatory uses that apply new data streams. These generally apply tiered approaches, where the type of information acquired increases in complexity from the lower to the higher tiers (e.g., RISK21 (Embry et al. 2014); US EPA NextGen project (US EPA 2014a, b)), which have been applied in case studies (e.g., Doe et al. 2016; Wolf et al. 2016)). This section highlights some key, data-driven publications that advance this area through analysis and integration of toxicogenomic, HTS, high-content data and/or in silico approaches for various applications in risk assessment.

Tiered testing Thomas et al. (2013a, b) used existing and previously published data from a variety of key TT21C testing approaches to produce a data-driven, tiered testing framework to prioritize further testing of environmental chemicals. This framework addressed the use of various new data sources including HTS and toxicogenomics, in vitro–in vivo extrapolation and exposure modeling. The first tier of the framework applies HTS and genotoxicity data in combination with in vitro–in vivo extrapolation, and pharmacokinetic and exposure modeling to calculate first order margins of exposure (or bioactivity–exposure ratios). A key concept the authors introduced is the differentiation of chemicals acting through selective (i.e., interacting with specific biomolecules) versus non-selective (causing general toxicity across bioassays) modes of action. The authors proposed that margins of concern be identified in parallel with consideration of the chemical's selectivity to determine if the second tier of testing is required. The second tier includes toxicogenomic analyses in short-term in vivo tests and mode of action studies of selective chemicals, in parallel with expanded pharmacokinetic evaluations and refined human exposure estimates. Margins of exposure are again calculated based on estimates of points of departure using BMD modeling of the toxicogenomic data, or mode of action studies, alongside consideration of hypothesized mode of action based on tier 1 and 2 screening. Margins of concern are determined to assess what chemicals require tier 3 testing. Through application of this tiered prioritizing, the authors demonstrated that many fewer chemicals would ultimately be subject to third tier using more conventional longer-term animal testing (Thomas et al. 2019).

Several investigators have focused specifically on combined approaches integrating computational toxicology and new data streams to develop advanced models for toxicity prediction. Indeed, studies have specifically shown that toxicogenomics data-based models outperform QSAR approaches alone (e.g., carcinogenicity: (Liu et al. 2011); hepatotoxicity: (Low et al. 2013)), suggesting that combined approaches may provide more sensitive and accurate predictions. Rusyn et al. (2012) proposed the determination of molecular bioactivity derived from HTS and in vitro toxicogenomic assays in combination with cheminformatics approaches (i.e., QSAR modeling) for toxicity prediction. These authors argued that these data sources could be used to generate 'hybrid QSAR-like quantitative models to predict human toxicity and carcinogenicity' that improve model prediction accuracy. Low et al. developed a hazard classification approach termed Chemical–Biological Read-Across (CBRA) to predict compound toxicity using data from both chemical and biological analogs (Low et al. 2013). Their CBRA approach yielded higher external classification accuracy for a panel of adverse effects (hepatotoxicity, hepatocarcinogenicity, mutagenicity and acute lethality) than other methods that used chemical descriptors alone or in combination with biological data.

An interesting demonstration of the use of chemical–biological read across was presented for substances of unknown or variable composition, complex reaction products, and biological materials (UVCBs), which present challenges for health assessment (Grimm et al. 2016). Using petroleum substances as the example UVCBs, this study explored similarities in UVCB bioactivity profiles in induced pluripotent stem cell-derived cardiomyocytes and hepatocytes. Concentration–response experiments were conducted for 21 petroleum substances from five product groups. High dimensional data included high-content imaging (in cardiomyocytes and hepatocytes) and transcriptomic data (derived using the high-throughput BioSpyder platform for the S1500 gene list on the hepatocytes for five substances). Analysis of bioactivity trends revealed similarity within groups and added confidence to the grouping of the petroleum substances. ToxPi scores based on points of departure from 15 experimental phenotypes were derived. Bioactivity ToxPi analysis revealed a high degree of similarity within product categories. The authors found that physico-chemical analysis was less able to differentiate between the product categories. However, bioactivity profiles were highly correlated with physico-chemical properties, and groupings were improved when these data were integrated. The transcriptomic analysis supported the groupings by bioactivity profiles and was identified as a promising avenue for providing additional mechanistic insight for toxicity evaluation. Overall, the authors demonstrated an experimental approach to potentially improve confidence in grouping UVCBs for read-across based on computational analysis of both high-content biological information and physical–chemical properties.

Adeleye et al. (2015) conducted a case study to explore how TT21C approaches may be used for chemical safety assessment. Using DNA damage response (mediated by p53 signaling) as the toxicity pathway, the authors developed a strategy to apply this pathway to risk assessment of the prototype genotoxin quercetin (a flavonoid). The authors specifically asked if the use of 0.5% quercetin in a body lotion by a consumer would adversely perturb DNA damage/p53 pathway responses. Their approach was based on the AOP concept, with formation of micronuclei in a cell as the adverse outcome. The authors examined 18-point dose–response curves in HT1080 (human fibrosarcoma) cells following quercetin exposure using fit-for-purpose HTS and high-content screening assays, as well as transcriptomics. The data were applied to computational systems biology pathway models to examine the quantitative relationships between key pathway elements and predict chemical concentrations that cause adaptive versus adverse responses. Interestingly, biomarker and gene expression changes did not occur at lower concentrations than those causing micronuclei. This was consistent with other genotoxins in the same model system (Clewell et al. 2014), and led the authors to posit that transcriptional activation for this particular pathway reflects response in the 'adverse' range. The authors compared in vitro points of departure (NOELs and BMDs) to outputs from biokinetic modeling and in vitro–in vivo extrapolation. They found that the steady-state Cmax plasma concentration was significantly lower than the concentration required to perturb the measured biomarkers and the adverse event (micronuclei). This led the authors to conclude that there is a low probability that use of the hypothetical product would result in systems level perturbation of the toxicity pathway. However, within skin, expected concentrations would be much higher than those inducing micronuclei in vitro, leading to the conclusion that additional work is needed in relevant model systems to address this finding. The work provides an effective example of the construct of an AOP-centric in vitro TT21C risk assessment. The authors emphasized that additional case studies on prototype chemicals are an effective approach to expedite the use of new data in risk assessment.

The above studies illustrate approaches to integrate data streams to significantly enhance analysis for a variety of regulatory applications. Limitations for these approaches are similar to those described above (relevance of models, available bioinformatics tools and resources, best practices and standards, linkages between molecular perturbations and adaptive/adverse effects, etc.). Increasing development and publication of AOPs will certainly be of benefit to these applications.

