



**Second Annual Conference on the State of the Science on
Development and Use of New Approach Methods (NAMs) for
Chemical Safety Testing**

Conference Summary

October 19-20, 2020

U.S. Environmental Protection Agency (EPA)

Webinar

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Day 1 Summary

Welcome and Charge to the Group

Maureen Gwinn (Director of EPA's Biomolecular and Computational Toxicology Division [EPA-BCTD] within the Office of Research and Development [EPA-ORD]) opened the webinar and introduced EPA Administrator Andrew Wheeler. In pre-recorded opening remarks, Administrator Wheeler welcomed everyone to the Second Annual Conference on the State of the Science on Development and Use of New Approach Methods (NAMs) for Chemical Safety Testing. In the first conference held in December 2019, over 500 people with a common interest gathered to implement Administrator Wheeler's vision to eliminate animal testing.

Administrator Wheeler gave examples of how EPA has since made progress to reduce, replace, and refine animal testing requirements. EPA established a [website](#) dedicated to NAMs information. In February 2020, EPA issued [guidance](#) deeming pesticide testing on birds unnecessary and waiving those requirements. EPA engaged stakeholders by hosting a public webinar and by convening a meeting of the Scientific Advisory Board (SAB). In June 2020, EPA released the [NAMs Work Plan](#), which outlines objectives, strategies, and deliverables to reach the goal of eliminating mammal testing by 2035. In July 2020, EPA released [guidance](#) reducing unnecessary testing on fish. In October 2020, EPA released [guidance](#) expanding waivers for dermal toxicity tests for pesticides. By the end of 2020, EPA will publish for public comment a draft method for making additions to the list of NAMs that EPA maintains under Section 4 of the Toxic Substances Control Act (TSCA). This conference will chart the path forward for reducing animal testing while upholding chemical safety.

M. Gwinn thanked Administrator Wheeler for his opening remarks and introduced Jennifer Orme-Zavaleta (Principal Deputy Assistant Administrator for Science of EPA-ORD) to provide the charge to the group. J. Orme-Zavaleta emphasized that Administrator Wheeler is very passionate about making progress on this issue. The goals of the Administrator's [directive](#) are threefold:

- 1) reduce requests for and funding of mammalian studies by 30% by 2025;
- 2) eliminate mammal testing by 2035; and
- 3) exclude EPA from approval processes on mammalian studies that are conducted after January 2035 as much as possible.

J. Orme-Zavaleta recounted last year's inaugural conference during which stakeholders convened to discuss how EPA would achieve these ambitious goals and create a work plan. The work plan product released in June 2020 outlines the following objectives:

- 1) evaluate regulatory flexibility for accommodating NAMs;
- 2) develop baselines and metrics for assessing progress;
- 3) establish scientific confidence and demonstrate application;
- 4) develop NAMs that fill critical information gaps; and
- 5) engage and communicate with stakeholders.

J. Orme-Zavaleta stated that EPA is aiming to release a progress report by the fourth quarter (Q4) of 2020. She encouraged attendees to check the [EPA NAMs website](#) for updates and to email NAM@epa.gov with any questions.

Implementation of Animal Testing Reduction at EPA

Russell Thomas (EPA): Overview of EPA NAMs Work Plan

Russell (Rusty) Thomas (Director of the Center for Computational Toxicology and Exposure within EPA-ORD) provided an overview of the June 2020 [NAMs Work Plan](#) and the strategies that have been developed by EPA to meet the goals of the Administrator.

Summary

- After the first EPA NAMs conference, held in December 2019, EPA's Office of Chemical Safety and Pollution Prevention (EPA-OCSPP) and ORD were tasked with developing the NAMs Work Plan to guide EPA toward meeting the ambitious goals laid out in Administrator Wheeler's [September 2019 Memo Prioritizing Efforts to](#)

Reduce Animal Testing. Thirty-four experts from across the EPA convened an internal workshop in January 2020 to begin the development of the work plan.

- The experts were divided into five subgroups reflecting the five objectives of the work plan. These objectives are detailed below.
- The subgroups reviewed the discussions from the 2019 NAMs Conference's breakout groups to help guide the development of each section.
- Each objective of the work plan has short- and long-term goals with specific deliverables and timelines.
- This work plan was developed using current information and understanding of the state of the science. As more information is reviewed, the work plan may need to evolve and adapt.
- Objective 1: Evaluating Regulatory Flexibility and Existing Statutes in Accommodating NAMs
 - o Review the existing regulations, policies, guidance, and statutes that may not allow for flexibility in applying NAMs. EPA will report on this review's findings in 2021.
- Objective 2: Developing Baselines and Metrics for Assessing Progress
 - o Build upon previously established baselines and metrics for animal use in OCSPP and ORD, progressively extending to each of the other offices. EPA will develop the summary metrics and report them through the NAMs website in Q4 of each year, starting in 2021, aligning with the annual NAMs conferences.
- Objective 3: Establishing Scientific Confidence and Demonstration of the Application of NAMs
 - o Characterize the scientific quality of the NAMs because they will need to be "as good as or better than" traditional methods. EPA will also work to develop scientific confidence in the NAMs across all agencies, develop reporting templates, and demonstrate the application of NAMs to regulatory decisions through case studies. EPA will engage the National Academy of Sciences (NAS) to develop a report on the uncertainties and utility of existing mammalian toxicity tests by Q4 2022. EPA will release a scientific confidence framework to evaluate the quality, reliability, and relevance of NAMs in the third quarter (Q3) of 2022. Case studies to evaluate application of NAMs to decision-making will be released biennially starting in 2022.
- Objective 4: NAM Development to Fill Scientific Gaps
 - o EPA will facilitate the joint planning of NAM development within EPA across offices and will encourage the development of NAMs by external parties, building upon current practices such as the Science to Achieve Results (STAR) Grant Program. Strategic Research Action Plans reflecting internal EPA research activities will be developed on a four-year planning cycle.
- Objective 5: Communication and Outreach with Stakeholders
 - o EPA has created a [centralized portal](https://www.epa.gov/nam) for the release of all EPA NAMs-related information:
 - o EPA will actively solicit feedback associated with all deliverables and objectives in real time through the website and through the new email address, NAM@epa.gov. The feedback will be reviewed quarterly.
 - o EPA will also hold public webinars associated with each deliverable release.

Additional points made:

- The December 2019 NAMs Conference breakout groups were designed to match the objectives outlined in the work plan.
- It is critical that all stakeholders use the email address (NAM@epa.gov) and the NAMs website to submit feedback, ideas, and more to EPA for this effort to be successful and beneficial for all groups involved.

Louis Scarano (EPA): Progress on Implementing the TSCA Alternatives Strategic Plan

Louis (Gino) Scarano (EPA Office of Pollution Prevention and Toxics [EPA-OPPT]) provided an update on the eight objectives in the *EPA Strategic Plan to Develop and Implement NAMs in TSCA*, with a focus on the first four:

- 1) implement NAMs;
- 2) maintain and update Section 4(h)(2)(c) list of TSCA-acceptable NAMs;
- 3) identify retrospective TSCA information;
- 4) identify available confidential business information received under TSCA;
- 5) use NAMs for prioritization;
- 6) develop an information technology (IT) platform;
- 7) collaborate with partners; and
- 8) launch a TSCA NAM website.

Summary

- The 2016 Frank R. Lautenberg Chemical Safety for the 21st Century Act began a drive toward reducing animal testing and promoting alternative testing methods. One of the requirements was to develop a [Strategic Plan](#) which was published in June of 2018.
- Objective 1 has seen progress through the lung effects project, which had a manuscript submitted in July 2020, and the skin sensitization project, which stems from a [draft policy](#) stating EPA will accept NAMs in place of the local lymph node assay (LLNA) test. In the lung effects project, one proposed tiered testing strategy involves insoluble polymer lung overload. These tiers start with measuring particle size distribution, then *in chemico* measurements such as biosolubility, followed by computational modeling, and then finally to strategic *in vivo* testing if the prior tiers indicate lung overload. A recent example where this tiered system was used identified a chemical substance as not being a concern for lung overload, leading to revocation of the Significant New Use Rule under TSCA for that substance. The skin sensitization project uses NAMs to study adverse outcome pathways (AOPs) by identifying key events stemming from interactions between the substance of interest and molecules that are associated with the allergic contact dermatitis adverse outcome.
- Progress on Objective 2 has moved steadily through the release of the list's first iteration in June 2018 with 39 NAMs, its [first update](#) in December 2019 with the addition of Appendix B and new NAMs (bringing the total to 85), and the beginning of the TSCA NAMs nomination form and process. Public comments led to the development of Appendix B, which contains "other useful information," such as tools and approaches to enhance the use of NAMs. EPA plans to release a draft proposal on this list's NAM selection process for public comment in 2020.
- Progress on Objectives 3 and 4 is occurring through Analysis of TSCA Available, Expected, and Potentially Useful Information (ATAEPI). Under ATAPEI, all study requests since the first one in 1979 are being reviewed. The preliminary results highlight the most commonly requested studies, such as the 28-day oral and 90-day inhalation tests for human health and the algae and fish acute tests for environmental organisms.
- The newly established Data Gathering and Analysis Division (DGAD) at EPA will focus on Objective 5 (use of NAMs for prioritization of TSCA chemicals).
- Objective 6 progress includes the deployment of new IT platforms, such as the International Uniform Chemical Information Database (IUCLID) 6.3, the Organisation for Economic Co-operation and Development (OECD)'s Quantitative Structure-Activity Relationship (QSAR) Toolbox 4.3, and EPA's development of a "sandbox" system for confidential business information via a local area network (LAN). EPA has also collaborated with the European Chemicals Agency (ECHA) and Canada to exchange public chemical data and methods using IUCLID and OECD Harmonization Templates.
- Objective 7 has progressed through a variety of partner and stakeholder collaborations, including a series of webinars with People for the Ethical Treatment of Animals (PETA) and the Physicians Committee for Responsible Medicine (PCRM), workgroups with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and OECD, and exchanges with other stakeholders.
- Objective 8 is complete with the launching of a [TSCA NAM website](#).

Additional points made:

- Between October 2015 and April 2020, OPPT received 49 chemical registration submissions from 24 companies, and 27 of those submissions relied exclusively on NAMs evidence for skin sensitization. This information will be used along with an evaluation of 51 TSCA chemicals using skin sensitization NAMs by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to refine the draft policy on skin sensitization.
- EPA is required to publish a list of TSCA-acceptable NAMs under Section 4. In developing this list, EPA will soon be releasing documentation describing how NAMs may be nominated to be placed on the list. NAM nominations should cover nominal information, developmental history, method description, relevance to TSCA chemical decisions, and reliability.

State of the Science in Development of NAMs

Andreas Bender (University of Cambridge): Using Chemical, Biological, and In Vivo Data for NAMs: Which Data Do We Have, and What Can We Do with It?

Andreas Bender (University of Cambridge) discussed the various *in vivo* data types that exist, what can be done with them, potential considerations for their use, and the potential path forward.

Summary

- A. Bender discussed key characteristics of chemical, biological, and phenotypic data available from *in vivo* animal data.
- There are essentially three types of data: chemistry, phenotypes, and targets or modes of action (MOAs) that are connected through other data, such as bioactivity, phenotypic response data, and AOPs.
- A. Bender reviewed three case studies.
- Case Study 1: Linking chemistry or an assay to an endpoint.
 - o Aimed at understanding the mechanisms behind structural cardiotoxicity and demonstrated a novel workflow to use data from different sources (ChEMBL, ToxCast, FAERS) to derive AOPs.
- Case Study 2: Linking gene expression or cell morphology data to an endpoint.
 - o The QSTAR Project is evaluating gene expression data in lead optimization. The main takeaway is that data require a clear rationale for interpretation. One cannot just generate data; the data need to be put into context.
 - o Gene expression data could be useful, but there are experimental factors that need to be accounted for, including cell lines, doses, time points, and other setup considerations, to ensure the relevance of the outcomes to an *in vivo* setting. Data analysis factors to be accounted for include whether the focus is on high coverage or specificity of the data, data standardization, and confirmation bias.
- Case Study 3: Using historical animal data for analysis.
 - o These data can improve estimates of treatment-related effects, demonstrate interspecies concordance, and predict useful time points.
 - o There are some limitations to animal data, such as lack of annotations and standardization and under-analysis of databases such as eToxSys.
- As long as it is difficult to understand existing animal data, it will be difficult to compare any new methods to them and even more difficult to replace them.
- Computational models are only as good as the underlying data. Data need to be useful for the problem at hand and developed within context.
- There will always be issues of data coverage with any shortcuts.
- Partnerships and consortia will be critical to help with identifying which readouts matter, sharing data, generating data, and developing agreements on the best practices for data analysis.

