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Progress in High-throughput *In Vitro* Toxicity Testing

By Robyn Prueitt, Ph.D., DABT

Many scientists are exploring ways to assess chemical hazards using innovative in vitro techniques that could one day replace traditional animal toxicology studies.

Over the last decade or so, there has been increasing recognition in the toxicology community of the need for less expensive, faster, and more robust approaches to the evaluation of chemical safety. Technological advances in molecular and systems biology have provided powerful new tools to probe how chemicals interact with biological systems. These methods are increasingly *in vitro*, which is Latin for "in glass," meaning in a test tube or petri dish rather than in the normal biological context of a whole animal. High-throughput *in vitro* screening techniques are now routinely used to study the patterns of responses of genes and protein pathways induced by chemical exposure that might be predictive of adverse health effects in humans. Chemicals can simultaneously interact with a single pathway or multiple pathways through different levels of biological organization (see figure), and deciphering the consequences of perturbations in these pathways can provide insight into a chemical's mechanism of toxic action.

…the U.S. EPA's Tox21 program will likely take decades to fully achieve its goals…

The *in vitro* techniques have many advantages compared to traditional animal toxicology testing, such as the use of no animals, more rapid results, a greater throughput for evaluating a large number of chemicals, lower cost, and the ability

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I N S I D E

Letter from the Editors

May 2015

Dear Colleague,

In this issue of *Trends,* we look at recent progress related to the development and application of *in vitro* toxicity testing methods. Given their speed and lower cost, *in vitro* techniques are increasingly being utilized as an alternative to traditional animal testing. However, limitations and uncertainties still remain, and *in vitro* systems can not yet fully replace the use of animals in biomedical research.

In the first article, we discuss high-throughput, *in vitro* screening techniques and key advantages and challenges compared to traditional animal toxicology testing. The second article examines the utility of *in vitro* testing for identifying chemical hazards and underlying causes in humans. Our third article addresses the potential power of *in vitro* toxicity testing for human health risk assessment, focusing on the need for careful study design and interpretation.

Gradient contributors to this issue include Dr. Robyn Prueitt, DABT, Dr. Lorenz Rhomberg, ATS, and Dr. Lisa Bailey. Joining us with a guest editorial on the harmonization of *in vitro* and *in vivo* findings for engineered nanomaterials is Dr. Philip Demokritou, a professor at the Center for Nanotechnology and Nanotoxicology at the Harvard T.H. Chan School of Public Health. Joining him is Dr. Joel Cohen, a toxicologist at Gradient.

We hope that this issue of *Trends* provides you with a better understanding of this topic.

Yours truly,

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Progress in High-throughput *In Vitro* Toxicity Testing

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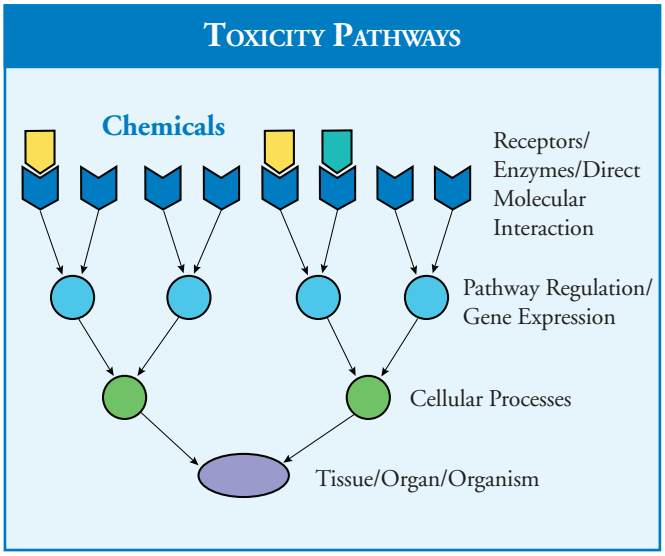
to measure potential health effects at relevant exposure levels for humans rather than the relatively high doses used in animal studies. Because of this, government and regulatory agencies are shifting the assessment of chemical hazards away from traditional animal toxicology studies toward mechanism-based biological observations largely obtained using the new technologies. To this end, in 2008, a collaboration of multiple federal agencies started a program called "Toxicology in the 21st Century" (known informally as Tox21). Tox21 is using high-throughput *in vitro* assays to screen 10,000 chemicals of public health relevance (based on their production, use, or exposure patterns) for potential toxicity to develop a cost-effective approach for prioritizing chemicals in need of toxicity testing.

