Novel Tools and Approaches Program

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Problem Statement

The Division of the National Toxicology Program (DNTP) has a goal of "leading the transformation of toxicology through the development and application of innovative tools and strategies." In vitro systems and short-term bioassays are part of the strategy for innovative approaches in assessing adverse biological events at critical chemical concentrations. In vitro systems in toxicology require continued improvement of their predictivity, timeliness, and human translation as non-animal approaches. Many in vitro methods lack the throughput, human health predictivity, or translational relevance needed to keep pace with current public health needs and expectations. The DNTP is challenged to identify and develop the best set of tools for more rapid and precise hazard assessment.

In promoting the use of novel tools and approaches that have a clear benefit to DNTP, we face critical challenges in instilling confidence in their application, predictivity, and scientific value within the regulatory and external toxicology communities. These challenges also present a unique opportunity for DNTP. To that end, the Novel Tools and Approaches (NTA) program will identify, evaluate, and implement novel tools and approaches that could contribute to advancing translatable, predictive, and timely toxicology in areas critical to the DNTP mission and to DNTP stakeholders. In the absence of identified tools to meet specific DNTP needs, the NTA program will foster the development of technologies to serve those needs and the toxicology community in general. DNTP is uniquely positioned to lead and innovate in key areas of technological advancement because of its resources, scientific outlook, and international perspectives in toxicology. In seeking to fulfill this goal of leading the transformation of toxicology, the NTA program will selectively invest in new tools that best align with the needs of DNTP.

Objectives

Although NTAs have substantial overlap with the more familiar concept of new approach methodologies (NAMs), NTAs can include modified animal use that is consistent with the 3Rs principles (replace, reduce, refine) to extend and confirm findings from in vitro studies, when necessary. Thus, the NTA program is responsible for managing and implementing innovative in vitro approaches to evaluate chemical hazard and dose-response in biological systems; to a lesser extent, the NTA program also aims to refine animal use by maximizing information gleaned from in vivo studies (e.g., assay/endpoint integration). These NTA data will be used to gain mechanistic insight into chemical toxicity, guide any follow-on animal studies, and better inform human risk assessment. Most of our NTA portfolio projects are within two focus areas: Bioassays and Advanced Biological Platforms (e.g., organ-on-a-chip), and Novel Technologies that generate high content data streams. The NTA program particularly values well-developed methods with demonstrated proof of principle and feasibility, and that are also rapidly adaptable to DNTP needs. The NTA program will also occasionally consider engaging nascent technologies that require complete characterization and validation but that have extraordinary potential

to benefit research projects within DNTP. Investing in developing technologies carries enhanced risk compared to adopting mature technologies for new applications within DNTP due to the possibility of technological failure or the eventual realization that the approach is unsuitable for meeting program needs. Outside expertise and resources can be called upon for additional input to inform and guide the NTA program, as needed.

The three main objectives of the NTA program in the two focus areas indicated above are to:

1. Identify and adapt promising new tools, technologies, and approaches that could enhance the efficiency and translational relevance of DNTP hazard assessments.
2. Ensure that novel capability development is aligned with contemporary problems that DNTP is attempting to solve.
3. Increase confidence in—and adoption of—novel methods within and outside DNTP and foster the development of novel nonstandardized methods in high-interest in vitro systems and short-term assays.

The NTA program proposes to focus on three specific research areas to help advance these objectives:

a. Spheroids and Organoids
    i. HepaRG 3D liver cell, metabolically competent spheroids
    ii. Neural stem cell spheroids for developmental neurotoxicology
    iii. Embryoid bodies driven to specific cell types to form organoids
b. Microphysiological Systems
    i. Organs-on-a-chip
    ii. Microfluidic chips
c. Novel Technologies with high dimensional data streams
    i. Opera Phenix high-content imaging system for cellular phenotyping
    ii. Tox21 high-throughput screening program and cross-partner projects
    iii. High-throughput transcriptomics (e.g., S1500+ platform)
    iv. Air-liquid interface (ALI) exposure system

