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The US Federal Tox21 Program: A Strategic and Operational Plan for Continued Leadership

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Background

The traditional approaches to toxicity testing have posed multiple challenges for evaluating the safety of industrial and environmental chemicals, pesticides, food additives, food contaminants, and medical products. There are tens of thousands of chemicals used across all sectors of the economy and there are many more present as contaminants in the environment and food supply. In addition, humans are rarely exposed to a single chemical, resulting in an incalculable number of chemical combinations to evaluate for potential human health concerns. Apart from specific classes such as pesticides and medical products, most chemicals have undergone limited, if any, traditional toxicity testing leading to a lack of adequate information to assess human health risks (NRC, 1984). The testing of such a large number of chemicals and mixture combinations across a comprehensive array of traditional animal-based tests raises significant ethical and resource issues. For medical products, substantial preclinical safety data is collected and used to evaluate potential human health concerns. However, despite the amount of safety data, unexpected adverse effects can still be observed in clinical trials or post-approval, suggesting that gaps remain in our understanding of human responses and the traditional toxicological endpoints measured in preclinical studies.

This paper does not necessarily reflect the policy of the US Environmental Protection Agency, National Toxicology Program, National Institutes of Health, or Food and Drug Administration.

In response to the challenges associated with the tradition-al toxicity testing paradigm, the National Research Council (NRC) produced the report entitled “Toxicity Testing in the 21st Century: A Vision and A Strategy” (NRC, 2007). This report recommended a fundamental shift from the traditional animal-based toxicity testing paradigm that relies on clinical and histopathological observations towards a predictive toxicology approach that relies on disruption of molecular events and cellular pathways identified using human-relevant *in vitro* assays and computational modeling. Based on the recommendations in this report, a joint effort was initiated through a Memorandum Of Understanding (MOU) executed by the U.S. Environmental Protection Agency (EPA)/Office of Research and Development/National Center for Computational Toxicology (NCCT), National Institutes of Health (NIH)/National Institute of Environmental Health Sciences/National Toxicology Program (NTP), and the NIH/National Chemical Genomics Center (NCGC), previously a part of the National Human Genome Research Institute and now a part of the National Center for Advancing Translational Sciences (NCATS) (Collins et al., 2008; Kavlock et al., 2009). This collaboration, informally called Tox21, was formed to address the goals of: (1) identifying mechanisms of chemically induced biological activity; (2) prioritizing chemicals for more extensive toxicological evaluation; and (3) developing more predictive models of in vivo bio-logical response. In 2010, this MOU was expanded to include the US Food and Drug Administration (FDA). In 2015, the Tox21 collaborators renewed their commitment to the program through a third five-year MOU between NTP, NCATS, EPA, and FDA. A collaboration of this type and duration is unique within the federal government.

Initial Successes of Tox21

Since its inception, Tox21 has been a productive interagency collaboration. It has advanced chemical testing by generating over 120 million data points on about 8500 chemicals using in vitro high throughput screening (HTS) assays (Tice et al., 2013). The combination of Tox21 and ToxCast data currently represents the largest generation of in vitro bioassay data for environmental chemicals (Richard et al., 2016; Tice et al., 2013). A large number of environmental chemicals tested in the Tox21 collaboration had little empirical data relevant to biological targets or possible adverse outcomes. The Tox21 data have been publicly released via multiple agency websites, highlighting the transparency associated with the Tox21 collaboration. Further-more, the Tox21 partners have instituted a timely delivery of the data into the public domain, with only a minimal holding period to allow for preparation of a manuscript describing the general findings from a particular screen. The publicly avail-able data have been used by national and international scientists and organizations for a broad range of purposes. For example, academic scientists have used Tox21 data to rank chemicals of concern at Superfund sites (Tilley et al., 2017) and the International Agency for Research on Cancer has used Tox21 data to help inform cancer hazard evaluations (Chiu et al., 2018).

