Identification and Characterization of Adverse Effects in 21st Century Toxicology

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ABSTRACT

The ILSI Health and Environmental Sciences Institute Project Committee on Distinguishing Adverse from Non-Adverse / Adaptive Effects held a workshop in May 2011 to discuss approaches to identifying adverse effects in the context of the 2007 NRC committee report titled “Toxicity Testing in the 21st Century.” At the workshop, scientists from industry, government, academic, and non-governmental organizations discussed case studies and questions regarding how data from new, high-content assays developed for screening can be used to identify adverse effects. This paper conveys the major points from discussions at the workshop, as well as from HESI committee meetings held the previous two years. In summary, future assessments will: (1) use in vitro and in silico data to predict later-occurring apical endpoints, (2) be based on Relevant Pathways of Toxicological Concern (RPTCs), (3) require a systematic effort to characterize the RPTCs, (4) evaluate toxicological responses on a time and dose-response continuum, and (5) need to describe the context of the responses for determining the correct point of concern or point of departure. For risk assessment and regulatory decision-making purposes, a framework will be useful for systematically analyzing data to distinguish adverse changes from those that are adaptive or not adverse. The workshop defined areas of research and analysis that will be needed to successfully use data developed from high-content, screening tools in a risk assessment paradigm that is based on characterization of adverse effects.
INTRODUCTION

The science supporting regulatory toxicology is undergoing a transformation that will change how toxicology testing, interpretation, and use of data in decision-making and public health protection will be performed in the decades ahead. The developing advanced technologies and high-throughput approaches for toxicity testing (NRC, 2007) will reduce animal use, produce legislative action around broader chemical testing needs leading to reform of TSCA, and improve efficiency of drug and chemical development. The new testing approaches and techniques are designed to identify markers or endpoints that are a departure from those apical endpoints associated with traditional toxicology testing (e.g., cancer, reproductive effects). However, these new early-stage endpoints present a challenge regarding how they will be interpreted biologically and integrated into current risk assessment practices and regulatory decision-making. A series of Forum articles was published in Toxicological Sciences in 2009-2010 outlining the challenges and potential solutions to the vision outlined in the 2007 NRC report (Andersen and Krewski, 2009, 2010; Boekelheide and Campion, 2010; Bus and Becker, 2009; Chapin and Stedman, 2009; Cohen Hubal, 2009; Hartung, 2009; MacDonald and Robertson, 2009; Meek and Doull, 2009; Walker and Bucher, 2009). These articles were useful for setting the stage and identifying many of the issues surrounding full implementation of the goal of the NRC report (Andersen and Krewski, 2009, 2010). The NRC identified one issue in particular as critical – that is, the need to determine what makes an effect "adverse" (Meek and Doull, 2009). This paper presents the work of a committee of the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) that was formed to address this key issue, as well as the results of discussions and a workshop held on this topic.

Existing toxicological test designs have been based on the identification of an adverse effect at a given dose, which can be used to define a point of departure for subsequent assessment of
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risk and regulatory decision-making. Given the rapidly expanding availability of predictive tools and technologies, there will be both a plethora of potential endpoints and effects, as well as an increasingly complex web of biological pathways that will need to be considered in order to determine which represent viable markers for safety and risk assessment. An integrative approach incorporating genomic and proteomic changes, pathway alteration analysis, and in vitro and in silico models that describe perturbations will identify new markers upon which biological and toxicological significance will have to be assessed. These new markers and alterations will need to be fully characterized, including dose-response, relation to in vivo physiological systems, and relevance to humans, before they can be used appropriately.

Differentiation between an adverse effect and an adaptive response is central to toxicology and a critical determination in the context of these new toxicity testing approaches. In anticipation of the need for rigorous scientific input into how new endpoints and markers of biological change may be incorporated into assessment of risk, a HESI committee on Distinguishing Adverse from Non-Adverse/Adaptive Effects was established in 2008 to engage scientists from government, academia, and industry in a dialogue. Specific goals included (a) develop an approach to evaluate the effects from new toxicity testing tools for integration into the safety assessment of chemicals and pharmaceuticals; (b) develop criteria to assist in differentiating adverse effects from other types of biological changes; and (c) review and revise the definitions of adverse and adaptive effects based on toxicological and biological considerations relevant to regulatory decision-making.

The HESI committee convened a workshop titled "Distinguishing Adverse from Adaptive Effects in the 21st Century" on May 10-11, 2011, at the US Environmental Protection Agency (US EPA) research facilities in Research Triangle Park, NC, USA. Workshop participants discussed
characterization of biological responses, integration of responses within a biological pathway, interpretation of different categories of data for safety assessment, and the potential development of a framework that recognizes, prioritizes, and uses all toxicity testing approaches and data in safety assessment. The following provides an overview of these discussions and their potential to inform risk assessment and advance regulatory approaches for protection of public health.

**Characterization of Biological Response**

From the time of Paracelsus, the effects of chemicals on biological systems have been characterized by the apical response observed. The apical response is the observable outcome in a whole organism such as a clinical sign or pathological state that is indicative of a disease resulting from exposure to a toxicant (NRC, 2007), and one which has been used in toxicology as an endpoint of relevance for designing and interpreting studies, comparing compounds, and determining appropriate human exposure limits. As science continues to progress, computational approaches and *in vitro* studies including high-throughput assays will enable the assessment of alterations of pathways and networks that have been described at the gene, protein, or metabolic level of organization. The present challenge is to determine the value and appropriate use for these data in the context of risk assessment. Regulatory decisions are typically based on an identified adverse effect. The "adverse effect" drives regulation of chemicals under US legislation, including the Toxic Substances Control Act (TSCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); and in Europe under the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) legislation. In addition, many procedures and processes that inform risk based-decisions are based on identifying an adverse effect, including benchmark dose calculations and doses allowed in pharmaceutical clinical trials. Definitions of "adverse effect" can be found in many laws,
regulations, and in the scientific literature (Boekelheide and Andersen, 2010; Goodman et al., 2010; Lewis et al., 2002; Dorato and Engelhardt, 2005; BfR, 2009; JMPR, 2006; NRC, 2007; US EPA, 2009). After evaluating the extensive literature, the HESI committee came to consensus on the following definition of "adverse effect":

"A change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences."