Rationale

The field of toxicology is moving toward more efficient and reduced use of animals, with a greater reliance on in vitro models, as well as analytical methods and innovative technologies to provide a rapid data source for DNTP. DNTP has a long history of using innovative tools to better solve toxicology problems in hazard and risk assessment. For example, DNTP has been involved in the Tox21 Program, which began over 15 years ago as an interagency collaboration to more rapidly predict (with improved accuracy) the toxicological effects of chemicals by integrating computational biology and robotic high-throughput screening for biological activity. Initial efforts of this program resulted in the development of a chemical library of about 10,000 compounds covering a sizeable range of chemical space. The chemical library was screened for activity in nuclear receptor and cellular stress response signaling pathways. By the beginning of 2021, just under one million data points generated under Tox21 Program activities have been deposited into PubChem, and the program has generated over 200 publications in more than 50 journals to date. Now, the Tox21 Program is in its third phase, investigating in greater detail toxicologic features such as metabolic competence and genome-wide transcriptional responses that are relevant to chemical exposures of environmental and pharmaceutical interest. Another example of the successful development by DNTP of a new technology platform that has now gained widespread

acceptance and use is the S1500+ high-throughput transcriptomic platform[1] that measures chemical exposure-induced gene expression changes in over 3,000 genes covering 96% of all known human cell-signaling pathways. The original paper describing this new platform has been cited 23 times in the past 2 years.

The program area of NTAs encompasses a variety of sophisticated data streams, biological assay systems, and technology platforms that share a deliberate but not exclusive emphasis on human-relevant in vitro models. The applications of NTAs have been outlined at various levels of complexity.[2] Initial levels begin with in silico and Quantitative Structure-Activity Relationship (QSAR) approaches for characterizing, identifying, and screening chemicals. A second level of complexity at the cellular level involves in vitro high-throughput screening platforms using human cells to evaluate a broad range of cellular responses. These generally involve specific assays based on modes of action (MOAs), such as nuclear receptor activation or generation of reactive oxygen species, and they provide quantitative estimates of human-relevant dose-responses. Projects with a higher level of complexity include such systems as multidimensional or multicellular models based on 3D spheroids and organoids comprising multiple cell types. The most complex in vitro models can include active microfluidic cell and tissue systems with liquid or pulsatile flow built into the models. Finally, short-term in vivo studies (e.g., 5-day bioassay) represent the highest level of complexity for NTAs that may be considered for use by DNTP. All these various data streams have the potential to increase understanding of mechanisms of toxicity and for defining dose-response relationships (including establishment of benchmark doses and "tipping points")—critical information for determining human risk from exposure.

At the time of its establishment, the NTA program was assigned a broad portfolio of capabilities that represent increasing complexity, spanning from in vitro methods to short-term in vivo tests. This portfolio represents a set of capabilities that provides DNTP with methods to rapidly produce suitable data for test article evaluation to apply to a number of critical decision points along the DNTP pipeline. The NTA program will also track progress and milestones for Tox21 projects that have DNTP leads. In addition to managing current projects in the portfolio, the NTA program will perform regular reviews of the entire landscape of emerging technologies and applications to identify new bioassays or biological systems, as well as novel technologies and unconventional approaches that could be useful to ongoing DNTP projects and the wider toxicology community. Fostering the adoption of these technologies, when the benefit to DNTP is clear, is also a responsibility of the NTA program.