AOP casestudies HTS and toxicogenomic data were originally emphasized as crucial sources of information for developing AOPs. To increase efficiencies in AOP development, efforts are underway to use publicly available high-content data to produce computational methodologies for AOP development. For example, Oki and Edwards (2016) mined HTS and in vivo animal data, and other disease phenotype information, using chemicals as common aggregators between datasets. Computational AOP networks were then defined based on this analysis, and two case studies (fatty liver disease and an aryl hydrocarbon receptor network) were explored in detail. The analysis confirmed that the networks included known nodes in these pathways, but also revealed novel outcomes and associations, emphasizing the value in integrating multiple data sources. Similar work leveraged ToxCast and TG-Gates data to generate computationally predicted AOP networks that could be used by domain experts to expedite formal AOP development (Bell et al. 2016). Sub-networks of a fatty liver computational AOP were analyzed for a reference chemical (carbon tetrachloride) and compared with published mechanistic descriptions. The authors concluded that the computational AOPs approximated the manually curated AOPs.

Other case studies explored the use of AOPs in different risk assessment contexts using various data sources. For example, Labib et al. (2016) applied murine pulmonary transcriptomic profiles to evaluate the weight of evidence in support of multi-walled carbon nanotube (MWCNT)-induced lung fibrosis. A hypothetical AOP was first proposed, and then lung gene expression profiles from time-series and dose–response studies on mice acutely exposed to three MWCNTs with different physical–chemical properties were analyzed. Key events within the AOP were supported by the toxicogenomic analysis (i.e., significantly perturbed pathways for all three MWCNTs were aligned with proposed key events) and were used to link MWCNT exposure to lung fibrosis. Pathway BMDs supported temporal- and dose-concordance across the hypothesized key events, with lower BMDs for pathways at earlier post-exposure time points. Moreover, transcriptional BMDs for key events were consistent with apical BMDs for alveolar septal thickness and fibrotic lesions. The study provides an interesting example of leveraging well-designed toxicogenomic studies to support a hypothetical AOP. The authors argued that the AOP and toxicogenomic profiles can be applied to provide a mechanism-based method for deriving acceptable levels of exposure to nanomaterials when other data are not available. Molecular modeling methodologies can also be used to develop an integrated strategy for toxicity prediction using an AOP framework. For example, an in silico molecular model of peroxisome proliferator-activated nuclear receptor γ (PPARγ) agonistic binding as a molecular initiating event leading to liver steatosis was developed that had a balanced accuracy of 81%, sensitivity of 85% and specificity of 76% (Al Sharif et al. 2016). These studies both demonstrate how

novel data streams can be used to support AOP development and for predictive toxicology purposes.

Perkins et al. (2015) explored the degree of scientific confidence and extent of completeness required for an AOP to be useful for different regulatory applications. Case studies spanned AOPs with low confidence (membrane disruption (narcosis) leading to respiratory failure), moderate confidence (hepatocellular proliferation leading to cancer (partial pathway)), and high confidence (covalent binding to proteins leading to skin sensitization and aromatase inhibition leading to reproductive dysfunction in fish). As in the earlier examples, the authors demonstrated how new data sources were useful in increasing confidence in the AOPs. Moreover, the authors found that with transparency and thorough assessment of the supporting evidence, even partial AOPs with unknown molecular initiating events, and all AOPs at all levels of confidence, had value in different regulatory applications.

Integrated approaches to testing and assessment (IATA) An IATA is an approach that integrates (and weighs) various sources of information (e.g., physicochemical properties, in silico models, grouping and read-across approaches, in vitro methods, in vivo tests and human data), and newly produced data when required, to inform regulatory decision-making (OECD 2016e). The various information sources are integrated to draw conclusions on the hazard and/or risk of chemical exposures. New data streams are expected to contribute significantly to IATA (OECD 2016a), which are intended to enable reduction and refinement in conventional animal testing and various support regulatory applications. In general, an AOP can serve as the basis for developing an IATA for that regulatory endpoint. There have been various advances in this area and focussed case studies.

An excellent discussion on the development and implementation of AOP-informed IATAs for chemicals or chemical groups has been published as the outcome of a workshop entitled "Advancing AOPs for Integrated Toxicology and Regulatory Applications" (Tollefsen et al. 2014). This workshop report describes how problem formulation based on the risk management scope and goals, selection of the AOP to inform the assessment, and evaluation of the data for the chemical of interest, influence the types of tests that will inform an IATA. The authors proposed a framework to guide this process and to determine what new data may (or may not) be needed for regulatory decision-making. They discuss the various data streams (and how these may be integrated) that can be used to inform IATA development and application. They present three case study examples of AOP-informed IATA approaches with different levels of scientific confidence that address different regulatory scenarios: (1) IATAs based on AOPs targeting hormone response (estrogen-, and androgen- and thyroid hormone-pathways) for chemical prioritization; (2) protein binding leading to skin sensitization for hazard identification and model development (more details below); and (3) acetylcholinesterase inhibition leading to coma and death, with various uses in risk assessment including classification and labeling. Within each example they define where new data sources can be mapped to an AOP to inform an IATA. An associated paper (Patlewicz et al. 2014a, b) outlined an IATA that applied the very well established AOP for skin sensitization The IATA focused on existing information as well as non-standard testing data (OECD 2016a). A pipeline was developed for its application to provide a systematic method to collate the data from the various sources. The pipeline was applied to assess skin sensitization potential for 100 chemicals. The authors found that by applying this AOP pipeline, in silico and chemico profiling data could predict skin sensitization with over 70% accuracy, which could be improved when information from other assays was included (e.g., mutagenicity data). In addition, a variety of papers have proposed frameworks to characterize the scientific confidence required for AOPs to meet different regulatory needs (e.g., Becker et al. 2015; Patlewicz et al. 2015)).

The OECD is taking considerable initiative in the area of IATA development, testing and application. In particular, the Cooperative Chemicals Assessment Programme (CoCAP) has a case studies project initiated in 2015 on the development and the application of IATAs: the reader is directed to the OECD website to explore the on-going publication of case studies submitted from member countries annually (http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm). The emphasis of this project at the OECD has been to demonstrate the practical applicability of alternative methods as part of IATA for different regulatory decision-making contexts and establish best practices and common considerations for the use of new methods for chemical assessment.

Overall, a number of IATA case studies have been developed to demonstrate methods to integrate data within an AOP framework. The scientific confidence for assessing AOPs and IATA has been debated and sound approaches for evaluation proposed, although this area is still very much evolving. Moreover, the levels of confidence required for various applications have been extensively discussed. Although significant progress has been made in the conceptualization of approaches, additional IATAs, DAs, and AOPs are required to provide guidance in applications in different regulatory contexts. These should be vetted by the international regulatory community prior to adoption, which is a primary emphasis of the OECD's program in this area. A main need in this area is further work to develop more 'endorsed' AOPs and IATAs, as there are only a few widely accepted models that have been published.