Additional points made:

- No method can save an unsuitable representation or model or remedy data irrelevant to a given question.

Kamin Johnson (Corteva): Transcriptome-Based Derivation of an In Vivo POD: Current and Future Utility

Kamin Johnson (Corteva) discussed a project his company has been working on for the past four years to study transcriptome-based points of departure (PODs) and their utility for predicting apical PODs. The presentation covered background on the use of transcriptome-based PODs in the pesticide regulatory process and compared traditional (apical) to transcriptome-based PODs. This work contributes to the EPA NAM work plan by establishing scientific confidence in NAMs and developing NAMs that fill critical information gaps.

Summary

- Transcriptome-based PODs present an opportunity to reduce the time and number of animals needed for risk assessment decisions. Agrochemicals typically have the most data requirements. The rodent carcinogenicity studies take two to three years to complete and are the rate-limiting step in the toxicity assessment process. General mammalian toxicology studies can use approximately 1,500 animals over four years to generate various apical POD values that are used to determine the final toxicity POD value used for risk assessment.
- The rationale for using molecular PODs to predict apical PODs stems from the hypothesis that a POD based on comprehensive molecular data will be protective of any downstream apical-effect POD. This hypothesis is

compatible with that of generic AOPs in which the precursors to an apical effect are chemical exposure and then a molecular change.

- Findings from rat liver data collected by the Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System (TG-GATES) consortium showed that the rat liver transcriptome became stable after ~24 hours. The concordance between the four-day transcriptome-based POD and the 29-day apical POD was nearly identical, suggesting that shorter-duration, transcriptome-based PODs can predict apical PODs that otherwise would have required longer *in vivo* studies. Other studies of varying lengths have found similar results across five Corteva chemicals, but some data also suggest that the liver is not always a useful surrogate for effects in other organs. Overall, there is a growing consensus that a transcriptome-based POD can estimate an apical POD.
- Transcriptome-derivation of the POD shows potential to waive the cancer bioassay study in the future. After more studies are conducted to determine concordance across MOAs and tissue types and upon acceptance from stakeholders, this method could reduce animal use by an order of magnitude (from 1,500 animals used over four years to just 150 used over six months).

Additional points made:

- The transcriptome POD derivation method was based on the workflow in the [Johnson et al. 2020](#) paper in *Toxicological Sciences*. The method requires generation of whole-transcriptome data and then use of BMDEExpress software to determine a single POD value.
- The Health and Environmental Sciences Institute (HESI) Emerging Systems Toxicology for Assessment of Risk (eSTAR) committee has pulled together a molecular POD team to develop a framework to derive an *in vivo* transcriptome POD for use in chemical risk assessment that will produce a human health-protective POD based on concerted molecular change. This committee includes members from industry, regulatory and governmental groups, academia, and consulting. K. Johnson encouraged attendees to join this committee and to register for its October 28-30, 2020 annual meeting.

Avi Ma'ayan (Mount Sinai Health System): Drugmonizome and Drugmonizome-ML: Integration and Abstraction of Small Molecule Attributes for Drug Set Enrichment Analysis and Machine Learning

Avi Ma'ayan (Mount Sinai Health System) discussed the programs Drugmonizome and Drugmonizome-ML, which have largely been developed for use in the pharmacology world but can be applied to systems toxicology. These app-based programs allow bioinformatics data to be used more broadly in toxicological decision making.

Summary

- A. Ma'ayan discussed the use of modelling and computational programs to study vast amounts of data covering thousands of chemicals and dozens of human cell lines.
- The genes that have been researched tend to be skewed toward certain "popular" genes, leaving many with no data at all. Similarly, in reviewing Tox21 chemicals, there are many chemicals with no data at all, whereas others have a wealth of data.
- Genes with less available data can be compared to similar genes with more data to estimate their gene expression. A. Ma'ayan presented a case study that reviewed which genes have been analyzed, using SARS-COV-2 as an example. A. Ma'ayan used Geneshot, a program that searches published literature to return which genes are mentioned and in what percentage of that literature. It also scores the similarity of less studied genes to those most studied, allowing researchers to identify genes that may function or be expressed similarly.
- Enrichr is a program that allows a researcher to find structurally similar genes to the gene of choice. Twenty-eight million gene sets have been submitted to Enrichr.
- Although RNA-seq co-expression and Enrichr co-expression may not be quite as precise as literature-based associations, they may nevertheless show novel associations.
- There are many methods that can be used to combine data sources and known information to strengthen understanding of compound effects. A. Ma'ayan's team has developed scripts to help gather these data from publicly available sources through machine learning.
- Drugmonizome is one of over 50 "Appyters" that have been developed. Appyters convert Jupyter notebooks into web-based bioinformatics applications. Drugmonizome is a new program developed to provide drug set enrichment analysis. This program can potentially predict drug targets, side effects, and more.
- The data processed in Drugmonizome can be used to make predictions about drugs, including (potentially) their side effects.

- A. Ma'ayan presented a case study using machine learning to predict novel drugs that could potentially be used in the treatment of COVID-19.

Additional points made:

- A. Ma'ayan demonstrated the L1000 Fireworks Display, a data map showing drug-induced transcriptomic signatures. In this visualization, points represent one drug on one cell type; those that do not have an established MOA are shown in gray.
- A. Ma'ayan discussed many programs and platforms that can be used to analyze gene expression data, including Geneshot, Kallisto, Enrichr, Drugmonizome, Drugmonizome-ML, Harmonizome, and more Appyters, as well as multiple data sources such as ARCHS4 and LINCS L1000.
- ARCHS4 RNA-seq data has been visualized by A. Ma'ayan's group to show gene-gene co-expression.

Ivan Rusyn (Texas A&M University): "Fit for Purpose" for Organotypic Models in Environmental Health Protection

Ivan Rusyn (Texas A&M University) discussed the use of tissue chips in tandem with modeling for regulatory decision making and presented three case studies on their application. Tissue chips are already used for internal decision making in the pharmaceutical industry. The science itself is fairly new, but increased funding over the past 10 years has increased research on tissue chips. Texas A&M University receives funding from the National Center for Advancing Translational Sciences (NCATS) to test and develop tissue chips. Overall, partnerships and investments in this area are growing.

Summary

- For each new tissue chip, over a four- to eight-month timespan, the Texas A&M University Tissue Chip Validation Center gathers documents and training for new technologies, identifies critical elements to replicate and conducts reproducibility testing, and defines the technology's area of use. There are a wide range of technologies under development, such as gravity flow cultures from the University of California, Berkeley and forced flow cultures from the University of Washington. The University of Pittsburgh's [Microphysiology Systems Database](#) houses all the information gathered and related publications.
- Going forward, tissue chip research should be "fit for purpose," meaning end users should describe the platform types they are most interested in prior to conducting experiments.
- Case #1 shows how tissue chips can be used to study reabsorption toxicokinetics in the kidney. Specifically, tissue chip data can be used to develop models using compounds with well-understood absorption compared to human urine data. The tissue chips are expensive and only replicate one element of a complex physiological system, so modeling is important.
- Case #2 shows how to derive a "safe dose" using NAMs. Ion channel blockage causes toxicity, and this blockage can be observed with *in vitro* methods. By testing 13 drugs *in vitro*, the "safe dose" was predicted within a factor of 10.
- Case #3 shows the importance of considering population variability during risk characterization by using modeling.
- Tissue chip science is not ready to eliminate the use of animals in the near future. There are promising organotypic models, but EPA needs to define the purpose. Instead of trying to completely replicate the human physiological system on a chip, the path forward is more likely a combination of physiologically based pharmacokinetic (PBPK) modelling, organotypic model-derived hazards, and data on mechanisms and toxicokinetics.

Additional points made:

- It is important to remember that the "best available science" can be or include non-animal tests. Many animal tests used by EPA are not actually required. Tissue chips themselves will not be used in isolation but will serve as another piece of evidence.
- NIH is making large investments in human-based models, while animal data are the comparator for NAMs at EPA. Animal cell-based micro-physiological systems are needed because this is where comparative data exists for building scientific confidence in new methods.
- NAS hosted a [workshop](#) in 2014 on the potential of the tissue chip for environmental health, which highlighted an information gap: the most effective use and purpose of the method.

Day 1 Closing Remarks

David Fischer (EPA)

David Fischer is the Deputy Assistant Administrator for EPA's OCSPP. He thanked the speakers and attendees from around the world. D. Fischer expressed appreciation for the effort that everyone made to participate in this important virtual conference. He noted that the presentations spoke to EPA's dedication to replacing animal testing with better science in NAMs. This research brings EPA closer to meeting Administrator Wheeler's goal of eliminating animal testing by 2035.

Day 2 Summary

Welcome

Anna Lowit (Senior Science Advisor for EPA's Office of Pesticide Programs [EPA-OPP]) welcomed everyone to the second day of the 2020 NAMs Conference. She noted that participants should use the Q&A box and that any questions not answered live would be answered by email after the conference. A. Lowit noted that the first day was very successful and productive. She noted that the presentations and recordings would be available on the conference website following the conclusion. She introduced Alexandra Dunn, the Assistant Administrator for OCSPP, to give her opening remarks. A. Lowit noted that Assistant Administrator Dunn is a proponent of the 3Rs: reducing, refining, and replacing animal testing. The 3Rs reduce animal use but also make scientific assessments more supportable and efficient.

Assistant Administrator Dunn (EPA-OCSPP) noted that a virtual conference was not the preferred option, but it nevertheless allowed for progress during a challenging health emergency. She thanked everyone for embracing this platform. Assistant Administrator Dunn confirmed that she is passionate about this agenda and noted that Administrator Wheeler's September 2019 memo set very ambitious goals for the Agency. The memo built upon progress that the Agency had been making toward the reduction of animal testing, as well as the improvement of the science that is relied upon for regulatory decision making. Assistant Administrator Dunn noted that EPA is working collaboratively with scientists from around the world and from across government, industry, and academia, as exemplified by the presenters. She again thanked everyone for their participation and reiterated the importance of submitted comments and ideas to NAM@epa.gov.

Addressing Current Limitations in NAMs

Chad Deisenroth (EPA): Retrofitting an Estrogen Receptor Transactivation Assay with Metabolic Competence

Chad Deisenroth (EPA-ORD) presented on the development of NAMs to fill information gaps, such as inadequate coverage of biological targets, limited capability to address tissue- and organ-level effects, lack of robust integrated approaches to testing and assessment, and minimal capability for addressing xenobiotic metabolism in *in vitro* test systems. Research on these gaps is housed under the four-year Chemical Safety for Sustainability Strategic Research Action Plan, which includes an objective to develop and apply methods to incorporate endogenous and exogenous xenobiotic metabolism into high-throughput *in vitro* assays.

Summary

- There is international recognition that *in vitro* metabolism systems should be considered, particularly for endocrine disruption. In response to this need, the NTP, NCATS, and EPA launched the Transform Toxicity Testing Challenge to identify innovative solutions to retrofit high-throughput assays for metabolism. There are parallel efforts in ORD to develop these systems, including intracellular and extracellular approaches, which can be integrated into a strategy to model *in vivo* metabolic bioactivation and detoxification.
- The intracellular approach to examine xenobiotic metabolism uses cell-based assays. It more closely models effects of target tissue metabolism and allows users to bypass the need for a DNA template by enabling user-defined composition and ratios of multiple input mRNAs. ORD used this approach to compare two activities across the three strongest *in vitro* systems. The intracellular approach suggests that the liver cell is not necessarily the gold standard and that other cells have unique metabolic processes.