Although Tox21 has made progress in its initial stages of developing assays and screening a subset of chemicals, there are several challenges in bridging the results from *in vitro* assays to the prediction of adverse health effects in a whole organism. One major issue is the lack of interacting systems *in vitro*, such as a circulatory system to deliver a chemical to different organs, or a metabolizing system such as the liver to transform chemicals into either more or less toxic forms. Because of this issue, the comparison of the dose of a chemical *in vitro* to that in a whole animal is not straightforward. Another challenge with *in vitro* assays is identifying when a chemical-induced perturbation to a gene or a pathway would lead to an adverse effect rather than signifying a neutral or adaptive response. Given these challenges, the U.S. EPA's Tox21 program will likely take decades to fully achieve its goals of understanding the mechanisms of toxic action of chemicals in the body and of greatly reducing the use of animals in toxicity testing (Tice *et al.*, 2013).

…new technologies allow for a reduction in the number of animals needed for toxicity testing…

A shorter-term application of high-throughput *in vitro* toxicity testing is incorporation of the resulting data into human health risk assessment. Another collaborative program of government agencies, "Advancing the Next Generation of Risk Assessment (NexGen)," is doing just that. As a start, NexGen is developing prototype health risk assessments with extensive evaluations of high-throughput assay data for well-characterized chemicals (*i.e.*, those with existing, robust data from traditional animal toxicity testing), such as ozone and bisphenol A. The goal of each prototype is to evaluate the utility of the data from new technologies for identifying potential adverse effects against the best available traditional data, thus providing insights as to whether future risk assessments can rely more heavily on *in vitro* data while maintaining quality and reliability.

Despite the benefits of high-throughput *in vitro* toxicity assays for informing assessments of chemical toxicity, *in vitro* systems cannot yet fully replace the use of animals in biomedical research. Such systems are usually most effective in the early stages of research as preliminary indicators of adverse effects; however, they remain limited in terms of their capabilities for generating reliable data regarding how a chemical can affect a complex, living organism. For example, *in vitro* approaches are widely used by the pharmaceutical industry during the drug development process in order to prioritize drug candidates in high-throughput screens for toxicity and to allow subsequent animal testing to focus on those candidates that are more likely to have low toxicity. Thus, although the new technologies allow for a reduction in the number of animals needed for toxicity testing, researchers will continue to use animals as a primary means for understanding how people may respond to a chemical exposure.



Chemicals can interact with a single pathway or multiple pathways through different levels of biological organization, and multiple pathways can lead to the same toxic effect in the target organ.

Adapted from: U.S. EPA. 2009. The U.S. Environmental Protection Agency's Strategic Plan for Evaluating the Toxicity of Chemicals. EPA/100/K-09/001.

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Tice, R.R., C.P. Austin, R.J. Kavlock, and J.R. Bucher. 2013. Improving the human hazard characterization of chemicals: A Tox21 update. *Environ. Health Perspect.* 121(7):756-765.

Causal Analysis Using *In Vitro* Data

By Lorenz Rhomberg, Ph.D., ATS

While progress has been made, much more research is needed to characterize the potential in vivo toxicity of chemicals entirely through causal pathways based on in vitro examination.