The Tox21 collaboration has resulted in the publication of over 200 scientific peer-reviewed articles in approximately 56 journals and these publications have been cited in more than 140 policy-related documents and expert panel reports including 80 reports by the U.S. National Academy of Sciences1. The Tox21 data coupled with computational models and additional data from the EPA’s ToxCast program and reference chemical curation by the

National Toxicology Program, are enabling high-throughput screening assays to be used for regulatory decisions in the EPA's Endocrine Disruptor Screening Program to both prioritize chemicals for further testing and to replace some Tier 1 screening assays (Browne et al., 2015; EPA, 2015). The application of Tox21 data to regulatory decisions has also been supported in a follow-up report released by the NRC entitled "Using 21st Century Science to Improve Risk-Related Evaluations" (NRC, 2017).

Lessons Learned

Part of the success of Tox21 includes a better understanding of the challenges that remain on the path to achieving the goals of the original 2007 NRC report. These challenges include technological and biological barriers associated with the current methods, as well as barriers in more efficiently translating the results into regulatory decisions. The technological and biological barriers include a lack of physiologically-relevant metabolic competence for many of the assays, the testing of only DMSO soluble chemicals, limited coverage of important cellular and intracellular processes, limited duration exposures, and the estimation of potency based on nominal chemical concentrations (Tice et al., 2013).

Additional barriers in translating the results from the Tox21 data into regulatory decisions and acceptance by the general toxicology community include insufficient communication, training, and education. The large amount of data, and technical complexity of the HTS data pose many barriers to understand-ing and accepting the data. In addition, translating the results from Tox21 data into decisions by regulators will require a pragmatic path forward for establishing scientific confidence in the in vitro assays and a better understanding of the qualitative and quantitative differences in uncertainty between the traditional methods and the new approaches.

Changes in Focus

The original focus of Tox21 was to develop and apply in vitro HTS methods for hazard identification and provide mechanistic insights into the perturbed pathways. To solve the challenges involved in efficiently and economically evaluating the safety of chemicals, develop more human-relevant test systems, bet-ter understand the mechanisms and pathways of toxicity, and more broadly achieve the goals laid out in the 2007 NRC report, Tox21 has developed a new strategic and operational plan that begins with expanding the focus of its research activities. The new activities are targeted to address key challenges in advancing toxicology testing in the 21st century, and if successful, will have substantial benefit to each organization regardless of differences in their unique missions. These new areas of focus are outlined briefly below.

Area of Focus 1: Develop alternative test systems that are predictive of human toxicity and dose response.

The overall aim of toxicity testing is identifying all the potential hazards that a chemical can elicit in an organism and characterizing the dose-response relationships for those hazards. New technologies and testing platforms are needed in Tox21 that more comprehensively capture the potential toxicological effects of chemicals, allow translation of molecular and

pathway perturbations to effects at the tissue-, organ-, and organism-level, and capture potential population variability in toxicodynamic responses. To more comprehensively capture potential toxicological effects, the Tox21 consortium will develop and characterize new technologies that provide multiplexed read-outs of chemically-induced changes in the global transcriptome. This work will include, for example, the use of targeted and/or global gene expression methods that allow for the analyses of thousands of genes directly from cell lysates. At the same time, the existing quantitative HTS efforts will be focused on developing assays for molecular events in high priority adverse outcome pathways (AOPs) and expanding technological capabilities to include previously inaccessible signal types (e.g., fast transient cellular changes such as ion channel signaling), high-content microscopy (e.g., micronucleus or staining for organelles or particular cellular proteins), and two-photon imaging of 3D cellular organoid structures.

Changes at the molecular and pathway level are often difficult to interpret with regards to their potential effects at the tissue-, organ, and organism-level. To date, Tox21 has used biologically simplistic test methods (e.g., receptors, stable reporter-expressing cell lines) that do not include primary tis-sue-specific cell context and many of the complex cell-to-cell interaction or feedback systems that exist at higher levels of biological organization. New alternative test systems are needed that incorporate the complexities found at these higher levels to augment the simpler HTS assays employed to date. To achieve this, the Tox21 consortium will leverage alternative species such as Danio rerio (zebrafish) (Padilla et al., 2012; Truong et al., 2014), as well as recent advances in organotypic culture models, microscale tissues, and microphysiological systems (Bhatia and Ingber, 2014; Low and Tagle, 2017). It is recognized that such approaches may have much lower throughput than current Tox21 assays and thus, for testing thousands of compounds, these technologies will be used in a tiered, follow-up capacity to bridge between the molecular perturbations identified in the high-throughput assays and the tissue-, organ-, and organism-level phenotypic outcomes.