- The extracellular approach for examining xenobiotic metabolism uses the media or buffer of cell-based or cell-free assays. This approach more closely mimics hepatic metabolism and the circulation of metabolites. An example of this approach is the alginate immobilization of metabolic enzymes (AIME) method, which uses lids fabricated with pillars going into a well microplate to capture liver metabolism along with hepatic S9 fraction and optimization cofactors like NADPH. To help improve cell viability, the S9 fractions are suspended in alginate hydrogel.
- The purpose of the AIME method is to evaluate cytochrome P450 metabolism. These experiments showed the AIME method is optimized for Phase I metabolism. Metabolic activity was validated diversely, and a two-hour incubation period was found suitable for parent compound depletion.
- The AIME method was used for a case study on 63 chemical compounds in an effort to retrofit it to estrogen receptor transactivation. The study reprioritized hazards based on metabolism-dependent bioactivity, demonstrated the AIME method's utility for identifying false positives and false negative effects, and enhanced *in vivo* concordance with the rodent uterotrophic bioassay. Chemicals with demonstrated estrogen receptor activity were used to test the method and develop an estrogen receptor QSAR method.
 - o To test the method, the false-positive test set used chemicals with metabolites known to spike bioactivity, and the results were compelling in distinguishing between parent compound and metabolite and in demonstrating bioactivation.
 - o The negative test set examined chemicals with metabolites that were not expected to be more bioactive than the parent compounds. The results demonstrated the expected results in most compounds and was also able to detect both bioactivation and bioinactivation.
 - o Collecting a full dataset across the 63 chosen substances will allow for extensive comparison of results to develop this method.

Additional points made:

- EPA is adapting the method to use with a bioprinter that can pattern the hydrogel into a microplate to achieve a more scalable application to high-throughput screening.

David Crizer (NTP): In Vitro Disposition of Tox21 Chemicals: Initial Results and Next Steps

David Crizer is a chemist in the Predictive Toxicology and Screening Group at NTP. He presented on the difference between the nominal (stated) concentration of a chemical applied to an *in vitro* system and the actual concentration absorbed by the cell. D. Crizer explained how to test and predict this difference.

Summary

- Current *in vivo* to *in vitro* extrapolation (IVIVE) predictions rely on the nominal concentration. If that nominal concentration fails to accurately represent the cellular concentration of that assay, the prediction accuracy may be affected. Uncertainty in the amount of chemical absorbed into the cell makes it difficult to say whether or how the administered chemical concentration or dose level relates to the observed results. Current steady-state IVIVE assumes that blood-to-tissue partitioning is equivalent to the cell-to-medium partitioning in an assay.
- Currently, there is a lack of empirical research into *in vitro* chemical partitioning. It is unknown how many chemicals in chemical databases, such as Tox21, have differential partitioning.
- Uncertainty in dose response poses a problem for making regulatory decisions.
- The hypothesis of this work is that the physicochemical properties of chemicals can be used to predict the difference between the nominal applied concentration versus the actual observed concentration within the cellular compartment.
- This project aims to analyze about 200 chemicals representative of the broad chemical space, about 92.5% of which are also present in ToxCast. A pilot test of 10 representative chemicals was conducted to refine the protocols. The pilot also consisted of a "cassette" test in which five chemicals were combined and tested in a single liquid chromatography-mass spectrometry injection to reduce testing time by roughly one third. It was shown that these cassette samples produced similar results to individual samples. The pilot showed that the nominal concentration did not equal the cellular concentration unless the surface area of the bottom of the dish was considered unavailable for bonding.
- The future of this project consists of testing minor changes to the protocol to continue to explore this subject.

Additional points made:

- When a chemical is introduced to a medium, several pathways are possible; it could sit in the medium, it could evaporate, it could be absorbed into the cell, or it could attach to the container walls.
- [Armitage et al. 2014](#) suggested that *in vitro* partitioning related strongly to the octanol-water partition coefficient ($\log K_{ow}$) and concentration of serum in the medium. Plastic binding was not as important in this case but is still a consideration. This model was used as a comparison to the work conducted here.

Developing Scientific Confidence in NAMs

Clemens Wittwehr (Joint Research Centre of the European Commission): An OECD Harmonized Template to Report NAM Results in Regulatory Environments: Principles and Practical Use

Clemens Wittwehr (Joint Research Centre of the European Commission) presented on the use of OECD Harmonized Templates (OHTs) to improve data reporting and exchange and to subsequently accelerate data acceptance. In 2012, when numerous templates for apical animal data had already been made available, the OECD identified a need to develop templates for mechanistic data and, as such, published a first version of OHT 201 in 2017. OHT 201 facilitates the reporting of NAM study results within an internationally agreed-upon format. An updated version of OHT 201 was officially released by the OECD on November 6, 2020.

Summary

- A unique template for mechanistic data was necessary because those data are not intrinsically linked to apical endpoints from *in vivo* animal tests. Mechanistic data investigate a sequence of events linking exposure to a biological response and frequently come from non-animal tests.
- IUCLID, which is available free of charge, is the most popular software for OHT implementation, and OHT 201 is available there.
- OHT 201 is designed to link chemicals to an intermediate mechanistic effect identified by a process-object-action ontology. The template is compatible with a variety of technologies including all classes of NAMs. OHT 201 will continue to be refined to best support the most popular technologies.
- OHT 201 can be used regardless of whether a NAM has an OECD test guideline supporting it. If there is a supporting OECD guideline, many fields are pre-filled.
- OHT 201 was made with the triangle of chemical safety in mind. The first point of the triangle represents a stressor. The second point represents a study method (e.g., *in vivo* or *in vitro* tests), and the third point represents a key event in an AOP. Once all three elements of this triangle use the same ontologies, stressor data (in OHT 201), key event data (in the AOP Knowledge Base), and method descriptions (in method databases) can be cross-referenced. Over time, OHT 201 may help researchers learn which methods are best for identifying different key events.
- The European Commission's Endocrine Active Substances Information System (EASIS) is expected to be published in 2020 and will then include 550 studies across 100 substances focused on intermediate effects using OHT 201, with more data added over time.

Monique Perron (EPA): Case Study #1: Integration of NAM Data for Evaluating Potential Developmental Neurotoxicity

Monique Perron (Senior Toxicologist at EPA-OPP) reviewed the work that EPA's OPP is conducting with ORD to test a weight of evidence (WoE) approach to incorporating NAM data in evaluating the developmental neurotoxicity of pesticides.

Summary

- Due to challenges and limitations in the developmental neurotoxicity (DNT) guideline study, there has been an international effort to develop *in vitro* assays that assess processes critical to development of the nervous system.
- These efforts have resulted in a battery of *in vitro* tests developed by ORD and investigators funded by the European Food Safety Authority (EFSA).
- Data from the ORD assays (microelectrode assay-network formation assay [MEA-NFA] and high content imaging [HCI]) were recently presented to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel in September 2020 using organophosphates as a case study.

- ORD assays can replicate key cellular events and processes relevant to DNT as demonstrated through the use of appropriate assay performance controls.
- ORD assays demonstrate reproducibility in both positive responses and the potency of those responses.
- Twenty-seven organophosphate chemicals in this set are differentially active in the MEA-NFA and HCl assay suites.
- IVIVE approaches for *in vitro* bioactivity observed in ORD assays result in estimated external dose values that are greater than, or in some cases approximate to, doses that inhibit acetylcholinesterase *in vivo*.
- Organophosphate data from these assays will be considered in combination with the results of assays sponsored by EFSA as part of an overall weight of evidence evaluation of the DNT potential of individual organophosphates.

Andrew White (Unilever): Case Study #2: Integration of NAM Data in a Next-Generation Risk Assessment for Cosmetic Ingredients

Andrew White (Unilever) presented on how NAMs can be used for consumer product risk assessments, which answer the generic question: Can we safely use x% of ingredient y in product z? A. White used a hypothetical case study for a face cream containing 0.1% coumarin to demonstrate how this would be done with the limitation of using no animal study data. This limitation was applied to “History of Safe Use” data, clinical data, animal read-across data, and *in silico* data based on animal use.

Summary

- In the face cream scenario, a reasonable estimation of daily likely exposure can be used in a PBPK model to inform the key parameters. The model shows the simulated plasma concentration of coumarin after dermal exposure, as well as uncertainty and population variability.
- After conducting exposure estimation, the next step is collating existing information from sources like ToxTree, the Molecular Initiating Event (MIE) Atlas, the OECD toolbox, and the scientific literature. In this hypothetical, the ToxTracker results were negative. There were some findings that reactive coumarin metabolite(s) could induce DNA lesions secondary to oxidative stress, and all binding and enzymatic assay results from a journal article were negative at 10 µM.
- An immunomodulatory screening assay found no effects at relevant concentrations.
- The results of newly developed cellular stress response assays to characterize non-specific biological activity suggested some dose response. However, in comparison to other known “high-risk compounds,” coumarin is not very active.
- In high-throughput transcriptomics (HTTr) for *in vitro* biological activity screening, the MCF7 POD data were not sufficiently robust to determine the margin of safety. The lowest POD values for the other cell models, HepG2 and HepaRG 2D, were used for the margin-of-safety calculation.
- Tier 2 refinement focused on examining metabolic pathways. The findings were within range of those from the HTTr test, showing low bioactivity.
- In conclusion, the risk assessment showed that the expected exposure to face cream was lower than all the PODs determined through the various tests and that coumarin is not genotoxic, does not bind to any targets of interest, and does not show immunomodulatory effects. This WoE suggested that 0.1% inclusion of coumarin in face cream is safe for consumer use.
- Risk assessments require many lines of WoE to make safe decisions rather than a couple of assays alone. The goal is to protect against hazards, not to define mechanisms. Diverse expertise, unbiased tools with biological coverage, uncertainty estimates, and more case studies are necessary to build confidence in applying NAMs to consumer product risk assessments.

Todd Stedeford (EPA): Case Study #3: Incorporating the Threshold of Toxicological Concern into Regulatory Decisions under the Amended Toxic Substances Control Act

Todd Stedeford (Toxicologist at EPA-OCSPP) presented the third case study, which covered the incorporation of the Threshold of Toxicological Concern (TTC) into regulatory decision making as dictated by TSCA.

Summary

- T. Stedeford gave an overview of TSCA and specifically covered regulation of new substances under Section 5 and existing substances under Section 6. He also discussed Section 4h, which deals with the reduction of testing on vertebrates.

- EPA assesses a wide variety of human health and environmental associations with known, intended, and reasonably foreseen conditions of use for new and existing chemical substances.
- For the types of data used for new and existing chemical substances, EPA requires producers to provide all data in their possession or control related to health and environmental effects. EPA may require the development of new information relating to the chemical substances.
- The amendment to TSCA in 2016 included a statutory mandate regarding testing on vertebrates. Such testing must consider all reasonably available information and encourage and facilitate scientifically valid test methods and strategies to reduce or replace use of vertebrate animals while providing information of equivalent or better scientific quality and relevance to support regulatory decisions.
- The TTC approach can fulfill many of these requirements under TSCA. EFSA and the World Health Organization have endorsed the TTC approach as a pragmatic, scientifically valid methodology to assess the safety of substances of unknown toxicity that are found in food.
- The TTC is a level of exposure determined to be of no appreciable risk to human health due to the absence of chemical-specific toxicity activity. TTC is regarded as an extension of generalized read across and chemical category approaches that are already used by EPA under TSCA and by other regulatory agencies, such as the Food and Drug Administration.
- TTC is a possible approach to screening some structural classes of new and existing chemical substances to make preliminary decisions. For example, TTC can be used:
 - o for evaluating exposure/release controls for new chemical substances and identifying the need for possible refinements in the absence of chemical-specific or analogue data;
 - o for risk-based prioritization of existing chemical substances; or
 - o as a non-animal approach for making initial decisions regarding the need for further data gathering.
- Several points warrant further evaluation. Oral TTC values are well established. However, TSCA risk assessments may include evaluations via other routes (e.g., dermal and inhalation), other endpoints (e.g., portal-of-entry effects), less-than-lifetime assessments, and ecological assessments.
- EPA is developing a collaboration with other organizations to determine opportunities for the use of TTC in regulatory decision making.

Additional points made:

- In the TSCA chemical hazard approach, the hierarchy of data preference for risk assessments is chemical-specific data followed by analogue data and then model data.

Day 2 Closing Remarks

David Dunlap (EPA)

David Dunlap (Deputy Assistant Administrator for Science Policy, EPA-ORD) thanked everyone for their participation. He noted NAMs research and development will be critical for reducing resource use and for rapidly gathering information on chemical safety to protect the public.

D. Dunlap highlighted current NAMs research and applications from earlier presentations. ORD is integrating metabolic competency into NAMs, an area of previous weakness. M. Perron (EPA-OPP) explained how NAMs can be used for developmental neurotoxicity testing. A. White (Unilever) demonstrated how NAMs can be used for cosmetic product risk assessments. T. Stedeford (EPA-OPPT) shared how OCSPP is using NAMs to determine TTCs in risk assessments.