Summary

The illustrative prototype case studies described above are useful in demonstrating how data from new test methods may be used in various applications in risk assessment and advancing the field. These case studies span all novel data sources, with integration of high-dimensional data increasingly recognized as a critical direction for risk assessment in the twenty-first century. Although regulatory uptake has been limited to date, there has been significant momentum in this area in the past several years. Increasing emphasis on case studies by the OECD and other international bodies, international efforts to develop best practices/guidance, and continued interaction and discussion between the research and regulatory communities are necessary to facilitate regulatory application.

Challenges and opportunities in implementation

Systematic review and evidence-based risk assessment

All areas of the life sciences see enormous increases in publications with more than half a million compiled in PubMed alone per year and estimated at least three times more in total. 'Toxicology' as a search term gives about ten thousand articles per year in PubMed. Clinical Medicine has first embraced this problem of information flooding: The Cochrane Collaboration and others have pioneered the creation of evidence-based medicine over the last four decades. Tools such as systematic reviews, quality scoring, risk-of-bias assessments, meta-analysis and others were developed to allow the objective and transparent condensation of all available evidence for a given question. The translation to toxicology as evidence-based toxicology (EBT) started only around the creation of the TT21C report (Hoffmann and Hartung 2006) with the first conference held in the same year, i.e., 2007 (Griesinger et al. 2009). The report makes no mention of EBT, but already in early responses the synergy of these concepts became clear (Hartung 2009a). EBT offers a framework for objectively assessing the current approach, help with the integration of existing information as data-stream and to renovate validation approaches (Hartung 2010) especially for pathway-based assays as mechanistic validation (Hartung 2013). The formal formation of the EBT Collaboration (http://www.ebtox.org) on both sides of the Atlantic starting in 2011 and the increasing utilization of systematic reviews in regulatory contexts (Stephens et al. 2016) followed. Tool development (Samuel et al. 2016; Hoffmann et al. 2017), harmonization of approaches and pilot projects have started, promising to further develop this powerful approach to handling diverse and conflicting evidence.

In parallel to TT21C, this conceptual framework developed (Hartung 2009b; Stephens et al. 2013; Hoffmann et al. 2014, 2016) as a door-opener and quality-assurance partner over the last decade. Not surprisingly, the follow-up NRC report from 2017 (US NRC 2017) includes now a chapter on "Interpretation and Integration of Data and Evidence for Risk-Based Decision-Making", which includes exactly these elements. Still, the importance of the EBT concept for implementing TT21C seems to be underestimated.

Quality assurance

A crisis of regulatory science in the 1970s, when FDA inspections found laboratories not to carry out and not to document properly studies used for regulatory purposes, led to the development of good laboratory practices (GLP). This was fundamental a few years later for the international collaboration via OECD and others introducing the mutual acceptance of data (MAD), which requires that data are produced according to agreed test guidelines under GLP in order to make them acceptable in another jurisdiction. However, GLP was developed around the predominant technology of its time, the animal test (Cooper-Hannan et al. 1999). The increasing use of other types of data further accelerated by TT21C requires similar concepts for these technologies, first of all the in vitro approaches (Pamies and Hartung 2017). This gave birth to good cell culture practice (GCCP) in the mid-1990s (Gstraunthaler and Hartung 1999), which were further developed under the auspices of the European Centre for the Validation of Alternative Methods (ECVAM) (Hartung et al. 2002; Coecke et al. 2005). Already during its development, GCCP was embraced by OECD's GLP (OECD 2004). More recently, good in vitro method practices (GIVIMP) for the development and implementation of in vitro methods for regulatory use in human safety assessment was adapted (OECD 2016b). In 2015, the GCCP collaboration was reactivated under the auspices of the Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University in order to update the guidance to the technological developments of the last decade, namely stem cell and organotypic culture conditions (Marx et al. 2016). Two workshops in the US (Pamies et al. 2017) and Europe (Pamies et al. 2017) set the base for the current drafting of GCCP 2.0. A key element of GCCP are reporting standards, which are another line of development under GCCP 2.0 (Leist et al. 2010). There are some parallel developments for both best practices and reporting standards and best practices for in vivo and in silico (Tropsha 2010) approaches as well. It should be noted, that regulatory acceptability of new approaches depends on the provision of quality assurance standards similar to GLP for animal

studies (Hartung 2009a). They are also a key element of EBT as only quality evidence can be condensed; this is why quality scoring of evidence is a critical element of systematic reviews (Samuel et al. 2016). Conversely, EBT has the potential similar to EBM for health care to guide good practice and reporting standards development. While not part of the original TT21C report, quality assurance approaches have emerged over the last decade as critical elements of TT21C implementation.

The human toxome project

At the core of TT21C is the concept of basing our testing on mechanism. The report conceptualizes this very much around the idea of excess activation or inhibition of physiological pathways, called toxicity pathways. This has spurred very much the concept of adverse outcome pathways (Ankley et al. 2010), which is a key element of TT21C implementation especially because of the enormous efforts to move this forward on an OECD level (OECD 2013). More than 200 AOPs are included in the current AOP-Wiki, which are created by expert-driven organization of existing knowledge typically on the level of modes of action, i.e., rather narrative and rarely quantitative.

The Human Toxome Project (http://www.humantoxome.org) takes this a step further. Acknowledging that our current knowledge is incomplete, biased and too often wrong (not reproducible), and seeing the need that ultimately molecular definition of toxicity pathways are necessary to create a sytems toxicology (Hartung et al. 2012) approach, the Human Toxome was initiated as an NIH Transformative Research Grant (Bouhifd et al. 2015a, b). The consortium included several members involved in the TT21C panel. The concept of molecularly defined "Pathways of Toxicity—PoT" was formed (Kleensang et al. 2014), simply defined as a molecular definition of cellular processes shown to mediate adverse outcomes of toxicants. Instead of a compilation of literature, the Human Project Toxome aims for an unbiased (untargeted) mapping of PoT from validated test systems and reference toxicants using multi-omics approaches (Hartung and McBride 2011). Each and every omics approach has too many measured endpoints relative to the affordable number of measurements and enormous resulting noise. The multi-omics approach aims to use orthogonal technologies assuming that perturbations, which show in the same test on different levels, are likely to be correct. Then, targeted measurements can verify these perturbations and experimental interventions can show causality.