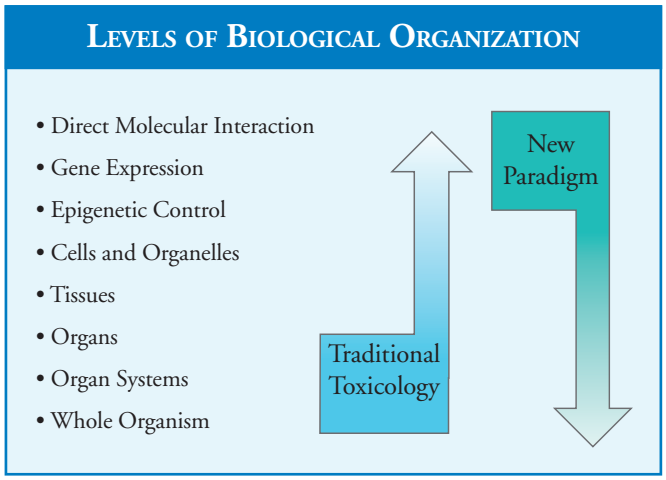
With *in vivo* animal testing, the primary outcomes are the apical toxic effects themselves – the dysfunction at the whole-organism level that is recognized in the form of defined adverse outcomes such as tumors, malformations, or organ failures. The traditional presumption is that any chemical toxicity will be similar enough in its operation among mammals that effects seen in tested animals may be expected in humans as well. As our knowledge has increased, however, we have more examples of agents that have dissimilar effects in different species, or for which susceptibility to given exposure levels differs between tested animals and humans owing to physiological, metabolic, and biochemical differences that are parts of the underlying mode of toxic action. We need to try to identify the roles of such influences by tracing backward through the causal chain, from the observed whole-animal outcomes to their underlying physiological changes, which in turn are produced by biochemical interactions (see figure). *In vitro* methods that examine some of these processes in isolation can be a valuable part of this process, but care has to be taken to consider how individual mechanistic elements interact to produce the overall apical effects being investigated.

…what will it mean to have good predictions of whole-animal outcomes that themselves are questionable predictors of human risks?

Another approach to using *in vitro* testing is gaining increasing interest, namely, the use of measurements of isolated biochemical processes or cellular responses as the primary observations, and then tracing their expected consequences (as they occur *in vivo* and interact with one another) to produce the ultimate adverse outcomes of concern. This reverses the direction of inference (see figure); instead of working from frank effects backward to their causes, this approach works from the potentially causal effects at lower levels of organization to project how they may act together to bring about overt adverse effects in the whole organism. Clearly, the success of this depends on how well the ultimate consequences of the observed biochemical and cellular responses can be identified and understood in dose-response terms, and we are only beginning to gain the sophisticated insights into disease-process evolution that will ultimately be needed.

Nonetheless, because high-throughput *in vitro* methods can examine many more chemicals, dose levels, and even the effects of chemical mixtures in a cost-effective way, the effort to make predictive use of such information is increasing – *e.g.*, seeking patterns of response among the many simultaneous tests in a microarray to serve as indicators of toxic mechanisms and ultimately of the ability of the tested agent to cause frank *in vivo* toxic effects in animals. This can be done in a screening mode, but an ultimate goal, not yet realized and perhaps distant, is to be able to identify the potential *in vivo* toxicity of chemicals solely through investigation of the *in vitro* examination of parts of their causal pathways.

To discover predictive patterns from high-throughput assays, algorithms have to be "trained" by observing correlations of patterns with *in vivo* whole-animal toxicity in experiments already done. The question for this approach is that, in view of the imperfect role of animal results in predicting human toxicity, what will it mean to have good predictions of whole-animal outcomes that themselves are questionable predictors of human risks?



Source: Rhomberg, L.R. 2010. "Toxicity testing in the 21st century: How will it affect risk assessment?" *J. Toxicol. Environ. Health.* B13:361-375.