D. Dunlap stated this larger NAMs effort will require continued collaboration across government agencies, industry, and research institutions. He encouraged attendees to submit comments on the work plan and feedback for the conference by emailing NAM@epa.gov and expressed anticipation for another year of progress by the third annual conference in 2021.

Appendix A: Acronyms

Acronym	Full Name
AIME	alginate immobilization of metabolic enzymes
AOP	adverse outcome pathway
ATAEPI	Analysis of TSCA Available, Expected, and Potentially Useful Information
BCTD	Biomolecular and Computational Toxicology Division
DGAD	Data Gathering and Analysis Division
DNT	developmental neurotoxicity
EASIS	Endocrine Active Substances Information System
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
eSTAR	Emerging Systems Toxicology for Assessment of Risk
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HCI	high content imaging
HESI	Health and Environmental Sciences Institute
HTTr	high-throughput transcriptomics
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IT	information technology
IUCLID	International Uniform Chemical Information Database
IVIVE	<i>in vivo</i> to <i>in vitro</i> extrapolations
K _{ow}	octanol-water partition coefficient
LAN	local area network
LLNA	local lymph node assay
MEA-NFA	microelectrode assay-network formation assay
MIE	molecular initiating event
MOA	mode of action
NAMs	new approach methods
NAS	National Academy of Sciences
NCATS	National Center for Advancing Translational Sciences
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
OCSP	Office of Chemical Safety and Pollution Prevention
OECD	Organisation for Economic Co-operation and Development
OHT	OECD Harmonized Template
OPP	Office of Pesticide Programs
OPPT	Office of Pollution Prevention and Toxics
ORD	Office of Research and Development
PBPK	physiologically based pharmacokinetic
PCRM	Physicians Committee for Responsible Medicine
PETA	People for the Ethical Treatment of Animals
POD	point of departure
QSAR	quantitative structure-activity relationship
Q3	third quarter
Q4	fourth quarter

Acronym	Full Name
SAB	Scientific Advisory Board
STAR	Science to Achieve Results
TG-GATES	Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System
TSCA	Toxic Substances Control Act
TTC	Threshold of Toxicological Concern
WoE	weight of evidence

Appendix B: Hyperlinks to Presentations and Other Materials

Hyperlink	Presentation Name
https://www.epa.gov/research/epa-new-approach-methods-efforts-reduce-use-animals-chemical-testing	Welcome and Charge to the Group
https://www.epa.gov/newsreleases/epa-takes-important-step-reduce-unnecessary-animal-testing	Welcome and Charge to the Group
https://www.epa.gov/chemical-research/epa-new-approach-methods-work-plan-reducing-use-animals-chemical-testing	Welcome and Charge to the Group
https://www.epa.gov/newsreleases/epa-continues-efforts-reduce-animal-testing-announces-guidance-fish-testing	Welcome and Charge to the Group
https://www.epa.gov/newsreleases/epa-announces-guidance-waive-toxicity-tests-animal-skin	Welcome and Charge to the Group
https://www.epa.gov/sites/production/files/2019-09/documents/image2019-09-09-231249.pdf	Welcome and Charge to the Group
https://www.epa.gov/chemical-research/epa-new-approach-methods-work-plan-reducing-use-animals-chemical-testing	Overview of EPA NAMs Work Plan
https://www.epa.gov/research/administrator-memo-prioritizing-efforts-reduce-animal-testing-september-10-2019	Overview of EPA NAMs Work Plan
https://www.epa.gov/research/epa-new-approach-methods-efforts-reduce-use-animals-chemical-testing	Overview of EPA NAMs Work Plan
https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/strategic-plan-reduce-use-vertebrate-animals-chemical	Progress on Implementing the TSCA Alternatives Strategic Plan
https://www.regulations.gov/document?D=EPA-HQ-OPP-2016-0093-0090	Progress on Implementing the TSCA Alternatives Strategic Plan
https://www.epa.gov/sites/production/files/2019-12/documents/alternative_testing_nams_list_first_update_final.pdf	Progress on Implementing the TSCA Alternatives Strategic Plan
https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/alternative-test-methods-and-strategies-reduce	Progress on Implementing the TSCA Alternatives Strategic Plan
https://doi.org/10.1093/toxsci/kfaa062	Transcriptome-Based Derivation of an <i>In Vivo</i> POD: Current and Future Utility
https://appyters.maayanlab.cloud/#/	Drugmonizome and Drugmonizome-ML: Integration and Abstraction of Small Molecule Attributes for Drug Set Enrichment Analysis and Machine Learning
https://upddi.pitt.edu/microphysiology-systems-database/	"Fit for Purpose" for Organotypic Models in Environmental Health Protection
http://nas-sites.org/emergingscience/meetings/bioplatform/	"Fit for Purpose" for Organotypic Models in Environmental Health Protection

Appendix C: Questions and Answers

Conference attendees submitted questions for the speakers at the end of each presentation. All questions were answered either during the presentation time slot or following the presentation via the Q&A box or email.

Implementation of Animal Testing Reduction at EPA

Russell (Rusty) Thomas (EPA): Overview of EPA NAMs Work Plan

Questions following the presentation included:

- **Rao Shaila (Gowan Company):** How would the NAMs help with regulatory submissions in the rest of the world where testing is a requirement?
 - o **Russell Thomas (EPA):** Most of the recent international guidelines allow for the inclusion of NAMs in the regulatory submissions, if not as a one-for-one replacement of the current animal-based tests, as supporting evidence that may be used to waive testing requirements.
- **Jennifer McPartland (Environmental Defense Fund [EDF]):** Are there other metrics for success aside from reduced animal testing, for example metrics related to improved public health decision-making reached in part through the use of NAMs?
 - o **Russell Thomas (EPA):** Right now, the goals and metrics are tracking the use of mammals in both research and testing within the Agency. I think you are referring to goals and objectives that are associated with the deliverables that will be outlined in the NAMs Work Plan.
- **Anonymous:** Has the National Academies committee been formed? If so, has it started working?
 - o **Russell Thomas (EPA):** Not yet. It is still in the formative stages.
- **Jennifer McPartland (EDF):** Is there a weblink to the National Academy of Sciences (NAS) committee on uncertainties around mammalian testing?
 - o **Russell Thomas (EPA):** Not yet, but once available it will be posted on www.epa.gov/nam and we intend to have workshops prior to the development of the committee so that we are all on the same page regarding the charge of that committee.
- **Katya Tsaoun (Johns Hopkins Bloomberg School of Public Health):** Are you planning to use evidence-based methods such as systematic literature maps and reviews in identifying scientific gaps? These methods also provide a guide for certainty assessment and one of your sister agencies at EPA's Integrated Risk Information System (IRIS) is pioneering these approaches which could add transparency and objectivity into the process.
 - o **Russell Thomas (EPA):** I think there will be some of these evidence-based methods and systematic literature reviews in identifying the scientific application and development of these new approach methods as well as comparing them to the traditional methods. There is not necessarily a component of the NAMs Work Plan that covers systematic literature review, but the Agency plans to continue using them not only in our traditional methods but also extending them to the way that we use NAMs for Agency decision-making.
- **Anonymous:** Could you elaborate on steps EPA is taking to figure out how to communicate cellular, *in silico*, and other NAMs results to ordinary people for whom the tests may sound bizarre?
 - o **Russell Thomas (EPA):** That is a good question, and it is part of the last objective, which is the communication and engagement of stakeholders. Obviously, stakeholder groups range from more technically oriented stakeholders to less. We plan to engage both scientific community members in how we are using some of these NAMs as well as laypeople. I think it is going to be a continual process to develop communication approaches that match both of those stakeholder groups and everyone between. Not everyone has the same level of understanding of NAMs at this point even if they were well versed in the traditional methods, so there is going to have to be a range of training materials and educational workshops to be sure that everyone is brought on this journey with us.
- **Laura Gutiérrez:** Is part of the Work Plan to validate existing method to study acute toxicities (as ocular toxicity) with Organization for Economic Co-operation and Development (OECD) guidelines for the use of pesticides?
 - o **Anna Lowit (EPA):** EPA's Office of Pesticide Programs (OPP) has a substantial effort to evaluate NAMs for the acute "6 pack studies" made up of the acute lethality studies for oral, dermal, and inhalation and the topical studies on eye irritation, skin irritation, and skin sensitization. The status of these

efforts can be found at: <https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/strategic-vision-adopting-new-approach-methodologies>.

- **Rao Shaila (Gowan Company):** Agreed, but some authorities do agree but some do need testing. Eventually we end up conducting studies despite waiver request. My question is how to approach this type of situation.
 - o **Russell Thomas (EPA):** I believe the case studies that are being developed by regulators, academics, and trade associations will be the path to achieve “evolution” in this space.
- **Rob DeWoskin (etiologic):** Is EPA collaborating with other regulatory agencies nationally and worldwide to advance NAMs?
 - o **Russell Thomas (EPA):** We have a series of collaborations through other organizations, such as OECD, working to apply NAMs where we can.
- **Anonymous:** Would NAMs be acceptable to EPA for consent orders and testing required under significant new use rules (SNURs)?
 - o **Gino Scarano (EPA):** The short answer is yes, and there will be an example in my presentation of that.
- **Anonymous:** Will the statistics on mammal use be normalized to research studies and regulatory dossiers submitted? Would NAMs usage/test waivers also be documented?
 - o **Russell Thomas (EPA):** It really depends on how you define a “study.” I'm not sure we can normalize it in an effective way based on studies and dossiers. Right now, the current metric is tracking the mammalian use in research studies within EPA.
 - o **Anna Lowit (EPA):** In the pesticides program, under 40 Code of Federal Regulations (CFR) part 158, like most other countries in the world there are defined regulatory testing requirements for pesticide chemicals. In our metrics and baselines for the reduction in animal use for pesticides will be based on the standard animal numbers used in the OECD studies. Our baseline is expected to be the standard number of animals used as our starting point. This link is to our new metrics page for the pesticide program: Links to OPP's waiver guidance and animal reduction metrics can be found at: <https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/strategic-vision-adopting-new-approach-methodologies>. NAMs test waivers will be documented. The pesticides program has guidelines publicly accessible, and we are in the process of developing more.
- **Anonymous:** Would you elaborate on how you plan to evaluate both mammal studies and NAMs? Would this include epidemiological studies?
 - o **Russell Thomas (EPA):** Yes, and I can elaborate. There are some clues already in the Work Plan through these different case studies that are being formulated with the EPA Office of Research and Development (ORD) and the regulatory partners. These are really the vehicle of how we plan to evaluate the NAMs and use them in terms of replacing some of the traditional animal tests. These could include epidemiological studies. The current case studies do not necessarily include them but are not necessarily ruled out in the future.
- **Anonymous:** Will there be a process to validate the NAMs and how would that look like?
 - o **Russell Thomas (EPA):** We are moving towards that. It would be established under the scientific confidence framework and not necessarily use the more traditional trial validation process. In order to meet the goals that we outline, the traditional validation process is not really appropriate or useful for validating all of the new approach methods and replacing all the traditional animal tests on a one-to-one basis. The scientific confidence framework really is how the Agency intends to establish confidence in these NAMs for the purpose of decision making.
- **Meg Whittaker (ToxServices LLC):** What consideration of mixtures toxicity will go into developing NAMs specifically for mixtures?
 - o **Russell Thomas (EPA):** Currently the work plan does not specifically address mixtures, but it certainly is part of the larger research program that ORD is developing. Trying to apply these NAMs to mixtures is not something specifically called out in the Work Plan.
- **Fatma Okuş:** Where *in silico* tools about toxicology are in NAMs. Can we trust them, how we can understand the reliability, especially publicly available ones?
 - o **Russell Thomas (EPA):** Hopefully in developing the scientific confidence framework, EPA is going to lay out how we would establish scientific confidence in them. Also, in the Work Plan, we intend to develop a report on the current uncertainty surrounding the traditional animal studies and use that to establish the expectations for the NAMs. It is through both objectives that we intend to develop scientific confidence in our new approach methods together with setting expectations based on our

traditional animal studies.