The project was developed around a pre-validated endocrine disruptor test, the MCF-7 proliferative response. Besides transcriptomics, the most matured omics technology, emphasis was given to metabolomics, which is closest to the phenotypic changes produced by toxicants (Bouhifd et al. 2013; Ramirez et al. 2013). It turned out that both the cell system as well as the metabolomics component of the project required extensive quality assurance still to be defined (Bouhifd et al. 2015a, b; Kleensang et al. 2016). The project also developed data-management tools such as the Toxome Collaboratorium (Fasani et al. 2015) and several tools incorporated into Agilent's GeneSpring software suite. Employing these, the untargeted PoT identification from omics data in MCF-7 cells stimulated by estrogenic chemicals was developed (Pendse et al. 2016a, b; Maertens et al. 2017). This work has laid the foundation for a systematic mapping of PoT, which would form then the basis for systems toxicology, i.e., the modeling of toxic perturbances to ultimately run virtual experiments to predict outcomes and assure that our understanding of physiology and pathophysiology is correct and reflected in the models (Hartung et al. 2017). These developments are taking the original concepts of a pathway-based toxicity testing of TT21C to the next level. However, a concerted effort of PoT mapping would need to be established to make this happen. The faster growth of the AOP concept prompted recent discussion to incorporate the Human Toxome experiences into Effectopedia (https://www.effectopedia.org), which aims for a more molecular definition of AOP. Furthermore, the ToxCast and Tox21 programs are increasingly embracing –omics approaches, which might make a stand-alone Human Toxome initiative superfluous.

Adopting an integrated and coordinated approach

A key component of any test system is the bioinformatics/computational systems biology approaches that will take quantitative data generated from the imaging, functional, and molecular assays and integrate these into a mechanistic framework for discerning biological activity and toxicant-induced effects. The goal of this modeling is to detect emergent disruptions of biological pathways to elucidate adverse responses. The adverse response is then used to identify a point of departure for the purpose of in vitro-to-in vivo extrapolation for human safety assessment targeted for end-users of this technology.

Over the past decade, various groups have accumulated extensive practical experience applying novel computational systems biology tools to synthesize various data streams to determine disruptions in biological pathways and adverse effects (Boekelheide and Andersen 2010; Boekelheide and Campion 2010; Bhattacharya et al. 2011, 2012; Boekelheide and Schuppe-Koistinen 2012; Andersen et al. 2013, 2015; Clewell et al. 2014; Deisenroth et al. 2014; McMullen et al. 2014; Bouhifd et al. 2015a, b; Clewell et al. 2016). In particular, developments focused on the definition of signatures of toxicity based on molecular endpoints (Andersen et al. 2013; McMullen et al. 2014) have been leveraged to

define adversity. At this point in time, it is clear that integrating tools to bring together various data streams, including imaging (in vitro pathology), molecular, and functional data, are both possible and needed. The data to be integrated includes morphological inputs (e.g., cell proliferation/counts; cell size; cellular differentiation; morphological biomarkers of effect; live cell imaging of cell movement), molecular inputs (transcriptomics; protein expression) and functional assessments (e.g., action potentials; beating rate; calcium waves; altered hormonal responses in co-cultures vs. mono-cultures).

Developing and implementing such an integrated computational systems biology framework for interpretation of these data streams has the goal of defining adverse effects, and will require the following steps.

- *Pathway analysis of response to model toxicants.* Determine key pathways perturbed by prototypical toxicants. Enrichment analysis (Subramanian et al. 2005; Pendse et al. 2017) and over-representation analysis (Kamburov et al. 2011) using databases such as Reactome, Kyoto Encyclopedia of Genes and Genomes, and Gene Ontology will be needed.
- *Establish quantitative relationships between phenotypic and molecular inputs.* Established tools for determining points of departure for in-life studies (i.e., lowest observed effect level and benchmark dose) and their adaptation to -omics technologies will be needed. The imaging endpoints will be used as landmarks to differentiate which changes in molecular endpoints are adverse (vs. adaptive).
- *Mode-of-action definition.* Assess the relationships between modes-of-action derived from molecular inputs. Machine learning will be needed to identify aspects of the molecular response that are most consistent qualitatively (in terms of the pathways induced) and quantitatively (in terms of the concentrations that induce them) across perturbations of the model system. The degree to which the most predictive pathways align with the known biology surrounding the model compounds and systems will provide further validation of the 3D technologies as toxicity platforms.

After developing the fundamental integrating tools and thought processes described above, prototype safety assessments of chemicals based on endpoint analysis and integration, including mode-of-action prediction, in vitro point-of-departure determination, and in vitro-to-in vivo extrapolation will be conducted to evaluate the performance of the approach.

Selection of prototype chemicals. A battery of prototypical toxicants to demonstrate the utility of a safety assessment strategy based on the integration of imaging, molecular, and functional endpoints will be needed. Model selection will depend on data availability, predictivity of models, cost, and other factors. Case study chemicals will be chosen with available exposure data and, when possible, in vivo data. Following the computational modeling with the prototype toxicants, additional test toxicants will need to be evaluated to exercise the model and determine its reliability and applicability domain.

Mode-of-action prediction. The strategy for identifying compound mechanism will involve a two-tiered approach. Determination of a point of departure requires a dense concentration response over the range of biological activity. For compounds lacking sufficient preliminary data, a range-finding study to determine what concentrations are active, using a small set of imaging, molecular, and functional endpoints will be required. Estimated active and cytotoxic concentrations will then be used to establish a dense concentration response range to define and validate appropriate endpoints for the analysis. Changes in phenotype will be related to molecular inputs using newly developed models, and mode of action will be determined through pathway analysis methodologies that integrate the various endpoints, as described above.

In vitro point of departure. A combined weight-of-evidence strategy will be used for identifying points of departure for in vitro models, considering imaging, molecular, and functional endpoints. The relative sensitivities of these endpoints will be determined, as well as their relationship to in vivo endpoints where applicable. A benchmark dose will be used in association with endpoints related to adversity to define concentrations demarking regions of safety.

*In vitro-to-*in vivo *extrapolation (IVIVE).* IVIVE will be performed (1) to evaluate the predictivity of the in vitro point of departure from the in vitro models for the in vivo point of departure or dose levels showing effects of well-characterized toxicants, and (2) to demonstrate the framework to predict in vivo safe exposure conditions based on the in vitro point of departure for less well characterized environmental chemicals. An interactive workflow will be used to optimize in vitro dosing and exposure conditions in appropriate in vitro models and collect time course samples from them. In vitro biokinetic and cellular dosimetry modeling will be needed to describe compound exposures over time in the in vitro models. The use of long-term cultures will require experimental and computational in vitro biokinetics and dosimetry support to assess dose–response in the in vitro setting so that these can be linked to relevant human exposure conditions (Kramer et al. 2015; Pomponio et al. 2015). The description of equivalent in vivo exposure conditions will use published whole body PBPK models if available or use a high-throughput IVIVE approach to estimate reasonable exposure ranges in vivo (Wetmore et al. 2015).