Public Health Context

Promising new and innovative approaches, along with evolving tenets and principles and an urgent need to translate study findings rapidly to human health applications, are profoundly impacting the field of toxicology. DNTP has historically sought innovative methods for addressing toxicological problems in hazard and risk assessment. We have a critical need for a portfolio of fit-for-purpose tools and approaches that can be easily and rapidly deployed to provide information on potential human hazards and risks of exposure. These approaches will aim to elucidate general MOAs and critical benchmark concentrations of effect, both of which are important for extrapolating observed effects to human exposure scenarios. Promotion and utilization of NTAs by DNTP will help to build confidence in these approaches within the wider toxicology community, thus enhancing the identification and

[1] Mav *et al*. (2018) A hybrid gene selection approach to create the S1500+ targeted gene sets for use in high-throughput transcriptomics. PLoS One 13(2):e0191105. https://doi.org/10.1371/journal.pone.0191105.
[2] Andersen *et al*. (2019) Developing context appropriate toxicity testing approaches using new alternative methods (NAMs). ALTEX 36(4):523-534. https://doi.org/10.14573/altex.1906261.

understanding of human hazard and exposure risk in general. Information stemming from data generated by NTAs can also guide decisions for further testing, such as whether DNTP should use assay systems/models that use longer exposure durations than can be achieved in in vitro or in guideline studies.

Alignment with Mission, Goals, Strategic Pipeline

The identification and acquisition of innovative technologies and measures to rapidly provide data that are relevant to human health is consistent with DNTP's vision and mission. Experience in applying NTAs is vital to training the next generation of translational toxicologists, as it will lead to incorporating in silico, in vitro, and short-term in vivo study data into risk assessment evaluations.

Stakeholder Interest and Engagement

The NTA program is charged with actively developing a strategy for a future portfolio that outlines specific directions for DNTP in the NTA space. DNTP anticipates an increased need to incorporate sophisticated in silico and in vitro methods in toxicological risk assessments for many environmental contaminants that have little or no biological data. This objective has been addressed within the Tox21 Program, of which DNTP is a primary partner. The Tox21 Program relies heavily on high-throughput and high-content in vitro assays and QSAR modeling strategies for characterizing compound toxicity; appropriately, several Tox21 projects are included in the NTA program's portfolio, with the National Center for Advancing Translation Science (NCATS), the U.S. Food and Drug Administration (FDA), and the U.S. Environmental Protection Agency (EPA) as major collaborating stakeholders. Because the innovative approaches environment is constantly evolving as new technologies and methods are developed, the stakeholders and intended beneficiaries of NTA projects are expected to be wide-ranging and changing, and interest/level of engagement will likely vary by agency and stakeholder group, as well as by project. Overall, the NTA program's investments will focus on complex in vitro platforms, in coordination with other programs, and more physiologically holistic short-term animal studies. The NTA program aims to enhance DNTP capabilities to address complex toxicology problems, thereby providing benefit to DNTP and the general toxicology community.

Steps Taken to Engage Stakeholders

Leaders of each project included in the NTA portfolio have identified their principal stakeholders and the level of stakeholder communication that currently exists. All these stakeholders are seeking the same new innovative, rapid, reliable, and translatable methods as DNTP. Thus, both formal collaborations (e.g., with NCATS or EPA) and routine two-way communications (e.g., discussions at scientific gatherings and presentations by DNTP scientists on their progress in implementing the use of novel tools and innovative approaches) are ways of keeping key members of these stakeholder communities informed and engaged.

Ongoing and Continuing Interactions

The NTA program's listing of all projects, which is included in the program's comprehensive Program Plan, identifies the specific stakeholders. Principal stakeholders for the projects in the NTA program's portfolio can be grouped into several categories, which are summarized below.

Stakeholder*	Category of Organization
EPA, FDA, NIOSH, Health Canada	Regulatory Agency
OECD, ECHA, EFSA, HESI, EC-JRC	International Advisory Committee
Zebrafish, animal alternatives community	Research Specialty Community
University of Ottawa, Oregon State University	Academic Laboratory
NCATS, NCTR, DoD	Government Research Center

* NIOSH = National Institute for Occupational Safety and Health; OECD = Organisation for Economic Co-operation and Development; ECHA = European Chemicals Agency; EFSA = European Food Safety Authority; HESI = Health and Environmental Sciences Institute; EC-JRC = European Commission-Joint Research Center; NCTR = National Center for Toxicological Research; DoD = U.S. Department of Defense.