- **Jennifer McPartland (EDF):** Relatedly, is EPA coordinating with federal research institutes in addition to regulatory agencies (e.g., National Institute of Health [NIH], National Institute of Environmental Health Sciences [NIEHS]), particularly regarding gaps and adverse outcome pathway (AOP) development?
 - o **Russell Thomas (EPA):** The short answer to this is yes. We partner with an NIEHS through the Tox21 consortium. Both in terms of trying to identify what those gaps are and how we approach them from research and science perspective, we are coordinating and working with our federal research partners on that.
- **Jason Fritz (EPA):** Outside of EPA's Office of Chemical Safety and Pollution Prevention (OCSPP), is there a current Agency activity (or case study) currently underway, where human health risk measures are based solely on NAMs endpoints?
 - o **Russell Thomas (EPA):** There is a case study outlined in the work plan in terms of using NAMs for biosolids with our partners in the EPA Office of Water.
- **Anonymous:** Even if EPA phases out, but the same chemicals would be required to be tested in other countries. It [is] super important that EPA encourages other countries to come up with similar vision.
 - o **Russell Thomas (EPA):** It is important for EPA to encourage other countries to come up with a similar approach as we are part of a global economy. Certainly, what we do is impacted by and impacts the requirements of other countries.
- **Katy Wolton (Syngenta):** Will there be guidance for NAMs developers in the meantime on how to assess the performance of their new method? Bearing in mind we do not necessarily want to compare performance to animal data.
 - o **Russell Thomas (EPA):** This is going to be covered by Gino Scarano in the next talk in terms of how NAMs developers will be asked to assess the performance associated with these methods.
- **Ravi Menon (Afton Chemical):** As part of the NAMs program, is there a plan to update traditional EPA quantitative structure-activity relationship (QSAR) tools such as Estimation Program Interface (EPI) Suite and Ecological Structure Activity Relationships (ECOSAR)? These tools, while helpful for preliminary screening, do not have robust domain information, that limits their use. These tools do not pass the OECD validation principles requirements. It would be good to improve/update these tools.
 - o **Gino Scarano (EPA):** This is not a part of my presentation, but it is a good point and something to consider.
- **Anonymous:** To continue the line of questioning that Fatma Okuş mentioned five questions ago on *in silico*, where *in vitro* or *in vivo* tools about toxicology are in NAMs. Can we trust them, how can we understand the reliability of the species selection? The assay amenability?
 - o **Russell Thomas (EPA):** I hope that this will be addressed in the NAS report that is planned as a deliverable, and in the scientific confidence framework on our new approach methods whether they are *in vitro* or reduced *in vivo* models.
- **Bill Eckel (EPA):** OPP's Environmental Fate and Effects Division (EFED) is currently working on QSAR for fish acute toxicity studies.
- **Anonymous:** For OPP and OCSPP, could you describe how staff is being trained to understand and use NAMs given that the science is changing so quickly? For example, how often is their training updated?
 - o **Anna Lowit (EPA):** That is a good question because it can be hard to keep up as the science changes so rapidly. Several times, we have had our staff attend training specific to some of the areas such as skin irritation studies and *in vitro* dermal absorption. We have had Clive Roper give a training on dermal absorption and inhalation 3D models. I would anticipate getting staff trained on developmental neurotoxicity assays. You make a good point that it is difficult to keep up, but we are focusing our training on those areas we believe to be close to regulatory use.
- **Rob DeWoskin (etiologic, LLC):** Are there any plans to validate improvement in risk decisions and regulatory actions with more field testing of body burdens and effects, understanding how difficult this is especially for chronic exposures?
 - o **Russell Thomas (EPA):** That is a good question to validate the improvements in risk decisions in regulatory actions. I think that involves more monitoring as well as epidemiological studies. Right now, that is not a part of the Work Plan, but we also have research activities related to some of those suggested activities.
- **Clive Roper (Charles River Labs):** I am happy to give folks training on *in vitro* dermal absorption and *in vitro*

respiratory inhalation.

Louis (Gino) Scarano (EPA): Progress on Implementing the Toxic Substances Control Act (TSCA) Alternatives Strategic Plan

Questions following the presentation included:

- **Jennifer McPartland (EDF):** Beyond the public comment period for the NAMs nomination and acceptance process, will there be opportunities for public comment on individual NAMs for potential listing?
 - o **Gino Scarano (EPA):** There is not currently a separate opportunity for submitting public comments specifically on individual NAMs. Those comments can be submitted along with the public comments for the NAMs nomination and acceptance process.
- **Ravi Menon (Afton Chemical Corporation):** The pre-manufacture notice (PMN) process does not require any data currently. EPA assesses the hazard and risk of a submitted new chemical using structure-activity relationships and other resources (confidential business information data for example). How will NAMs development be integrated with the PMN review process?
 - o **Gino Scarano (EPA):** NAMs information will be integrated into that process as it is collected.
- **Xianglu Han (Lanxess):** Is there any scientific basis of setting the cutoff for insoluble polymers as a water solubility of 100 mg/L? If the water solubility is larger, is it true that there would be no testing requirement? For nanoparticles, there is a definition of dissolution in water. What is the definition of water dissolution for a polymer?
 - o **Gino Scarano (EPA):** The proposal does not apply to nanomaterials. The 100 mg/L water extractability cutoff was based on EPA's published general water solubility classifications (i.e., moderate solubility: >100 mg/L – 1,000 mg/L). Though these values were not established for evaluating the solubility of particles for lung overload, we used them as a conservative cutoff for extractability, per OECD TG120. If the high molecular weight polymer has a value greater than 100 mg/L, then the substance would not be considered within the category.
- **Rovida Costanza (CAAT Europe):** Tier III should be *in vitro* testing with 3D reconstruction of human airway tissue, before *in vivo*.
 - o **Gino Scarano (EPA):** Thank you for your suggestion.
- **Anonymous:** Were there mostly Good Laboratory Practice (GLP) laboratories?
 - o **Gino Scarano (EPA):** Most were GLP laboratories and some were internal company laboratories.
- **Donna Macmillan:** How were the 51 substances chosen? Was a balanced dataset considered?
 - o **Gino Scarano (EPA):** Many more chemicals were nominated than the 51 eventually selected. In some cases, it was difficult to procure the chemical, so it could not proceed in the study. Additional chemicals were dropped for reasons not recalled.
- **Joseph Manuppello (Physicians Committee for Responsible Medicine):** TSCA directs anyone submitting voluntary information to first attempt to develop that information by an alternative method identified by EPA. We have noted numerous examples of PMN submitters including the results of recent animal tests, including tests for which alternatives have been identified. How is EPA communicating this TSCA provision to chemical sponsors and reviewing compliance?
 - o **Gino Scarano (EPA):** We have communicated this in our outreach and presentations made to the public and stakeholders.
- **Anonymous:** Does OPP provide similar NAMs use information (how many are registered with or without NAMs each year) about pesticides under Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)?
 - o **Gino Scarano (EPA):** You can find the number of *in vitro* studies submitted to OPP in the last 2 years at this site (<https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/adopting-21st-century-science-methodologies-metrics>).
- **Ishita Virmani (RECETOX):** Can anyone submit a NAMs?
 - o **Gino Scarano (EPA):** Anyone with sufficient familiarity with TSCA, such as EPA, other United States federal agencies, international agencies, groups advancing alternatives to animal tests, TSCA submitters (regulated industry), consulting firms specializing in TSCA support, and companies with assays, models, and other tools seeking to repurpose and commercialize for TSCA compliance.
- **Rocky Goldsmith (EPA):** What does “Number > 650” in slide 26 refer to?
 - o **Gino Scarano (EPA):** That represents the approximate total studies as preliminary findings are gathered. For slide 26, over 650 studies have been requested for any health endpoint and the top

five requested studies are shown in the pie chart.

- **Jim Baldassari (FMC Corporation):** Does EPA recommend running OECD 442C and 442D before 442E as part of the two out of three method, or can any order be done?
 - o **Gino Scarano (EPA):** For the two out of three method, the assays can be run in any order. See the draft policy (<https://www.regulations.gov/document?D=EPA-HQ-OPP-2016-0093-0090>) for additional information.
- **Anonymous:** Related to the EPA Office of Pollution Prevention and Toxics (OPPT) receiving dermal acute studies, OPP just released a draft policy that allows for waiving these studies since they did not contribute to risk decision-making. Could this apply to TSCA chemicals? TSCA does not have specific requirements, but companies are doing these studies. Can OPPT communicate that it does not require these studies?
 - o **Gino Scarano (EPA):** Correct, there are no data requirements and EPA does not have control over what companies submit. If it is something required for a different country and it was performed, it has to be submitted under TSCA. Communication is important, but most companies know EPA does not require any studies.

State of the Science in Development of NAMs

Andreas Bender (Cambridge): Using Chemical, Biological, and In Vivo Data for NAMs: Which data do we have, and what can we do with it?

Questions following the presentation included:

- **Anonymous:** How have you concluded that the NovaScreen (NVS) assay was related to mitral valve incompetence? What was the endpoint measured in this assay?
 - o **Andreas Bender (Cambridge):** I would need to check that with the student.
- **Fatma Okuş:** Is it appropriate to understand the reliability of a tool from its database (e.g., *in silico* toxicology tool)?
 - o **Andreas Bender (Cambridge):** Yes absolutely, the underlying data is key to any prediction or analysis. I think some Applicability Domain concept is key here, i.e., understanding which say recall or positive predictive value (PPV) a certain similarity to existing datapoints gives us.
- **Anonymous:** Many of the Tox21 and ToxCast assays have activity due to activity burst? What precautions must be taken into account for the cytotoxicity?
 - o The speaker was unable to answer this question.
- **Anonymous:** How quick is it to process and interpret the omics data?
 - o **Andreas Bender (Cambridge):** Today's computers can be sufficiently quick, and there are hardly computational issues. The question is how to analyze it to see something meaningful. The intellectual question is more of a consideration than the computational.
- **Anonymous:** How did you determine the association of adverse events to the various assays of ToxCast/Tox21 (e.g., the assay NVS_NR_hER measuring ERalpha)? What was the logic behind associating it with mitral valve prolapse?
 - o The speaker was unable to answer this question.
- **Karma Fussell (Nestle):** With large datasets like omics, several groups have demonstrated the enormous effect that decisions on digital data management and analysis processes have on the final results, such that ten experts get ten different differentially expressed gene lists, or ten different interpretations of pathways affected. Knowing this and in the context of regulatory decision making where there can only be one version of "true results," how useful are gene expression NAMs?
 - o **Andreas Bender (Cambridge):** It is an important question. We need to bring people together to have an agreement among companies or other consortia to have a standard protocol. I think there can very well be signals for most endpoints we are interested in. There are times where it will be very helpful, but we need to establish those standardized protocols.
- **Dave Number (Honeywell):** Using a frequentist approach to analyzing single nucleotide polymorphisms (SNP) data sometimes lead to non-replicable results. I was wondering if you can elaborate on your Bayesian approach to gene expression data. What are you using for your prior odds?
 - o **Andreas Bender (Cambridge):** We did not do this ourselves using Bayesian methods, but you can e.g., estimate points of departure using gene expression data this way: <https://academic.oup.com/toxsci/article/176/1/236/5818890>. I think this will be a later

contribution by Unilever as well

- **Rocky Goldsmith (EPA):** Can you imagine the federated model approach such as Machine Learning Ledger Orchestration for Drug Discovery (MELLODDY) for anonymizing data between partners useful beyond pharma players (e.g., agriculture, petroleum)?
 - o **Andreas Bender (Cambridge):** What you can anticipate, and what we also generally see, is better chemical space (or generally input data) coverage, so better recall for models if you use them in this way. I think this is consistently shown, also with what Lhasa has shown from student-teacher models and the like. There are a few problems including assay conditions, which might not be compatible, and trust between partners (i.e., how do I trust other people's data quality?). My preference would be joint precompetitive data generation, otherwise, you end up with really clumped data, whether in chemistry or readout space. Of course, "many clumps are at least better than fewer clumps;" that is also true.