All prediction frameworks have a domain of applicability; however, determining this domain for biological systems is often not straightforward. To ensure the validity of the prototype safety assessments, a prototype training set of chemicals will be needed that is data-rich and conservatively included in the domain of applicability of the models. If, as expected, the models are predictive using these well understood chemicals, additional test chemicals can be studied to broaden the assessment of the applicability domain of the models and the fidelity of their predictions. This process is expected to be iterative, with continued feedback and improvement of the predictive capacity of the models and identification of adverse effects and points of departure.

The development and implementation of these modeling tools will provide the basis for combining disparate technologies into a quantitative assessment of adversity. By comparing in vitro imaging, molecular and functional data streams, and/or in vivo counterparts into a mode-of-action framework based on well understood prototype toxicants, confidence in an integrated definition for adversity will develop. Subsequently, as experience builds, less well characterized toxicants can be confidently evaluated with detailed in vitro dose- and time-responses analyses, providing sufficient data to distinguish points of departure for in vitro-to-in vivo extrapolations and chemical safety assessments.

Evolution of governmental agency initiatives

NCCT roadmap

The US EPA's National Center for Computational Toxicology (NCCT) has been developing a roadmap to assist in the further implementation of the NRC vision for toxicity testing in the 21st Century (Thomas et al. 2019). US EPA efforts in computational toxicology during the period 2004–2007 moved toward integration of advances in chemical, biomedical, computational, and informatics sciences to efficiently and economically evaluate the safety of chemicals. The early versions of this program applied new, first-generation approaches for chemical characterization, toxicity testing, IVIVE analyses and exposure modeling, producing various data streams to shift away from dependence on animal testing (Richard et al. 2016). The program is now crafting a blueprint for the future, looking to the early successes and moving on to dealing with remaining challenges for identifying biological targets and estimating relative risks posed at realistic exposures. The emphasis remains on the use of computational modeling and high-throughput approaches to transform components used to understand potential health risks of chemicals—chemical characterization, hazard evaluation, toxicokinetics and exposure assessment (Fig. 7). Surrounding these components are cross-cutting efforts in characterizing uncertainty and variability, development of software and information technology tools that facilitate translation, outreach and training activities, and establishing scientific confidence for different regulatory decisions.

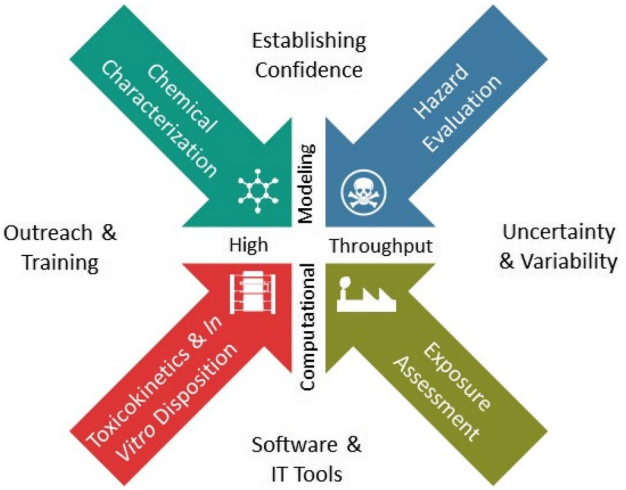

Fig. 7 Key elements of the EPA CompTox Blueprint over the next 5 years. CompTox emphasizes computational modeling and high-throughput approaches to connect and transform the traditional components of chemical risk assessment. Cross-cutting efforts in characterizing uncertainty and variability, development of software and information technology tools, outreach and training, and establishing scientific confidence will be essential to enable translation of these new approach methodologies to regulatory decision-making (Thomas et al. 2019)

Chemical characterization Development of a high-quality, structure-based cheminformatics platform has been essential for supporting computational chemistry and structure-based modeling activities. (Q)SAR models have been built and provided through the EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard/) for a range of physicochemical properties, toxicity, and environmental fate endpoints (Mansouri et al. 2016a, b; Mansouri et al. 2018). Embedded in the "Predictions" tab of the Dashboard was the ability to predict hazard and physicochemical properties using the Toxicity Estimation Software Tool (TEST) suite of QSAR models (https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test). Systematic read-across approaches also utilize the chemical structure information to predict a range of hazard-related effects for data-poor chemicals (Shah et al. 2016a, b). In traditional read-across, chemical structure together with expert judgment based on physicochemical properties, metabolism considerations, and toxicological mechanisms, when available, were used to identify appropriate analogs, which are then used to infer the effects of a target chemical (Wu et al. 2010; Wang et al. 2012). The reliance on expert judgment to address uncertainties has hindered its use for regulatory acceptance (Patlewicz et al. 2015; Patlewicz and

Fitzpatrick 2016). The investment in systematic read-across approaches can quantify the uncertainty and provide benchmarks to assess whether other contexts of similarity (e.g., physicochemical, metabolic or biological as assessed using HTS data) reduce the uncertainty (Helman et al. 2018). In addition, the program is developing chemical structural descriptors to assist systematic chemical categorization and prediction of both hazard and exposure-related properties. ToxPrint chemotypes have been used to identify structure–activity enrichments in HTS assays related to neurotoxicity (Strickland et al. 2018) and hepatic steatosis (Nelms et al. 2018), while these and other structural descriptors have been used to predict functional use and weight fractions in personal care products (Isaacs et al. 2016). Investment in computational chemistry and structure-based approaches will help in leveraging chemical information to associate chemical structures with hazard, toxicokinetic, and exposure characteristics.

Hazard evaluation The evolution of the initial stages of ToxCast and Tox21 led to appreciation of key limitations of the suite of HTS assays (Tice et al. 2013). These shortcomings included inadequate coverage of biological targets/pathways, reduced or idiosyncratic xenobiotic metabolism compared to in vivo situations, limited ability to evaluate volatiles or chemicals not soluble in dimethyl sulfoxide and challenges in translating perturbations at the molecular level to possible tissue-, organ-, and organism-level effects.

To address some of these challenges the initial tier of testing requires a broader-based screen that can capture potential hazards. Two approaches are now moving forward to assess broader biological landscape. In the first, RNA-seq-based multiplexed read-outs of gene expression interrogate the effects of chemical treatment across the entire transcriptome using with automatable, high-throughput transcriptomic measures captured directly from cell lysates in 384-well format (Yeakley et al. 2017). This approach, termed high-throughput transcriptomics (HTTr), sets the stage for cost-efficient screening of thousands of chemicals in concentration–response format. A second approach uses high-content imaging of cultured cells stained with multiple dyes to measure the effects of chemical treatment on subcellular organelles and structural features (Bray et al. 2016). With these high-throughput phenotypic profiling (HTPP) tools, hundreds of cellular features are assessed in a cost-efficient, 384-well format with customized image analysis algorithms. Over the next several years, the HTTr and HTPP approaches will be applied to multiple cell types to provide data across a much greater biological space thanassessed with the current ToxCast assays. Attempts to use these data streams for defining predictive points-of-departure from in vitro high content imaging-based assays are already in the early stages of development (Shah et al. 2016a, b; Wink et al. 2018).