Translation of toxicity testing into risk assessment requires an understanding of population variability and identification of potentially susceptible populations. Most of the HTS assays employed in Tox21 do not capture potential population variability in toxicodynamic response. Efforts by members of the Tox21 collaboration and partners have begun to address this challenge (Abdo et al., 2015; Eduati et al., 2015; O'Shea et al., 2011). The Tox21 consortium will expand on previous efforts and work to develop and integrate induced pluripotent stem cell (iPSC) technology into the new alternative test systems and high-through-put screening platforms to incorporate variability in chemical responses from genetically diverse populations.

Area of Focus 2: Address key technical limitations of current in vitro test systems.

As mentioned above, there are technical limitations to the cur-rent in vitro test systems. Some of these limitations are listed among the lessons learned in this article (see above). The Tox21 consortium will work to adapt existing methods or to develop new methods that will allow the consortium to systematically address the main technical challenges that face in vitro test systems. For example, the Tox21 partners have already begun to identify potential

solutions to the metabolic competence limitation through both in-house efforts and using a global science crowd-sourcing initiative2 (DeGroot et al., 2018; Ramaiahgari et al., 2017).

Area of Focus 3: Curate and characterize legacy in vivo toxicity studies.

The toxicological community as well as the organizations involved in Tox21 have data from legacy in vivo toxicity studies. These data form the basis for how we currently understand the potential effects of chemicals and provide a rich resource with which to help interpret the in vitro test systems and link the effects observed at the molecular level to those observed at the tissue-, organ-, and organism-level. The data will also be useful for characterizing the qualitative and quantitative variability associated with traditional in vivo toxicity studies in order to understand how they may differ from the new in vitro test-ing approaches (Browne, et al., 2015; Hoffmann et al., 2010; Kleinstreuer et al., 2015). The interagency Tox21 consortium will work together to identify and curate these legacy, non-proprietary toxicity studies, enter data into a computable form, and harmonize ontologies used to characterize the toxicity studies.

Area of Focus 4: Establishing scientific confidence in vitro test systems and integrated assay batteries.

A standard approach for the validation of in vitro test systems for hazard identification (e.g., OECD, 2005) has been used by U.S. Federal agencies and international organizations over the past decade. However, this approach has proven to be largely unsustainable; taking many years to complete, requiring significant resources, and typically focusing on a one-for-one replacement of a specific regulatory endpoint of interest (Griesinger et al., 2016; Judson et al., 2013). Although regulatory agencies are open to using new methods and integrated assay batteries such as the Tox21 HTS assays, for a range of decision contexts, the agencies must have confidence that the new approaches provide data that are reliable, reproducible, and relevant to the intended context of use. A new area of focus for Tox21 is to perform research that informs the development of an evaluation frame-work for the definition of performance standards which can be used to establish confidence in the new approaches. The under-lying research will need to support a framework that is generalizable and scalable for key events and pathways that range from data-rich (e.g., estrogen receptor) to data-poor.

Area of Focus 5: Refine and deploy in vitro methods for characterizing pharmacokinetics and in vitro disposition.

Current approaches to extrapolating estimates of in vitro potency to external doses assume that the nominal concentrations used in the in vitro assays are equivalent to plasma concentrations and that the in vitro toxicokinetic assays and the in vitro-to-in vivo extrapolation (IVIVE) modeling sufficiently capture the complex toxicokinetic behaviors of industrial and environ-mental chemicals as well as pesticides and pharmaceuticals. However, it has been well established that active and passive disposition of chemicals in in vitro assays (e.g., binding to plastic, transport inside or outside the cell, binding to media proteins) may significantly bias potency estimates for some chemicals (Blaauboer, 2010). In addition, the in vitro toxicokinetic assays and IVIVE modeling approaches do not work well for certain chemicals or chemical classes when compared with traditional in vivo pharmacokinetic studies (Wambaugh et al., 2015). To overcome these challenges and to advance the

utilization of Tox21 data, a new area of focus for Tox21 will be on new methods and computational modeling approaches that bet-ter predict the relationship between target tissue concentrations and external doses of chemicals. In addition, the Tox21 consortium will begin to collect the experimental and computational data necessary to incorporate in vitro disposition into estimates of effective potency and efficacy.