Kamin Johnson (Corteva): Transcriptome-Based Derivation of an In Vivo Point of Departure (POD): Current and Future Utility

Questions following the presentation included:

- **Chris Vulpe (University of Florida):** What are your thoughts about using a similar approach for ecotoxicity endpoints?
 - o **Kamin Johnson (Corteva):** The AOP concept of molecular change leading to apical effects applies to all species. In my opinion, the transcriptome approach to POD derivation can be used in any species. If interested, there are studies in fish published by others that have examined this question.
- **Anonymous:** How do you know that a gene has a treatment related change? Are there specific signatures that particular agents leave on a gene? How do we know that the cellular mechanisms for repair do not limit the expression of the gene?
 - o **Kamin Johnson (Corteva):** Statistical tools, such as the Williams trend test and the 1.5-fold change test, help identify treatment related changes. Cellular mechanisms may limit expression, but we focus on changes in bioactivity and any gene can drive that type of departure.
- **Atish Patel (FMC Corporation):** Were the same doses used to compare 24-hour transcriptomic versus 29-day apical PODs? The fold difference can also be due to dose selection.
 - o **Kamin Johnson (Corteva):** In the data I showed, the same dose levels were used for the 24-hour microarray and 29-day apical studies. Appropriate study design is important to accurately derive a POD. Data that I did not have time to show suggest that a study design with more dose groups (but smaller group sizes) is ideal.
- **Anonymous:** Did I hear that in one of the presented cases, the 90-day showed no transcriptional changes in the liver but liver tumors in the chronic study?
 - o **Kamin Johnson (Corteva):** Liver gene expression at 90-day study was null, but tumors in kidney in a 2-year study.
- **Xianglu Han (Lanxess):** I missed some [of the] presentation, so [I am] not sure if this has been addressed. If transcriptome POD is recommended as POD for risk assessment, do you recommend transcriptome data be used as the basis for Globally Harmonized System (GHS) classifications? I feel the hazards used in risk assessment and in GHS classifications should be identified in a consistent manner.
 - o **Kamin Johnson (Corteva):** In my opinion, if the regulatory paradigm requires identification of hazards for protection of human health, using transcriptome data to identify the hazard is difficult for most hazards.
- **Atish Patel (FMC Corporation):** If you show that the liver is not always a useful surrogate and that the transcriptomic POD from target organ is also sometimes $>10\times$ of apical POD, do you anticipate that a $10\times$ UF will then be applied to the transcriptomic POD, thus impacting how the molecule is in turn used? What work is being done to correlate apical to transcriptomic PODs to gain further confidence in this method to reduce the uncertainty? If a $10\times$ UF is applied to a transcriptomic POD, how different is this to using duration based UFs ($6\times$ versus $3\times$) to extrapolate from short-term (28-90 days) studies where we can still get some target organ or apical toxicity information?
 - o **Anna Lowit (EPA):** As a risk assessor, we acknowledge the need for considering uncertainty factors and such this issue will be important. The first thing we must do is the science. When we think about policy considerations before we establish the science, it ties us in knots. We do not know how

applying uncertainty factors or extrapolation factors will look. We want to work with stakeholders on the science, create case studies, and consider options.

- **Anonymous:** What do you think are the next steps to encouraging this type of data to be generated and accepted as a surrogate to conducting long-term rodent bioassays?
 - o **Kamin Johnson (Corteva):** I think there are a few major questions that need to be addressed: 1) what is the appropriate study design in terms of dose level selection, group sizes, group spacing, and derivation of the transcriptome POD; 2) is linking the gene expression data to a mechanism required or is a “bioactivity”-based POD sufficient; and 3) how flexible is the current regulatory system to change that may not specifically identify a hazard (e.g., in a risk-based system, does labeling a molecule as a carcinogen or a reproductive toxicant actually protect human health?).
- **Anonymous:** How often are you not able to accurately predict (no more than a 3× fold difference) the animal POD using the transcriptome POD (i.e., what is the rate of false negative data)?
 - o **Kamin Johnson (Corteva):** For the molecules we have examined to date, about 75% of these have a transcriptome POD within 3× of the apical POD. My hypothesis is that the lack of concordance may be more due to inherent error (lack of exact reproducibility) in estimating the “true” apical or transcriptome POD.
- **Anonymous:** Do you think this approach could be applied to reproductive and developmental toxicity? What are the first steps we could take in this field?
 - o **Kamin Johnson (Corteva):** Yes, I think it can be applied to any toxicity. We have data which I did not show that this approach works for a developmental toxicant acting via aromatase inhibition.
- **James Jacobus (Minnesota Department of Health):** Have you analyzed the testis data in more detail to understand why this cancer study POD was overshoot by 20-fold using the transcriptome POD approach?
 - o **Kamin Johnson (Corteva):** We have not in detail. It may be that the initiating for the toxicity is not the testis but another organ, which we did not examine. It may be the higher dose levels used in the transcriptome study compared to the cancer bioassay were not ideal to generate the most accurate transcriptome POD. These are hypotheses that require additional studies to answer.
- **Karma Fussell (Nestle):** Has the Health and Environmental Sciences Institute (HESI) team established a harmonized workflow for data analysis to determine the POD by each member?
 - o **Kamin Johnson (Corteva):** This is an important question we are working on that is a long-term goal. There are methods in literature for a harmonized workflow. For regulatory use, we need a consensus on what methods to use.
- **Mark Jankowki (EPA):** Can you comment on when/if proteomics should be used in concert with transcriptomics? Do you see proteomics as promising as well?
 - o **Kamin Johnson (Corteva):** We have only examined transcriptomic data in our studies. Other types of omics (proteomics; lipidomics; metabolomics) may also be informative. We have not examined any of these other data types. Proteomics may be more technically challenging than transcriptomics. Proteomics are noisier and more expensive, but they add to the quality of the predictions if combined with transcriptomics. Alone, predictions with transcriptomics are more robust than predictions with proteomics.
- **Chris Vulpe (University of Florida):** Have you tried using these approaches on CRISPR screens? There are large databases of CRISPR data for example at Broad or Sanger.
 - o **Kamin Johnson (Corteva):** Yes, these are great datasets to integrate. We created gene set libraries from this data for Enrichr. We also use these data to validate predictions, and to prioritize drugs.

Avi Ma'ayan (Mount Sinai): Drugmonizome and Drugmonizome-ML: Integration and Abstraction of Small Molecule Attributes for Drug Set Enrichment Analysis and Machine Learning

Questions following the presentation included:

- **Logan Everett (EPA):** For All RNA-seq and CHIP-seq Sample Search Space (ARCHS4), has there been any effort to clean up the annotations for these data sets that came from Gene Expression Omnibus (GEO)? In my experience, GEO has many miRNA-seq and ChIP-seq studies misannotated as traditional RNA-seq, and I have seen these pulled into past versions of ARCHS4. These seem likely to inflate gene-gene global correlations in the repository.
 - o **Avi Ma'ayan (Mount Sinai):** The best effort that I am aware of is MetaSRA <https://metasra.biostat.wisc.edu/>. We do some filtering, so not all studies are included in the gene-

gene correlation matrices.

- **Patricia Ruiz (Centers for Disease Control and Prevention [CDC]):** How these tools compare to commercially available systems biology tools, e.g., Ingenuity, MetaCore?
 - o **Avi Ma'ayan (Mount Sinai):** Since these tools are beginning to become more mature and have been widely used in Pharma, as Novartis and Johnson & Johnson, showing they are competitive with those commercial options. These are open and free as well.
- **William Irwin:** Is there an association with cholesterol lowering drugs and poorer outcomes for Coronavirus Disease 2019 (COVID-19) cases?
 - o **Avi Ma'ayan (Mount Sinai):** There are mixed reports, but there is strong evidence that this pathway is central for the virus life cycle.
- **Patricia Ruiz (CDC):** Could you expand on current chemical space available at the tools presented (e.g., environmental chemicals)?
 - o **Avi Ma'ayan (Mount Sinai):** We just looked at it between the Library of Integrated Network-Based Cellular Signatures (LINCS) L1000 data and about 400 of them overlap with the Tox21 compounds. We look forward to future data being analyzed in this way.
- **Annie Jarabek (EPA):** How is curation documented?
 - o **Avi Ma'ayan (Mount Sinai):** The curation involves Extract, Transform, Load (ETL) processes of taking data from a public database and transforming it into an abstract representation. These are documented as Jupyter notebooks on GitHub and now also as ETL Apyters:
<https://appyters.maayanlab.cloud/#/?q=ETL>.
- **Anonymous:** You have demonstrated workflows using a variety of technologies. Do you think the regulatory use and transformation of this data should be used and adapted into agile workflows on the fly? Or should we be building large infrastructures as SaaS. In research mode, one discovers and applies and publishes, but in regulatory mode, we try to build elaborate frameworks. How should we focus our efforts? [Should we] make modular workflow tools and draw assumptions with one-off questions when they arise, or build complex models that predict endpoints that never end?
 - o **Avi Ma'ayan (Mount Sinai):** I think you still want to have an elaborate framework that is considered the state-of-the-art pipeline and is continually improving, but I am not familiar enough with the domain to determine with certainty.

Ivan Rusyn (Texas A&M): "Fit for Purpose" for Organotypic Models in Environmental Health Protection

Questions following the presentation included:

- **Karma Fussell (Nestle):** Have toxicological mechanistic data collected using organotypic models become reproducible enough that they can be used as part of standardized regulatory safety testing?
 - o **Ivan Rusyn (Texas A&M):** That is a good question. Currently they are not very reproducible to use for regulatory safety, but if standards are applied, they can transform the field.
- **Rocky Goldsmith (EPA):** We create organotypic organ-on-a-chip solutions, and leave the "whole" system, how will we "apply" these tools to mixtures that do not reach the target tissues being assayed at the same relative levels as they would have through biology? This also specifically hits hard on racemic mixtures. Take a technical grade pesticide with specific stereoisomeric ratios; their relative abundance on a tissue-on-a-chip will not be "biologically-relevant." How do you anticipate this could be addressed sooner than later (i.e., estimates at target tissues relevant for emulating mixture) so that we do not continue to generate cloudy data fraught with misrepresentation of isomeric ratios. How do we make organotypically relevant concentrations for mixtures (modeling)?
 - o **Ivan Rusyn (Texas A&M):** The mixtures challenge runs first into the throughput challenge. I am personally less concerned about "like human" than throughput.
- **Rocky Goldsmith (EPA):** Just because these assays, primarily designed for pharma, could be used to assay, does not mean that in unique chemical space areas (i.e., heavily halogenated) they will still be amenable to that assay. How have partners addressed that problem early in the context of non-pharma chemical space, for each of the organotypic "end-points"?
 - o **Ivan Rusyn (Texas A&M):** The challenge is not drug versus non-drug, but how can one use it with the diversity of chemicals and concentrations. I do not believe that current tissue chips are only for pharma. Most I have seen are not "disease model" type, so they can be used. I have yet to see a model that is reasonable throughput/cost and availability to be applicable to EPA.

- **Jennifer McPartland (EPA):** What has been gleaned thus far in evaluating “tissue on a chip/microphysiological” systems alongside other more typical *in vitro* cell-based reporter assays, transcriptomics, etc.? If this type of evaluation is not yet underway, are the plans to do some comparative analyses along these lines? (I appreciate this is a complicated question.)
 - o **Ivan Rusyn (Texas A&M):** Most comparisons are needed in the first step. If someone publishes a model, the question is whether that will then work in another lab, not necessarily including gene expression. Their publications in that space provide a direct comparison. There is not convincing evidence that we need more complicated models. We must be ready to test thousands of compounds. The throughput can become a showstopper for elegant models.
- **Rob DeWoskin (etiologic):** Are you seeing increasing regulatory acceptance of NAMs that are validated against human data rather than animal data? Are these comparisons with human data becoming more of a trend?
 - o **Ivan Rusyn (Texas A&M):** There is little “regulatory acceptance” to begin with at the moment, in my opinion. However, both regulators and the industry repeatedly point out that a comparison to animal data is most straightforward and desired to understand the “value proposition” of NAMs for decision-making.
- **Satinder Sarang (Shell):** How does metabolism compare to traditional cell-lines, do you see a change in metabolism over time? How well does the effective concentration(s) *in vivo* compare to actual effective dose in organotypic cultures for pharmaceutical-like chemistries and petrochemicals?
 - o **Ivan Rusyn (Texas A&M):** It depends on the cell type, and there are several liver tissue chip models that have demonstrated metabolism over 14-28 days in culture. I have no data on the latter question.
- **Annie Jarabek (EPA):** When you say the Agency needs to articulate what it needs, do you mean specific endpoints in target systems?
 - o **Ivan Rusyn (Texas A&M):** There are two points: (1) the Agency would be best served articulating what “-icities” are a priority so that NAMs developers understand what needs to be “replaced”, and (2) for chemicals where there are data gaps, it may be good to articulate what the most impactful gaps are.