In a second tier restructured ToxCast assays will allow small sets of chemicals to be run in orthogonal in vitro assays to confirm the interactions with the biological target or MOA inferred from first tier. The portfolio of assays for orthogonal confirmation may include existing ToxCast or new fit-for-purpose assays (McMullen et al. 2018). In a third tier, chemicals with a verified interaction with a biological target or pathway would be linked with a likely adverse outcome using the AOP framework (Ankley et al. 2010). If the biological target or pathway perturbed by the chemical is not associated with an available AOP, existing knowledge of the target or pathway will be used to guide development of novel models that can inform creation of new AOPs.

Apart from the development of the tiered testing framework, the diversification of chemical space evaluated in the HTS assays is being expanded by assembling a library of water-soluble chemicals and developing novel air–liquid exposure systems to expose cells in concentration response to volatile chemicals (Zavala et al. 2018). A two-part strategy categorized as ‘extracellular’ and ‘intracellular’ approaches will tackle issues related to lack of metabolic competence. With an ‘extracellular focus’, chemical metabolism can be designed into the media of cell-based assays or the buffer of cell-free assays. Multiple directions are being pursued to provide the relevant metabolic activity to the assay media or buffer. One promising approach embeds S9 or microsomal fractions in an alginate matrix and attaches the matrix to plastic protrusions on custom designed multi-well plate lids. The protrusions extend down into the well of the plates and allow chemical metabolism without the S9 or microsomal fractions interfering with assay readouts or causing cytotoxicity. In an ‘intracellular’ strategy, chemical metabolism occurs inside the cell and effectively models target tissue metabolism. In one approach, chemically-modified mRNAs corresponding to different xenobiotic metabolizing enzymes are synthesized and transfected into target cell types singly or in multiplexed ratios that mimic specific target tissues, e.g., liver (DeGroot et al. 2018). These extracellular and intracellular approaches will be applied to retrofit the in vitro assays in the first and second tier of the testing framework.

Toxicokinetics and in vitro disposition In the future, EPA will be extending the domain of applicability of the high-throughput toxicokinetic (HTTK) models across a broader range of environmental chemicals and incorporating additional assays and in silico tools that address known limitations in existing approaches (Pearce et al. 2017a). For example, studies have been performed high-throughput bioavailability measurements using the Caco-2 model (Hilgers et al. 1990) to examine the contribution of bioavailability to the lack of correlation for some chemicals (Wambaugh et al. 2018).

Further refinement and development of computational chemistry and structure-based modeling of tissue partitioning, and volume of distribution will address challenges in assessing steady-state kinetics in IVIVE models. These computational chemistry approaches build on previous efforts in the pharmaceutical industry to estimate tissue partition coefficients using physicochemical properties (Schmitt 2008; Pearce et al. 2017b) and allow the development of dynamic toxicokinetic and physiologically based toxicokinetic (PBTK) models for individual chemicals. Non-steady state toxicokinetic models can capture important aspects of toxicokinetics, such as the time needed to reach steady state for a diverse range of chemicals and estimate of tissue dosimetry (e.g., maximal and/or time-integrated concentration) in critical time periods of developmental susceptibility. While in vitro toxicokinetic methods provide significantly faster alternatives to traditional toxicokinetic testing, these methods still require the time-consuming and sometimes difficult development of chemical-specific methods for chemical concentration analysis. For this reason, in silico approaches based upon chemical structure features and physico-chemical properties can predict in vitro toxicokinetic data (Ingle et al. 2016). These new in silico models allow toxicokinetics, exposure, and hazard to be combined for large screening libraries such as Tox21 (Sipes et al. 2017), whereas methods limited to in vitro-measured toxicokinetics deal only in hundreds of chemicals at a time (Wetmore et al. 2012, 2015).

The shift to in vitro models for hazard characterization has necessitated an understanding of in vitro disposition, i.e., the fate and movement of a chemical within an in vitro assay (Blaauboer 2010; Fischer et al. 2017, 2018). Most POD analyses from in vitro assays have relied on nominal concentrations as the basis for estimates of in vitro potency; however, taking into account binding to plastic, intracellular transport, and lipid association in the assay situation itself can result in a significantly different potency estimate for a chemical (Mundy et al. 2004; Meacham et al. 2005; Croom et al. 2015; Kramer et al. 2015). To overcome this challenge, efforts are now underway to measure directly any differences between nominal and cellular concentrations for a set of chemicals to determine whether in vitro disposition can be modeled using computational approaches (Thomas et al. 2018). A summary of databases for used for hazard characterization is sprovided in Supplemental Table 1.

Exposure assessment To estimate exposure for a broad range of chemicals, relatively simple computational models have predicted median exposure rates for the total US population (Wambaugh et al. 2014). However, the uncertainty around the exposure predictions is relatively large. Although more complex exposure models may reduce uncertainty, they require detailed parameterization of the weight fraction and off-gassing of the chemical in hundreds of products in conjunction with detailed human activity characterization, which is difficult to scale to thousands of chemicals (Isaacs et al. 2014). Based on the finding that consumer product usage was a significant source of exposure (Wallace et al. 1987; Wambaugh et al. 2013), improved databases of chemicals known to be in consumer products can be developed to parameterize more complex exposure models and to reduce uncertainty in exposure predictions (Isaacs et al. 2016). The updated database will include new sources of data from safety data sheets and reported chemical functional uses (Dionisio et al. 2018). In addition to the data mining and curation activities, computational models under development will predict likely uses for a chemical based on structure (Phillips et al. 2017). There is a key behavioral economics piece to this puzzle, i.e., what consumer products are being purchased, brought into the home, how used, frequency of use, and in what combination (Egeghy et al. 2016). Portions of this information are routinely collected by retailers and market research firms for business purposes; however, this information is generally not available for ExpoCast applications. Future efforts need to focus on acquiring these data to evaluate current and ongoing population-level consumer product use patterns. The ultimate goal of these efforts is predict screening-level rates of exposure for any chemical structure by integrating formulation science, behavioral economics, and mechanistic fate and transport modeling to delineate linkages among inherent properties, functional role, product formulation, use scenarios, and environmental and biological concentrations (Egeghy et al. 2016).