Primary use of *in vitro* testing for decision-making in risk assessment will require a high degree of reliability, with well understood false-positive and false-negative rates and confidence that all of the apical whole-organism impacts of concern are captured and identified by the array of *in vitro* methods to be applied. Until this happens, whole-animal *in vivo* testing will have the dominant role in toxicity testing. But even now, *in vitro* methods can be valuable adjuncts to identify underlying mode-of-action and metabolic processes, and their dose-dependence, for the more informed interpretation of how whole-animal data apply to human risk evaluation.

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In Vitro Toxicity Testing: Practical Implications

By Lisa Bailey, Ph.D.

In vitro testing holds great promise as a meaningful risk assessment tool provided that studies are able to utilize relevant dosing, and the data are carefully interpreted.

Since *in vitro* toxicity testing is inherently mechanistic, it is becoming increasingly important in providing a better understanding of mechanism and mode of action (MoA) in risk assessment, including mechanisms of adaptive and adverse responses. An understanding of the molecular basis for toxicity can inform risk assessment by providing a strong foundation for extrapolation of chemical toxicity to other species and prediction of the biological plausibility of adverse effects in humans.

…it is critical that doses more likely to be experienced by humans are tested in vitro.

Incorporation of *in vitro* testing into risk assessment in a meaningful way requires several important considerations. One is that the *in vitro* studies need to be conducted at doses relevant to those experienced *in vivo*. Different doses have different mechanisms of action owing to adaptive and protective effects at low doses *vs*. saturation of those protective mechanisms at higher doses. For example, positive *in vitro* genotoxicity results at doses higher than what can be found *in vivo* should logically be considered irrelevant for risk assessment purposes. This is because detoxification, DNA repair mechanisms, and other adaptive responses are overwhelmed at high doses, leading to cytotoxicity and mutations which may not reflect *in vivo* responses at lower doses. Therefore, it is critical that doses more likely to be experienced by humans are tested *in vitro*. Defining No Adverse Effect Levels (NOAELs) *in vitro* is also important for a full understanding of the dose-response relationship for the mechanisms of toxicity and the basis for possible thresholds at low doses.

Despite their high sensitivity (particularly gene expression data), a second important consideration that can complicate the interpretation of *in vitro* toxicity data involves their low specificity as compared to whole animal data. Perturbations of molecular pathways at low doses may very well reflect adaptive (and not adverse) responses. Although potentially more difficult to interpret, these data provide possible keys toward understanding the underlying molecular motifs that regulate adaptive cellular responses to low levels of chemical stressors.

The U.S. EPA's Draft Framework for a Mutagenic Mode of Action for Carcinogenicity (U.S. EPA, 2007) provides an example of where the informed application of *in vitro* data may help resolve uncertainty regarding MoA. This framework was recently dropped from the U.S. EPA's agenda due to debate within the agency regarding how to apply the age dependent adjustment factor (ADAF) of 10 for chemicals with a mutagenic MoA, how to define a mutagenic MoA, and whether a threshold for mutagenesis is possible. *In vitro* gene expression data may help resolve this debate since they can provide information about what cellular defense mechanisms are operating at low doses to protect against mutagenesis; moreover, data for higher doses can be used to probe pathways where these protective mechanisms become saturated and mutagenesis can occur, thus potentially providing a stronger scientific basis to support a threshold for mutagenesis. A recent genomics analysis for naphthalene (Clewell *et al.* 2014) does just that. With this information, adjustments for early life exposures can be based on data rather than on uncertainties.

In vitro toxicity testing is becoming a necessary and powerful part of the weight of evidence for chemical toxicity and risk assessment. It is critical, however, that its use be anchored to relevant doses and careful interpretation of the data.

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By The Way…

About 65,000 dietary supplements are on the market, consumed by more than 150 million Americans, according to a 2013 Canadian government study.

Source: http://www.cbsnews.com/news/herbal-supplements-industry-lashes-out-at-fraud-claims/.