Addressing Current Limitations in NAMs

Chad Deisenroth (EPA): Retrofitting an Estrogen Receptor Transactivation Assay with Metabolic Competence

Questions following the presentation included:

- **Jay Petrick (Bayer):** Why were Phase II cofactors excluded?
 - o **Chad Deisenroth (EPA):** There's certainly an interest to include them. It was a hurdle to optimize Phase I cofactors without negatively affecting cell viability. We considered this an acceptable first step, but [I] agree that Phase II cofactors eventually need to be included.
- **Atish Patel (FMC Corporation):** How do you account for solubility differences in +/-S9 conditions and how does it impact the interpretation of the results?
 - o **Chad Deisenroth (EPA):** The expectation is that the S9 is already solubilized when put in the alginate. Then, alginate gets cross linked inside a matrix. Our real concern is diffusion of compounds across the barrier. Our analysis shows some passive diffusion of chemicals to feed metabolites produced. No issues with solubility have been observed.
- **Anonymous:** As Dr. Chad Deisenroth explained it well, the *in vitro* assay sometimes may produce the best results because the complications of S9 and bioactivation in these cell assay systems.
- **Anonymous:** Have these studies been conducted using human S9?
 - o **Chad Deisenroth (EPA):** The initial development was with human S9. Unfortunately, when applying it to guideline estrogen receptor (ER) assays, we did find some estrogenic effects in the S9, so we could not apply it to this specific assay.
- **David Saltmiras (Bayer):** For the activated metabolites, are you aware of any data for *in vivo* half-lives?
 - o **Chad Deisenroth (EPA):** To some degree, we are making hazard identification with only bioactivity. We do not know what mixture of metabolites is produced from the parent compound. The identity of the metabolites is somewhat unknown, but the literature helps us have a decent idea.
- **Anonymous:** Does the S9 system include sulfation and glucuronidation?

- The speaker was unable to answer this question.
- **Paul Carmichael (Unilever):** Could you comment further regarding the utility of this very neat too beyond the ER transactivation assay?
 - **Chad Deisenroth (EPA):** The applications should work on any assay within this microplate well format. His lab focuses on endocrine related assays, but it is still relevant to tier I assays.
- **Susan Borghoff (ToxStrategies):** Is it true that glucuronidation or sulfation enzymes, cofactors are no in the S9 system but are major metabolic systems involved with genistein and BPA that would decrease their bioactivity. Are you discussing how to ensure Phase 2 enzymes are incorporated?
 - **Chad Deisenroth (EPA):** Yes, the lack of phase II activity is a noted limitation of the current Alginate Immobilization of Metabolic Enzymes (AIME) method and likely impacts the bioactivity of some chemicals. We are actively working on approaches to include phase II activity
- **Martin Phillips (EPA):** Any plan to apply the same metabolic system approach to the Attagene assays so we can see the effects on the transcriptome?
 - The speaker was unable to answer this question.
- **Paul Carmichael (Unilever):** Are there any plans to ensure broader adoption (e.g., in bioactivation/detoxification in POD setting with high-throughput transcriptomics)?
 - **Chad Deisenroth (EPA):** We are working on promoting adoption of the method by Cross-Origin Resource Sharing (CROs), but [we] have not addressed application specifically to the Attagene assays.
- **Sayak Mukherjee (Battelle):** How are you relating the *in vitro* concentration of primary and secondary metabolites to the *in vivo* concentrations that one might see?
 - **Chad Deisenroth (EPA):** They are not related at this time, but [it] is an important point for risk characterization.
- **Helen Tinwell (Bayer):** Have you applied this method to other *in vitro* assays (e.g., H295R steroidogenesis assay)?
 - **Chad Deisenroth (EPA):** Not yet, but we have our sights set on other endocrine assays, particularly steroidogenesis.
- **Anonymous:** Could you elaborate more on the challenges to transfer the AIME method to the bioprinting?
 - **Chad Deisenroth (EPA):** We are in the early stages of the project, but are actively evaluating hydrogel and crosslinking options, speed, and pressure of dispensing, and technical variability in standard microplates.
- **William Irwin:** What is the maximum S9 exposure time that can be used (i.e., is 2 hours long enough)?
 - **Chad Deisenroth (EPA):** We have evaluated out to 8 hours, but activity of S9 is quickly lost post-thaw. Metabolic profiling experiments are classically run for 30-60 minutes, so 2 hours was a reasonable time point for efficiency. This likely is not applicable to all chemicals, particularly those with slower reaction kinetics.
- **Christopher Choi (Takasago International Corporation):** I understand the use of a cell-line to artificially express the receptors to evaluate the binding potential, however, there is a disconnect as these receptors are not endogenously expressed. How can we go about filling in the gaps in our understanding the usefulness of these assays?
 - **Chad Deisenroth (EPA):** The VM7Luc4E2 cell line uses endogenous ER to transactivate artificial response elements. There is a case to be made for biological relevance, as far as that argument stretches for engineered, transformed cell lines, but the high throughout is the primary point of utility. The application of orthogonal assays with cellular and structural complexity of the native tissue and organ would perhaps be the better way to address biological relevance and toxicodynamics.
- **Anonymous:** How does the mRNA direct method compare to the liver on the stick method for predicting bioactivation?
 - **Chad Deisenroth (EPA):** Good question. The formal analysis on the comparison between the two methods has not been conducted. The effects are more mixed in the mRNA liver mix transfection where the kinetics of parent and metabolite binding are likely overlapping to a greater extent than the AIME method.
 - **Christopher Choi (Takasago International Corporation):** i.e., just because it binds, does not always mean that it will result in downstream activities (e.g., G-coupled protein activities).

- **Nicole Kleinstreuer (NIH):** Regarding the reliability of the Tox21 assays, as integrated into the ER pathway model, this model has been validated against the regulatory reference standard of the guideline uterotrophic assay and found to be robust, predictive, and reliable.
- **Anonymous:** I am wondering if there were controls run to determine potential background activity associated with S9. In other projects examining sequential or co-incubations of S9 and ER CALUX, the S9 was reported to have some innate activity.
 - o **Chad Deisenroth (EPA):** Indeed, this was the case with human S9 fractions where there was considerable intrinsic estrogenic activity. The background ER activity for rat S9 was captured in the negative solvent controls run on each plate.
- **Shoba Iyer (CalEPA):** What are the advantages of retrofitting immortalized cells with metabolic capacity as compared to testing chemicals in primary cell cultures instead?
 - o **Chad Deisenroth (EPA):** Primarily the speed and throughput, but legacy data and acceptance of specific guideline *in vitro* assays also plays a role. Primary cells are also a good option, but [they] present their own challenges and domains of applicability for screening.
- **Barbara Losey:** For all speakers: Can you provide a list of citations on the NAMs website of publications related to the work presented here today?
 - o **David Crizer (National Toxicology Program [NTP]):** Chad Deisenroth, Danica E DeGroot, Todd Zurlinden, Andrew Eicher, James McCord, Mi-Young Lee, Paul Carmichael, Russell S Thomas, The AIME Platform Retrofits an Estrogen Receptor Transactivation Assay with Metabolic Competence, Toxicological Sciences, kfaa147, <https://doi.org/10.1093/toxsci/kfaa147>

David Crizer (NTP): In Vitro Disposition of Tox21 Chemicals: Initial Results and Next Steps

Questions following the presentation included:

- **Anonymous:** Are the NTP methods validated and GLP quality?
 - o **David Crizer (NTP):** They are not to that extent.
- **Martin Phillips (EPA):** Have you considered using nuclear magnetic resonance (NMR) to quantitate using stable-labeled compounds versus radiolabeled ones? Could increase the number of compounds you can “ground-truth.”
 - o **David Crizer (NTP):** That is not something we have considered at this point, but that is a good point.
- **Mark Jankowski (EPA):** Are you counting (flow cytometry?) the number of cells to calculate the total volume of cells for determining chemical concentration in cells?
 - o **David Crizer (NTP):** I am not sure how Josh Harold did that. We would have to reach out to him to see how he exactly tackled that.
- **Rocky Goldsmith (EPA):** For the chemistries you have looked at, are there specific physicochemical parameters or molecular descriptors, or fingerprints that suggest one could identify chemicals in “well-behaved” or “poorly behaved” chemistries in the *in vitro* context? Molecular features or properties that could suggest assay amenability and flags?
 - o **David Crizer (NTP):** We have not gotten to that point yet. We are focusing on really nailing down the analytical work first before we dig into those trends into what properties matter and how chemicals partition. This is something that we plan to dig further into in the next pilot and hope to publish that larger study.
- **William Irwin:** What is the effect of xenobiotic efflux pumps, which can be upregulated?
 - o **David Crizer (NTP):** We do not have enough data at this point to comment on that, which is why we are going to a larger pilot with more concentrations.
- **Anonymous:** Why wouldn't there be binding at the bottom of the wells?
 - o **David Crizer (NTP):** These MCF7 Cells are confluent at the bottom of the wells, and it is also 100% covered, or at least close to 100% covered. The chemical is not going to move through the cells and then into the plastic, so we feel good with that assumption.
- **Xianglu Han (Lanxess):** Has the EPA or NTP considered developing an *in vitro* system to account for metabolism of chemicals by the human gut microbiome? For oral exposure of chemicals, this is upstream of other steps, such as tissue absorption of chemicals. Ideally, in many cases, the gut microbiome metabolites should be used for other *in vitro* systems to study chemical toxicity.
 - o **David Crizer (NTP):** I am currently not aware of ongoing work on this at this time. I do however know that there is certainly interest in this area.

- **Sayak Mukherjee (Battelle):** Does your work validate Armitage model? Do you expect to see departures from Armitage model and why?
 - o **David Crizer (NTP):** So far this seems like there is good agreement, but we do think there are other things out in the literature that are important factors that are not included in the Armitage model. That is why we were trying to see if it is possible to develop a more involved model that considers physicochemical properties.
- **Costanza Rovida (Center for Alternatives to Animal Testing Europe [CAAT-EU]):** Have you considered intra-cellular distribution of chemicals?
 - o **David Crizer (NTP):** Currently we are not looking at intra-cellular distribution of chemicals.
- **Natalia Ryan (Syngenta):** What are the existing quality control (QC) grades for the 200 chemicals? Will your work have any impact on explaining QC grades, or improving the grades in the future?
 - o **David Crizer (NTP):** For all the 200 chemicals that I have checked they passed analytical QC based on the scoring in Tox21 database. Most of which have a QC grade of A. At this time, it is not expected that this work will have any impact on QC grades.

Developing Scientific Confidence in NAMs

Clemens Wittwehr (Joint Research Centre [JRC]): An OECD Harmonized Template (OHT) to Report NAM Results in Regulatory Environments: Principles and Practical Use

Questions following the presentation included:

- **Patrick Phibbs:** For OPP and OPPT speakers: Assuming 201 is approved in November 2020, how long would it take for the EPA offices to decide whether they will accept data presented in that format? How do they let regulated parties know?
 - o The speaker was unable to answer this question.
- **Anonymous:** This approach feels very human-centric. How do you envision this applying to ecological applications for AOP? Should species domain be included in the OHT?
 - o **Clemens Wittwehr (JRC):** It is neither human nor ecologically centric. It is just focused on NAMs methods. You should not link mechanistic data to be siloed in one area or another.
- **Karma Fussell (Nestle):** Is there any plan to either amend the OECD TG guidance with the standardized OHT 201 templates or have them co-located with the TG on the OECD website?
 - o **Clemens Wittwehr (JRC):** OECD needs to develop guidance on when to follow which test guideline.
- **Anonymous:** How are dose response dependency in induction of mechanistic effects and thresholds, and toxicokinetics taken into account? Is there simply an inference that any response, irrespective of dose or toxicokinetics is linked to an adverse outcome?
 - o **Clemens Wittwehr (JRC):** For regulatory (validated methods) dose response information from a test item/chemical will be compared with a reference item that gives a known response in the method. Only when the measured response is above a certain limit a test item would be considered 'positive' for a key event. In case of non-validated method, a test item could be considered positive (e.g., when the response is significantly higher than the background). Depending on the metabolic competence of test system used in the method toxicokinetics can be taken into account as well. Most *in vitro* test systems do not have metabolic competence, in which case toxicokinetics information must be obtained elsewhere. The template asks for information on how the result is obtained (data calculation and statistics and evaluation/data interpretation criteria) and for the metabolic competence of the test system. This should enable the recipient of the data to judge in how far the results confirm the mode of action of the test item and its contribution to an adverse outcome.
- **Carol Marchant (Lhasa Limited):** Is the ontology linking to OHT 201 already in place or is this work to be done?
 - o **Clemens Wittwehr (JRC):** This is already complete, and the ontology terms are selected.
- **Barbara Birk (BASF):** How is Good *In Vitro* Method Practices (GIVIMP) included in the OHT Process?
 - o **Clemens Wittwehr (JRC):** GIVIMP and OHT 201 are inseparable. We were working closely together so that if you want to report data under the paradigm, you could use OHT 201.
- **Yadvinder Bhuller (Health Canada):** Is the OHT 201 also designed for *in silico* approaches (e.g., read across, QSAR)?
 - o **Clemens Wittwehr (JRC):** Yes, it is technology independent. However, since we know *in vitro* is so

popular, we have added elements to help with that technology specifically.