To provide experimental data on chemicals in the indoor environment, new analytical chemistry methods, such as non-targeted analysis (NTA) and suspect screening analysis (SSA), are being used to characterize the chemical composition of indoor media, such as house dust (Rager et al. 2016), and various items people frequently encounter (e.g., household products and articles of commerce) (Phillips et al. 2017) and drinking water point-of-use water filters (Newton et al. 2018). The new analytical methods have identified many chemicals not previously known to be present in those items and can provide semi-quantitative estimates of concentration of their relative mass fraction. The NTA and SSA efforts are supported by the work on the cheminformatics infrastructure, while the strengths and limitations of the technology are being evaluated through activities such as the EPA Non-Targeted Analysis Collaborative Trial—ENTACT (Sobus et al. 2018).

Finally, advances in exposure sciences will also address chemical mixtures. From a toxicological standpoint, testing all mixture permutations of even a hundred chemicals is prohibitively expensive. However, exposure monitoring and modeling combined with advanced data mining methods can identify certain prevalent mixtures of chemicals that either

occur frequently in the environment (Tornero-Velez et al. 2012) or within biomonitoring data (Kapraun et al. 2017). In the future, a few prevalent mixtures will be evaluated using high-throughput toxicity testing approaches, enabling design of more efficient and focused approaches for examining the risks of various mixtures.

FDA roadmap

Since joining the Tox21 consortium in 2010 (Hamburg 2011), the US Food and Drug Administration has further strengthened its commitment to TT21C through the Agency's recent predictive toxicology roadmap (USFDA: US Food & Drug Administation 2017). The overarching goals of this initiative are to foster the development and evaluation of new approach methodologies, with a view to incorporating these methodologies into regulatory review. FDA expects this initiative to ultimately expedite product review and help avoid risk products from reaching consumer markets. The roadmap is currently undergoing public consultation to ensure it meets its intended purpose (USFDA: US Food & Drug Administration 2018).

ICCVAM roadmap

The Interagency Coordinating Committee on the Validation of Alternative Methods, which works with 16 federal agencies in the United States led by the US National Institute for Environmental Health Sciences, has recently developed a roadmap for establishing new approaches to evaluate the safety of chemicals and medical products (ICCVAM 2018). The purpose of this initiative is to promote the use of twenty-first century science for public health protection. One of the specific goals is to encourage the use of new approach methodologies (NAMs) by federal agencies and regulated industries by the following means.

1. Provide clear language regarding the acceptance of NAMs.
2. Collaborate with international partners to facilitate global harmonization and regulatory acceptance.
3. Explore processes to incentivize and promote the use of NAMs.
4. Identify appropriate metrics for prioritizing activities, monitoring progress, and measuring success.

Although challenging, the focus on measuring success is key to demonstrating in concrete terms that the new approaches to toxicological risk assessment will provide significant savings in both cost and time, and ultimately lead to enhanced public health protection from potential risks of chemical substances (including medical products) to which people are exposed.

Canada's chemicals management plan post 2020

The Canadian Environmental Protection Act (CEPA) is the primary federal statute under which environmental substances are regulated in Canada. A major accomplishment under the current version of CEPA, in effect since 1999 but currently in the process of being updated (Canada House of Commons 2017), was the categorization of 23,000 existing substances registered on Canada's domestic substances list (DSL) and the subsequent focus on the approximately 4300 chemicals that were identified as priorities for evaluation under the Chemicals Management Plan (CMP) between 2011 and 2020. With this comprehensive chemicals program well in hand, Health Canada is currently developing an expanded vision for its Chemicals Management Plan (CMP) post-2020. While this vision is currently under active development, NAMs such as high-throughput screening (HTS) and toxicogenomics are anticipated to play an important role in the scientific toolbox being developed for use in future screening and evaluation of environmental substances. The use of bioactivity–exposure ratios (BERs), representing the ratio between doses demonstrating bioactivity in high-throughput in vitro assays and predicted human exposure levels (see Fig. 8), as well as other computational and high content approaches are being explored as potentially useful tools for priority setting and decision-making. The CMP Risk Assessment Toolbox (Health Canada 2016) identifies types of approaches that may be used to address substances or groups of substances of varying complexity in a fit-for-purpose manner and includes complex assessment for substances or groups of substances that may require cumulative assessment approaches; substances or substance groupings that require de novo and in-depth risk assessments; and substances or substance groupings for which a broad-based or streamlined hazard or exposure analysis may be conducted often based on low potential for exposure and conservative scenarios. Moving forward, the NAMs being developed and tested will add to the Risk Assessment Toolbox for broad-based approaches and support integration of emerging data into the more complex risk assessment approaches through the development of integrated approaches to testing and assessment (IATA). Under the CMP, novel approaches to evaluate the environmental or human health risk of substances are described in science approach documents (SciADs) to demonstrate the approach to be used in future assessments or prioritization exercises. Publications to date include the threshold of toxicological concern (TTC)-based approach for certain substances, ecological risk classification approach, biomonitoring-based approaches and an approach to substances with low human health hazard concern (Health Canada 2018). In advancing its vision for chemicals management post-2020, the Government of Canada continues working closely with the

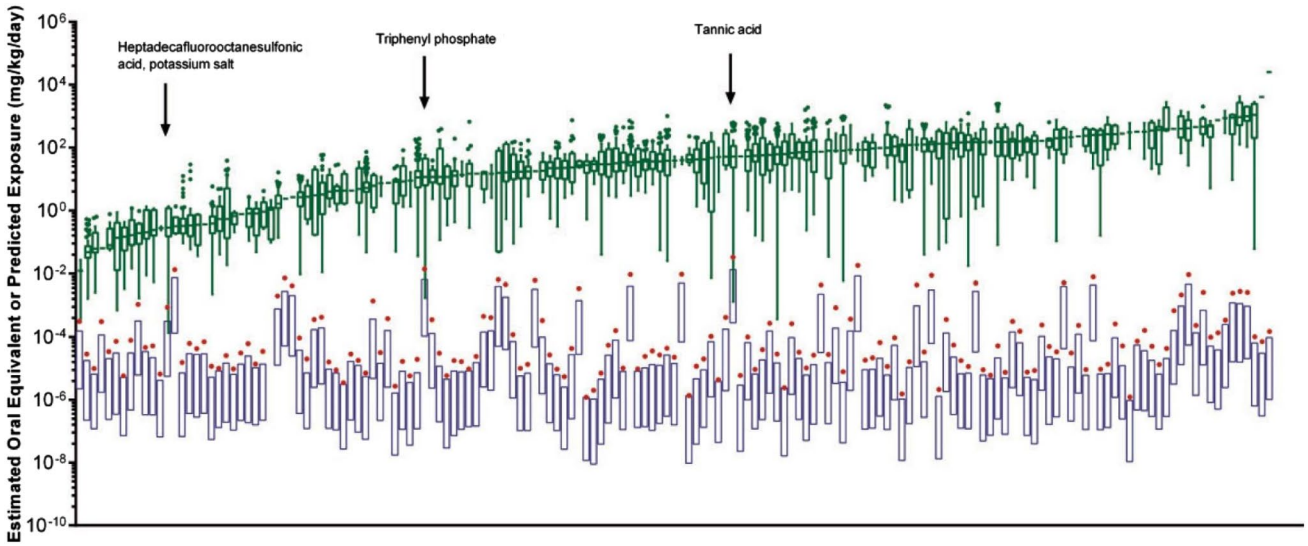

Fig. 8 Comparison of bioactivity patterns for 163 ToxCast chemicals with high-throughput exposure modeling results (Wetmore et al. 2015)

international regulatory and research communities to build confidence and harmonize the expanded application of emerging technologies in chemical risk assessment.