DNTP is also an important stakeholder for the NTA program, as the NTAs that are adopted and adapted by the program must be directly relevant and applicable to DNTP, the primary end user of the data generated through these NTAs. Continued communications between project leads and key members of their specific stakeholders are expected, and these communications will be shared by project leads with members of the NTA program, as needed.

Milestones and Metrics

Milestones and metrics are currently project-specific (Figure 1). Within our portfolio, we have projects that have recently been completed and are awaiting manuscript or report finalization, thus requiring no further expenditure of resources except for personnel time (e.g., Developmental Transcriptomics in Rat Pups Exposed to Chemicals In Utero). Other projects in the portfolio are ongoing and have built-in "go/no-go" milestones that extend over the projected lifetime of the project (e.g., Systematic Evaluation of the Application of Zebrafish in Toxicology); these projects are monitored at 6-month intervals to ensure efficient use of DNTP resources and adequate progress toward completion. Finally, the portfolio also includes newly proposed projects with clearly defined durations (<3 years) and progress milestones that have been approved by the NTA program and are awaiting programmatic approval and/or resource allocation (e.g., Cell Line Selection for High-throughput Transcriptomics; Embryoid Bodies for Investigating the Use of Transcriptomics to Predict Teratogens and Developmental Toxicants).

Focusing the NTA portfolio on the three key areas of spheroids and organoids, microphysiological systems, and high dimensional data streams described above, under the objectives, is expected to require up to 2 years, as current projects reach completion and new projects that fall into one of these three focus areas are proposed. Specifically, projects utilizing spheroids and high-content data streams are already ongoing and should mature within this 2-year time frame. The efforts aimed at fully developing our capabilities in organoids and microphysiological systems will likely require a longer timeline due to the complexity of these systems, the sophisticated biology that underlies them, and the need to collaborate with partner agencies such as NCATS and EPA; nevertheless, measurable progress is expected within a 3- to 5-year time frame.

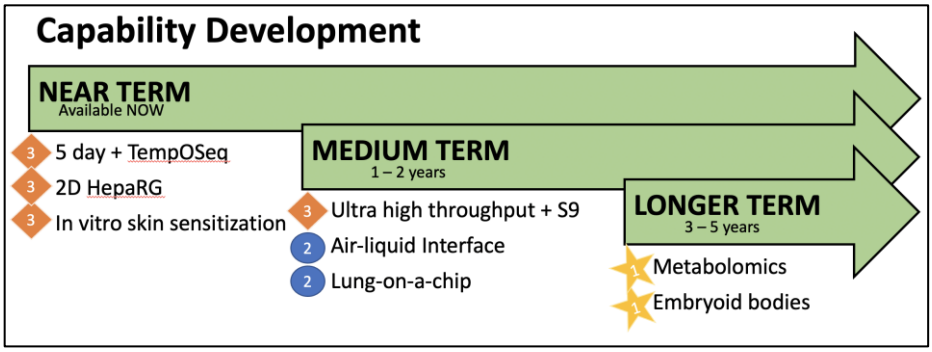

Figure 1. Timeline for Reaching Maturity for Eight NTA Capabilities.

Numbers associated with each capability link that capability to one of the three objectives; additional time after project completion may be required for manuscript publication and project close-out.

Value Proposition and Summary

DNTP values toxicological methods that produce high-quality, statistically valid, human-relevant, and actionable data for protecting public health. DNTP guideline toxicology studies have been recognized as the gold standard for setting regulations. Historically, these studies have served as the standard approach in toxicology, connecting pathological, biochemical, molecular, and behavioral changes to adverse effects of test articles. The scale and complexity of such guideline studies have increased the time required to produce final study reports and other publication products (e.g., manuscripts). Novel tools and approaches in toxicology are designed to provide more rapid assessments of hazards and to contribute greater insight into MOAs, leading to higher predictive capability and enhanced human relevance. Such approaches are not yet highly standardized and involve a certain amount of risk in providing benefits that carry the promise of advancing the DNTP mission and the field of toxicology as a whole.