What's New at Gradient

Awards and Announcements

- **Lisa Bailey**, **Laura Kerper**, and **Lorenz Rhomberg** tied for the *Best Abstract Award for 2015* by the Society of Toxicology (SOT) Risk Assessment Specialty Section (RASS) for their abstract "Hypothesis-Based Weight-of-Evidence Evaluation and Risk Assessment for Naphthalene Carcinogenesis."
- **Michael Peterson** and **Thomas Lewandowski** were awarded one of the *Top 10 Risk Assessment Abstracts* by the SOT RASS for their abstract "Using PBPK Modeling to Evaluate the Concurrent Effects of Perchlorate, Other Goitrogens, and Iodine on Thyroid Status."
- **David Mayfield** and **Lorenz Rhomberg** were awarded *Outstanding Presentation* by the SOT Mixtures Specialty Section for their abstract "Examination of Multiple Dose-Response Analysis Methods for Estimating Dermal Cancer Risks for PAH Mixtures."
- **Kurt Herman**, **Ari Lewis**, and **Tim Verslycke** have been promoted to the position of Principal at Gradient.
- **Leslie Beyer** and **Kim Reid** have been promoted to the position of Principal Scientist at Gradient.
- **Dallas Wait** has been appointed as Vice Chair of the U.S. EPA's Environmental Laboratory Advisory Board (ELAB).
- **Lorenz Rhomberg** has been appointed to the Economy-Wide Modeling Panel, a committee of the U.S. Environmental Protection Agency's Science Advisory Board.
- **Lorenz Rhomberg** has been appointed to the Editorial Board of the journal *Regulatory Toxicology and Pharmacology.*
- **Matthew Mayo** has been appointed to the Town of Lancaster's Zoning Board of Appeals.
- **Matthew Mayo** is now a Licensed Professional Geologist in the state of Louisiana.

Publications

Beck, B.D., **M.R. Seeley**, and E.J. Calabrese. 2014. Use of toxicology in the regulatory process. *Hayes' Principles and Methods of Toxicology, Sixth Edition*. (Eds.: Hayes, A.W.; Kruger, C.L.), CRC Press, Boca Raton, Florida, p35-87.

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Guest Editorial: Harmonizing *In Vitro* and *In Vivo* Study Findings for Engineered Nanomaterials

By Philip Demokritou, Ph.D. and Joel Cohen, Sc.D.

More development and standardization are needed before in vitro assays can become a reliable tool for hazard screening of nanomaterials.

With the ever-increasing number and variety of engineered nanomaterials (ENMs) and nano enabled products (NEPs) entering the consumer market, human exposures are inevitable. Thus, there is interest in the development of inexpensive and accurate methods for correlating ENM properties with toxicity across their life cycle (LC). Currently, whole-animal inhalation studies are considered the "gold-standard" for pulmonary toxicology. Standardized guidelines for subacute, acute, and subchronic testing (OECD Test Guidelines 403, 412 and 413) have been developed to ensure comparable and consistent results across studies, although further refinement is still necessary to improve the detection of acute pulmonary effects (Landsiedel *et al.*, 2014a).

…comprehensive guidance on how to integrate in vitro results into nanomaterial testing strategies is limited.

Due to the high cost and laborious nature of *in vivo* toxicity studies, the variety of ENM properties and transformations across their LC, and ethical considerations of animal welfare, most efforts to date have focused on the use of *in vitro* methods for screening nanoform powders. However, comprehensive guidance on how to integrate *in vitro* results into nanomaterial testing strategies is limited. Furthermore, findings from *in vitro* studies across the literature remain poorly comparable. Such discrepancies may be attributable to (1) different sensitivities of various cell lines, (2) the influence of cell culture media on particle-particle and particle-cell interactions, (3) variable preparation of ENM suspensions that may influence particle agglomeration and bioactivity, and (4) lack of dosimetry considerations to account for the time required for particles in suspension to be delivered to cells in culture (Cohen *et al.*, 2014).