EU-ToxRisk initiative

The elements of TT21C have received considerable support in Europe, with, for example, the EU-ToxRisk initiative seeking to "drive the required paradigm shift in toxicological testing away from 'black box' animal testing towards a toxicological assessment based on human cell responses and a comprehensive mechanistic understanding of cause-consequence relationships of chemical adverse effects" (EU-ToxRisk 2019). This initiative is organized around the following four thematic areas: (1) database requirements and design; (2) systems toxicology/biology, adverse outcome pathways; (3) test system evaluations; and (4) risk assessment and uncertainties. Oversight for this innovative program is provided by an international Scientific Advisory Board, comprising leading experts in toxicological risk assessment. Broad stakeholder involvement has been achieved through the engagement of a broad range of public, private and academic partners. The overarching goal of EU-ToxRisk is to "deliver testing strategies to enable reliable, animal-free hazard and risk assessment of chemicals".

Conclusions and future perspectives

Over a decade has passed since the publication of the NRC report on toxicity testing in the 21st Century in 2007. This report espoused a new approach to toxicity testing, taking advantage of new developments in the toxicological and risk sciences to markedly increase the throughput of traditional, largely mammalian-based toxicity testing practices. Key elements of the TT21C vision were the use of high-throughput in vitro screening assays and computational toxicology to characterize the toxic potential of the large number of agents present in the human environment that could pose potential health risks. Central to the vision was the focus on identifying, and avoiding, critical toxicity pathway perturbations that could to adverse health outcomes.

The TT21C report included a long-term plan for implementing this vision, spanning a period of 20 years, with provision for mid-course corrections. The present review both takes stock of progress since 2007 and suggests refinements to the original long-term plan. Overall, progress in implementing TT21C appears to be ahead of schedule in many areas. The capacity to conduct HTS assays in robotics mediated laboratories, such as in the US National Chemical Genomics Center, far surpasses what was imagined possible in 2007. Moving forward, refinements to HTS screening strategies, including expanding the range of human cell lines available for use in screening as well as the spectrum of relevant in vitro endpoints to be evaluated, will serve to guide the selection of the most appropriate test batteries to support human health risk assessment. Evidence of regulatory acceptance of increasing use of new approach methodologies for toxicity testing is provided by a recent announcement by the US Environmental Protection Agency to stop conducting or funding toxicity studies on mammals by 2035 (Grim 2019).

While progress has been made in mapping toxicity pathways by individual research centers, much remains to be done in this regard over the next decade. Like mapping the human genome, mapping the human toxome will likely

require a 'big science' effort to bring this initial work to a successful conclusion. This will be a critical success factor for TT21C, as the development of sensitive and specific strategies for identifying toxicity pathway perturbations—the cornerstone of TT21C—requires an in-depth understanding of the human toxome and embedded toxicity pathways.

The focus on toxicity pathway pathways has served to underscore the relevance of molecular and genetic epidemiology, which provides an opportunity to investigate toxicity pathway perturbations directly in humans under real-world conditions of exposure using appropriate biomarkers. The distinction between mechanistic toxicology and molecular epidemiology will become increasingly blurred as the focus shifts to charting toxicity pathway perturbations in human tissues, whether in vitro or in vivo.

Advances in exposure science, including those documented in the 2012 NRC report exposure science in the 21st Century (ES21C), have also contributed to the realization of the vision for TT21C. Biomonitoring data characterizing human exposure to environmental agents using biomarkers of exposure in target tissues, is becoming increasingly available internationally. The increasing availability of biomonitoring equivalents (BEs) provides useful benchmarks for identifying populations at risks, where exposure biomarkers exceed the corresponding BEs.

The emergence of high-throughput biomonitoring platforms offers the promise of rapidly evaluating exposure to large numbers of agents, spanning a large component of the human exposome. The combination of high-throughput in vitro testing and high-throughput biomonitoring provides a new foundation for much more rapid risk assessments and subsequent risk decisions.

Progress in both computational toxicology and computational exposure assessment has served to support the TT21C objective of reducing reliance on costly and time-consuming animal tests. Sophisticated algorithms for structure–activity analysis are increasingly being used to support read-across within the EU REACH program (https://www.ulreachacross.com). Computational exposure assessment algorithms, such as those used in the US EPAs CPCat Database (https://actor.epa.gov/cpcat/faces/home.xhtml) comprising a library of exposure data on over 49,000 chemicals in consumer products, have also served to provide rapid exposure without the need empirical measurement.

Advances in toxicology, epidemiology and exposure assessment has inspired the development of a framework for the next generation of risk science under the US EPAs NexGen project. Incorporating advances in risk assessment methodology, such as in vitro to in vivo extrapolation; this framework integrates new scientific developments in multiple disciplines to provide a comprehensive blueprint to guide twenty-first century risk assessment. Experience with this framework has documented that many of the principles and procedures embedded in TT21C and ES21C are beginning to find their way into practice. Although much has been accomplished since the 2007 NRC vision for the future of toxicity testing, much remains to be done to fully operationalize this vision. Progress to date has confirmed the integrity and fidelity of the NRC vision, which is well on its way to becoming a reality. A critical success factor will be mapping the human toxome, which will provide the depth of understanding needed to complete the transformation away from evaluating apical endpoints in toxicity testing to preventing toxicity pathway perturbations.

Acknowledgements The authors gratefully acknowledge the input of Drs. Patrick McMullen and Miyoung Yoon to parts of this article. The authors would also like to thank Drs. Maureen Gwinn and Sean Collins for helpful comments, which served to improve the original draft of this article. We also thank Abdallah Alami for assistance in the final formatting of the manuscript and supplementary material. D. Krewski is the Natural Sciences and Engineering Research Council of Canada Chair in Risk Science at the University of Ottawa.

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