This NTA program aims to support DNTP by (1) investigating the relevance to DNTP scientists of capabilities that may already be available within the National Institute of Environmental Health Sciences or at external laboratories, as well as by (2) supporting the development and validation of new capabilities identified by DNTP scientists as meeting evolving programmatic needs. For example, in silico toxicology[3] and in silico genetic toxicology[4] approaches were recently developed under a large collaboration with DNTP participation. The S1500+ high-throughput transcriptomic platform was developed within DNTP in collaboration with many external government and commercial scientists for applications in rapid toxicity screening and benchmark dose assessment.[5,6] Existing technologies, such as the Duplex Sequencing approach to error-corrected next generation sequencing, which can rapidly provide detailed molecular information on mutational spectrum and frequency in any cell type in humans and rodents, are being applied to existing DNTP projects to define their programmatic benefit, a prelude to their adoption as a DNTP tool. The two main goals of the NTA program listed above are

[3] Myatt *et al*. (2018) In silico toxicology protocols. Regul Toxicol Pharmacol 96:1-17. https://doi.org/10.1016/j.yrtph.2018.04.014.
[4] Hasselgren *et al*. (2019) Genetic toxicology in silico protocol. Regul Toxicol Pharmacol 107:104403. https://doi.org/10.1016/j.yrtph.2019.104403.
[5] Bushel *et al*. (2020) Comparison of normalization methods for analysis of TempO-Seq targeted RNA sequencing data. Front Genet 11:594. https://doi.org/10.3389/fgene.2020.00594.
[6] Mav *et al.* (2018)

directed at moving the field of toxicology forward on the basis of cost/benefit analysis (resources needed vs. benefit to the program/field of toxicology) and relevance to the specific mission of DNTP, recognizing that many NTAs may exist that, although extremely beneficial in other settings, do not directly benefit DNTP's mission. Thus, the NTA program will serve as a checkpoint in evaluating NTAs to help focus adoption efforts on the most beneficial NTAs. In this regard, the NTA program will interface with other programs (e.g., the Carcinogenicity Health Effects Innovation program, Occupational and Inhalation Exposures program, Emerging Contaminants and Issues of Concern program) to introduce promising new capabilities for applications in areas deemed programmatically important and a good fit for the technology, as well as to seek input on potential benefits of engaging in technology development. Thus, the program value proposition will be continuously driven by internal DNTP project needs, as well as DNTP stakeholder needs (for externally facing capabilities and usage).

The 28 projects in the existing NTA portfolio are of varying size and complexity. One can ask whether the existing portfolio is sufficient and appropriate to deliver the benefits that DNTP requires to continue moving into the future and retaining a leading role in the international toxicology community. Some NTA portfolio projects are limited and piloted in scope, whereas others represent longer term commitments over multiple years. Members of the NTA program have carefully considered the challenge that will be required to focus the portfolio in the near term on fewer projects that fall within each of the three technology areas identified by the NTA Program Plan: spheroids/organoids, microphysiological systems, and high-content data streams. DNTP has ongoing efforts in these three key areas, and focusing on these activities represents a prime strategy to concentrate resources that provide maximum impact and benefit to DNTP. The NTA program aims to achieve this focused portfolio within the next 2 years as we seek to maximize our contribution to the field of toxicology. The efforts aimed at developing our capabilities in organoids and microphysiological systems will likely require a longer timeline, but measurable progress is expected within a 3- to 5-year time frame.

In the future, as new projects are submitted to the NTA program for consideration, we will be challenged to retain our focus on those new technologies and approaches that best align with the DNTP mission and provide the necessary capabilities to meet our needs. Our goal will be to build a cohesive program for the future while allowing for innovation and refocusing our portfolio when doing so benefits DNTP. The greatest measure of the NTA program's value will be the adoption of DNTP-promoted NTAs among members of the wider toxicology community.