Changes in Structure

To accommodate the new strategic direction and the expansion in focus of the Tox21 collaboration, a greater integration among Tox21 partners is required. This presents multiple challenges as each partner has different organizational and programmatic drivers. To overcome this issue, a new structure has been implemented for Tox21 (Fig. 1). The central functional group in the new structure is the cross-partner project. Cross-partner projects are defined research activities that fall into one of the five areas of focus and must have project support from two or more Tox21 partners. The cross-partner projects have three-year terms and are reviewed annually by the Tox21 leadership enabling a more formal research planning and execution process. The cross-partner projects are supported by infrastructure teams that are tasked with maintaining specific activities that are central to most of the research projects. The initial infrastructure teams and cross-partner projects are listed in Table 1. Public abstracts are available on the Tox21 website.

Summary

The Tox21 collaboration has provided both the data and the science to begin implementation of the National Research Council's vision of toxicity testing in the 21st century (NRC, 2007). Since its inception, the Tox21 program has led the world in the development and use of high-throughput screening assays and has provided publicly accessible data for the biological activity of ~8500 chemicals (Kavlock et al., 2012; Tice et al., 2013). These data have provided a foundation on which a pivot in chemical hazard identification and in vitro dose-response assessment is now beginning to influence the fields of toxicology and risk assessment worldwide (Chiu, et al., 2018; EPA, 2014). It is time to expand the focus of the Tox21 consortium to include other data and methods necessary to fully advance toxicity test-ing into the 21st century.

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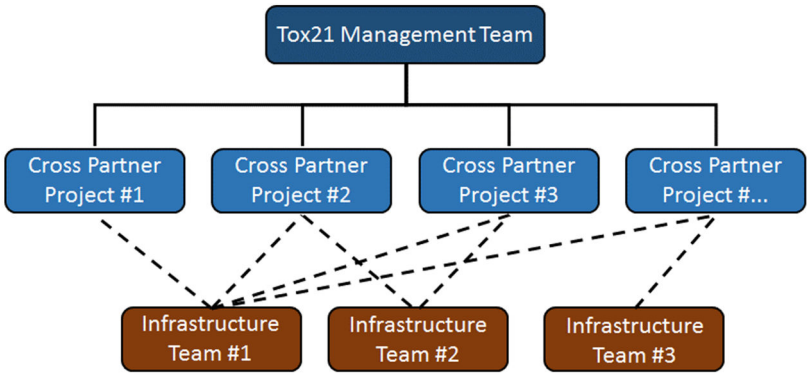

Figure 1.

New Tox21 organizational structure. The central functional group is the cross-partner project. The cross-partner projects are overseen by the Tox21 management team and supported by infrastructure teams that are tasked with maintaining specific activities central to most of the research projects.

Table 1.

Initial Cross-Partner Projects and Infrastructure Teams for Tox21

Cross-Partner Projects	Infrastructure Teams
In Vitro Disposition of Tox21 Chemicals	Chemical Library Management
Developing Performance Based Standards for Tox21 Assays	Communications
Development of a Reference Chemical Dataset for Interpretation of High-Throughput Transcriptomic Screening Data	Assay Evaluation and Screening
Incorporating Genetic Susceptibility into Developmental Neurotoxicity Screening	
Development of a High-Throughput Assay to Identify 5-α Reductase Inhibitors for Orthogonal Evaluation in an Androgen-dependent Human 3D Prostate Tissue	
Cell Line Selection for High-Throughput Transcriptomic Screening	
Predictive Modeling of Developmental Toxicity with Human Pluripotent Stem Cells	
Development of a High-Throughput Assay to Identify Acetylcholinesterase Inhibitors	