- **Ravi Menon (Afton Chemical):** Have you looked at using OHT 201 for reporting QSAR results, for predicting bioconcentration factors (BCF) in fish for example?
 - o **Clemens Wittwehr (JRC):** OHT 201 is technology independent. Because most NAMs are *in vitro*, we have added elements to the template to help with *in vitro* methods specifically. However, you can report QSAR results with OHT 201 by using free texts and attachments. Do not forget that OHTs are updated every year – so if the need to accommodate QSARs in a more comfortable way increases, these needs will be taken into account.
- **Anne Gourmelon (OECD):** How does OHT 201 match with the OECD Guidance Doc 211 on the description of non-standard guidelines?
 - o **Clemens Wittwehr (JRC):** OECD 211 is about the method description (e.g., what you must do when executing the method). This relates to the triangle of chemical safety. The OECD 211 document relates to how you should describe the method in right upper angle of triangle, while OHT is represented in left upper hand triangle.
- **Kellie Fay (EPA):** How do you resolve user-selected ontology terms (process, action, or object) that are the same but stem from different ontologies? I believe this is an issue with the AOP wiki.
 - o **Clemens Wittwehr (JRC):** That is exactly what we are trying to address right now. [It is] challenging, but crucial.
 - o **Kellie Fay (EPA):** I would love to hear your ideas on how to resolve this issue at some point. I believe there are some tools which map across ontologies, but [I am] unsure how good they are.

Monique Perron (EPA): Case Study #1: Integration of NAM Data for Evaluating Potential Developmental Neurotoxicity

Questions following the presentation included:

- **Anonymous:** Where can we find the number of pesticides registered with the EPA per year?
 - o **Monique Perron (EPA):** Information about pesticide registration can be found at: <https://www.epa.gov/pesticide-registration/about-pesticide-registration>.
- **Francesca Pistollato:** What about times of exposure in these *in vitro* assays?
 - o **Monique Perron (EPA):** The microelectrode array network formation assay (MEA NFA) is exposed to chemicals from day 0-12 with measurements taken days 5-12 to recapitulate neuronal network formation. For the HCl suite, exposure times vary by specific assays ranging from approximately 24 hours to 5 days.
- **Katy Wolton (Syngenta):** Did you learn anything about the mode of action (MoA) of the organophosphates (OPs) using the NAMs batteries? Could you expand on this?
 - o **Monique Perron (EPA):** Currently we still do not know what the MoA is for any potential developmental neurotoxicity (DNT) effects. The results observed in these assays were chemical specific, so you did not see a trend necessarily for the disruption of certain critical processes over others. As such, the results appear to be chemical dependent.
- **Anonymous:** Why do some of the most toxic OPs in humans (phorate, etc.) not appear to have a human-computer interaction (HCI) signal?
 - o **Monique Perron (EPA):** Phorate was positive in some of the HCI assays with effects on rat synaptogenesis/maturation assay endpoints. However, I would note that when referring to the human toxicity of OPs typically one is referring to the neurotoxic effects related to acetylcholinesterase inhibition. The MoA(s) for the developmental neurotoxicity outcomes have not been established.
- **Rocky Goldsmith (EPA):** How have these methods changed the way you establish common mechanism groups, if at all?
 - o **Monique Perron (EPA):** To date, these methods have not changed the way we establish the common mechanism group.
- **William Irwin:** Any mitochondrial tox assays, they have good concordance for developmental toxicity at least?
 - o **Monique Perron (EPA):** The OPs tested have data in other ToxCast assays (e.g., for mitochondrial toxicity and developmental toxicity) and some potency comparisons have been performed; however, additional examination of the responses across other assay targets would need to be performed. For this case study, we focused only on the DNT NAMs results.

- **Afolarin Ogungbemi (Helmholtz-Centre for Environmental Research):** Based on your data, do you see a difference between the effect or action of oxon metabolites and parent compound of OPs?
 - o **Monique Perron (EPA):** There was no clear global pattern of effect on the basis of oxon/non-oxon with respect to DNT NAMs bioactivity; however, you can find discussion of the results obtained for each oxon/parent pair (when available) in EPA's issue paper that was presented to the FIFRA Scientific Advisory Panel (SAP) in September 2020: <https://www.epa.gov/sap/fifra-scientific-advisory-panel-meetings>.

Andrew White (Unilever): Case Study #2: Integration of NAM Data in a Next Generation Risk Assessment for Cosmetic Ingredients

Questions following the presentation included:

- **Paul Carmichael (Unilever):** What do you see as the major (surmountable) shortcomings of the approach? What work is underway to address these?
 - o The speaker was unable to answer this question.
- **Anonymous:** Given differences in POD from different cell lines, could you tell me your criteria for selecting the cell lines you used?
 - o **Andrew White (Unilever):** Some cell lines were chosen so that they can be used as a benchmark against other work being done. Some cells lines were chosen for their ability to indicate metabolism.
- **Scott Jenkins (EPA):** Even though the *in vitro* PODs are higher than the steady state concentration prediction, how do you think about the fact that users of the face cream could be exposed for months to years while the *in vitro* data is based on several hours of exposure?
 - o **Andrew White (Unilever):** Good question, there is still additional work to define how relevant single dose short timescale data is to longer term exposures. Two things help provide some support we compared to a steady state repeat dose scenario in the Physiologically Based Pharmacokinetic (PBPK) model. Also, there is now the Accelerating the Pace of Chemical Risk Assessments (APRCA) publication that indicates for 90% of compounds assessed the *in vitro* data was conservative compared to the repeat dose test. This does not mean we are satisfied and expect to utilize more organotypic models with repeat dose in the future where necessary
- **Anonymous:** In doing risk assessment, would you combine existing/old animal data together with *in vitro/in silico* data exclusively?
 - o The speaker was unable to answer this question.
- **James Dawick (Innospec Inc.):** Did you include any *in vitro* assays to look at reproductive or developmental toxicity of coumarin as part of this Next-Generation Risk Assessment (NGRA)?
 - o **Andrew White (Unilever):** Not as part of this study, [and] we realize this is a gap for developmental and reproductive toxicology (DART) that needs developing further.
- **Katya Tsaïoun (Johns Hopkins Bloomberg School of Public Health)** Is there a definition of weight of evidence that the EPA is using? I am seeing a variety of ways different groups use the term.
 - o **Andrew White (Unilever):** There are questions outstanding on extrapolation of short-term assays to long-term *in vivo* exposures and the biological coverage on these. Some of these have ongoing activities to map cell lines and biological space, also some additional tier 2 lower throughput organotypic assays will aid in refining the exposure extrapolation.
- **Anonymous:** How can you be confident that the set of tests you choose provides an adequate biological coverage?
 - o **Andrew White (Unilever):** There is still further work ongoing to help provide more evidence and confidence for this across a larger range of compounds. The question of biological coverage and what is sufficient is still being addressed not just by us but across different groups. Also, we do not think this set is absolute and will be added to either due to identified concerns or where additional tools will provide added value.

Todd Stedeford (EPA): Case Study #3: Incorporating the Threshold of Toxicological Concern into Regulatory Decisions under the Amended Toxic Substances Control Act

Questions following the presentation included:

- **James Jacobus (Minnesota Department of Health):** Since you are using an exposure-based approach are you taking into account how additional exposure routes such as diet increase daily exposure totals for an

individual?

- **Todd Stedeford (EPA):** EPA/OPPT has not implemented the Threshold of Toxicological Concern (TTC) approach for its risk assessments. The presentation was an overview of the TTC approach and its potential applications under TSCA.
- **Anonymous:** How do you define “a scientifically validated test method?” Is TTC used in TSCA high priority substance (HPS) regulation?
 - **Todd Stedeford (EPA):** We have not used it yet; I was providing some ideas about where it could be used. This is something that we are going to investigate through that collaboration and see if it really fits for purpose NAMs that we can use under new chemicals or existing chemicals.
- **Anonymous:** How do you define “a scientifically validated test method?” Is there any TTC case study for TSCA HPS regulation?
 - **Todd Stedeford (EPA):** TSCA Section 4(h)(2)(C) requires EPA to develop a list of alternative test methods or strategies that are “scientifically reliable, relevant, and capable of providing information of equivalent or better scientific reliability and quality to that which would be obtained from vertebrate animal testing.” EPA is currently developing criteria that will be used for identifying NAMs for placement on the list. EPA/OPPT has not developed a TTC case study for a chemical substance identified as a high-priority substance.
- **Jennifer McPartland (EDF):** Where can we get more information about the 2020 collaboration focused on TTC under TSCA?
 - **Todd Stedeford (EPA):** That is all the information that is available now is what I stated. It is really going to be a collaboration of external partners also relying on our experts in the office of research and development.
- **Ravi Menon (Afton Chemical):** Do you recommend use of EPA tools such as the Chemical Screening Tool for Exposures and Environmental Releases (ChemSTEER) and the Exposure and Fate Assessment Screening Tool (E-FAST) for exposure/release and risk assessment for PMNs? Are there plans to update these tools?
 - **Todd Stedeford (EPA):** EPA/OPPT routinely uses ChemSTEER and E-FAST to estimate exposures and releases. These tools are updated periodically. Updated tools and models are available at the following: <https://www.epa.gov/tsca-screening-tools/using-predictive-methods-assess-exposure-and-fate-under-tsca#fate>.
- **Helen Goeden (Minnesota Department of Health):** The U.S. Food and Drug Administration (FDA) has been working on a major 'upgrading' of TTC for several years. Is EPA aware of their efforts?
 - **Todd Stedeford (EPA):** Yes, EPA/OPPT is aware of these efforts.
- **Rocky Goldsmith (EPA):** Is there a way to capture TTC in QSAR, such as Quantitative Structure Threshold of Toxicological Concern Relationships? Can a structural basis be developed?
 - **Todd Stedeford (EPA):** EPA/OPPT has not explored this possibility.
- **Jennifer McPartland (EDF):** Has the collaboration not yet started?
 - **Todd Stedeford (EPA):** It has not started yet, but we anticipate a kickoff later this month.
- **Ravi Menon (Afton Chemical):** Can the TTC approach be integrated with EPA tools such as ChemSTEER?
 - **Todd Stedeford (EPA):** ChemSTEER generates screening-level estimates for environmental releases of and worker exposures to a chemical manufactured and used in industrial and commercial operations (i.e., workplaces). These exposure estimates are integrated with hazard information in the health or ecological risk assessment. EPA/OPPT has not yet explored integrating the TTC approach into ChemSTEER or into the risk assessments.
- **Karma Fussell (Nestle):** Has EPA considered using *in vitro* NAMs to navigate the TTC decision tree?
 - **Todd Stedeford (EPA):** EPA/OPPT has not explored this possibility.
- **Jennifer McPartland (EDF):** As a follow up, is there a way we can obtain more information about how folks might be able to participate in the TTC collaboration in advance of the meeting scheduled for next month?
 - **Todd Stedeford (EPA):** EPA/OPPT provided follow-up information to this request after the meeting.
- **Gianluca Selvestrel (Mario Negri Institute for Pharmacological Research):** On the last presentation slide, which [was] the reference for ecological TTC and subcategorization into “MoA-like” substances (HESI project)?
 - **Todd Stedeford (EPA):** Slide note 2 from the last slide was, “HESI, Animal Alternatives in Environmental Risk Assessment Committee, available at: <https://hesiglobal.org/animal-alternatives-in-environmental-risk-assessment/>.”