In addition to discrepancies across *in vitro* studies, the capacity for *in vitro* results to quantitatively predict *in vivo* ENM toxicity has to date been unsatisfactory. For example, correlation of *in vitro* test results with No Adverse Effect Levels (NOAELs) from regulatory inhalation studies has so far been unsuccessful (Landsiedel *et al.*, 2014b). This may be partly attributed to particle transformations in the micro-environments of cell culture media enriched with serum proteins *versus* the alveolar lining fluid of the animal lung. Future research will be necessary to determine the extent to which the protein corona and particle kinetics observed in an *in vitro* system can influence toxicity compared with microenvironments encountered *in vivo*.

Furthermore, many *in vitro* studies apply unrealistically high ENM doses that are not representative of realistic exposure levels across their LC, and moreover are often not of the same scale as *in vivo* inhalation exposures (Cohen and Demokritou, 2015). In some instances, exposure concentrations administered *in vitro* in a "bolus" format would induce "overload" *in vivo*, where pulmonary clearance becomes severely impaired.

Recently, *in vitro* and *in vivo* dosimetry methodologies (Cohen *et al.*, 2014) have been developed that enable toxicologists to identify and achieve equivalent levels of particle deposition in both the animal lung and *in vitro* cellular systems (Cohen and Demokritou, 2015). Such methodologies can facilitate determination of equivalent effective dose values across these two biological systems. Meanwhile, evidence continues to grow demonstrating that *in vitro* dosimetry can affect hazard ranking for low aspect ratio ENMs (Pal *et al.*, 2015).

In summary, it is clear that *in vitro* assays require further development and standardization before they may be considered a reliable tool for hazard screening of nanomaterials. Further research is needed to identify relevant cell types and mechanistic pathways, and special consideration of *in vitro* dosimetry is of paramount importance in order to produce *in vitro* effects that are predictive of *in vivo* effects.

Philip Demokritou is a professor at the Center for Nanotechnology and Nanotoxicology at the Harvard T.H. Chan School of Public Health in Boston. Joel Cohen is a toxicologist at Gradient. They can be reached at pdemokri@hsph.harvard.edu and jcohen@gradientcorp.com.

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Cohen, J.M. and P. Demokritou. 2015. Dosimetry for *in vitro* nanotoxicology: Too complicated to consider, to important to ignore. *Nanoparticles in the Lung: Environ. Exposure and Drug Delivery* (Eds.: Tsuda, A.; Gehr, P.), CRC Press, Boca Raton, FL, p267-293.

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What's New at Gradient

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Upcoming Presentations

Salt Lake City, UT. May 30-June 4, 2015. American Industrial Hygiene Conference & Exposition.

- **"Interpretation of Risks at Exposures Above and Below OELs."** L.A. Beyer.
- **"Challenges in the Derivation and Application of OELs for Developmental and Reproductive Toxicants."** T.A. Lewandowski.
- **"Measuring Workers' Exposure to Metals in Smelter Materials Used as Fill and Assessing Potential Risk."** L.A. Beyer, M.R. Seeley, G.I. Greenberg, B.D. Beck, S. Thakali, and F. Melnikov.

Denver, CO. May 31-June 3, 2015. American Association of Petroleum Geologists Annual Convention and Exhibition.

- **"Critical Review of Research on Potential Upward Migration of Hydraulic Fracturing Fluid and Brine."** M.P. Tymchak and S.A. Flewelling.
- **"Evaluation of Human Health Risks *Via* Drinking Water for Spills of Hydraulic Fracturing Fluids."** S.A. Flewelling, M. Sharma, D.E. Merrill, A.S. Lewis, and J.T. Rominger.

Chicago, IL. July 11-14 2015. Institute of Food Technologists Annual Meeting.

- **"Risk Assessment of Contaminated Coffee to Determine the Need for a Product Recall."** L.A. Beyer, M.L. Hixon, D.B. Mayfield, M.K. Peterson, and E.M. Dubé.

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