As more and more biological information is obtained from 'omic studies or in vitro assays, it becomes difficult to consider this information in the context of the paradigm of "adverse effect" developed over the last 50+ years of toxicity testing and research. It therefore is necessary to assess the impact of changes in a set of genes or proteins in a particular pathway and develop an understanding of how that pathway impacts the function of the organism in a quantitative way.

The issue of adaptive response was also addressed and a consensus definition was reached:

"In the context of toxicology, the process whereby a cell or organism responds to a xenobiotic so that the cell or organism will survive in the new environment that contains the xenobiotic without impairment of function."

A number of research efforts sponsored by regulatory agencies, government consortia, and cross-institutional initiatives (Dix et al., 2007; Schmidt, 2009; Suter et al., 2011) have the
potential to change the way toxicity testing is conducted consistent with the NRC vision (NRC, 2007). The paradigm in which a mode of action is determined for a single chemical, followed by development of predictive assays for that mode of action, is shifting toward a new paradigm where screening assays will be performed that lead to prediction of a mode of action for a compound with targeted testing to validate the hypothesis (Figure 1). Fulfillment of this paradigm requires sufficient understanding, in part through continued research, of the intermediate steps between the screening assay(s) that indicates a molecular initiating event and other assays that characterize additional key events in a mode of action that will lead to an adverse outcome.

Placement of the Effect within a Biological System

Systems biology is a foundation of this new paradigm. An understanding of the biology underlying adverse effects that accounts for homeostasis based on repair mechanisms and other adaptive responses will be needed to make the vision of the new paradigm a reality. In addition, computational modeling of these pathways and networks will be critical to formulate a hypothesis, design targeted tests, and establish a collection of modes of action and adverse outcome pathways for agents of interest. With this knowledge, risk assessment of drugs and chemicals can be conducted with a scientific foundation, with greater certainty and applicability to human biology. Although this may not be achievable in the short term (Krump et al., 2010), efforts such as the US EPA's computational toxicology program and the explosion of bioinformatics tools indicate that the vision will eventually become reality.

The concept of the toxicity pathway is an important part of the NRC report (NRC, 2007). The pathway concept suggests that toxicity results when a chemical reaches and interacts with an initial key target, thus beginning a series of biological events that can ultimately result in the
development of an adverse outcome. The toxicity pathway is a cellular response pathway that when sufficiently perturbed can result in an adverse effect (NRC, 2007; Boekelheide and Andersen, 2010). Pathways that are associated with toxicity processes can be called Relevant Pathways of Toxicological Concern (RPTCs). The number of RPTCs is unknown, although it is possible that a relatively small number may describe the majority of toxic responses. At low doses, changes in a RPTC may reflect adaptation of the cell, while at higher doses the changes may be adverse. A critical near-term need in toxicology is to determine which pathways are RPTCs and describe them in a quantitative manner so that the changes in these pathways can be used to assess risk. Understanding the key nodes in the RPTCs and the dose-transition points will be an important part of this learning. Boekelheide and Andersen (2010) provide an example of how understanding the RPTCs for carcinogenicity could change the way carcinogenicity assessments are conducted. The authors point out the need for understanding pathway dynamics and the role computational methods will play in developing these new approaches.

There are a number of overlapping and sometimes confusing aspects of these processes, including the molecular initiating event, toxicity pathway, mode of action, and adverse outcome pathway. The different concepts are typically associated with different levels of biological organization and can overlap with both adaptive responses and adverse effects. The molecular initiating event is the initial point of chemical-biological interaction within the organism that starts the pathway. Additional events further along the pathway that lead to and are associated with the adverse outcome are referred to as key events. The molecular initiating and key events are empirically observable precursor steps that are a necessary part of, or markers for, a mode of action (Boobis et al., 2008; US EPA, 2005a). A classic molecular initiating event is binding to a nuclear receptor which leads to the key events of enhanced metabolizing enzyme synthesis,
increased oxidative stress, and cell proliferation (Klaunig et al, 2003). The molecular initiating event, when combined with a series of key events, is typically described as a mode of action (Boobis et al., 2008; US EPA, 2005a). These descriptions have been further expanded to include adverse outcome pathways which combine the existing knowledge linking the molecular initiating event to an adverse outcome at the individual or population level and may include exposure (Ankley et al., 2010).

The identification of an adverse outcome after exposure to a xenobiotic has been a mainstay for assessing risk to inform risk management decisions. The adverse effects used for these decisions have tended to be the apical outcomes after exposure, such as tumors, permanent changes in the target tissue, or specific transient changes in the target tissue directly associated with the ultimate outcome of concern. These endpoints have been considered the Relevant Response for Regulation (RRR), which is the basis for a risk assessment. The US EPA established a mode of action following the IPCS Mode of Action/Human Relevance Framework (US EPA, 2005a; Boobis et al., 2006) for the herbicide and inorganic arsenic metabolite, dimethylarsinic acid (DMA) (US EPA, 2005b). The basis for the DMA mode of action was the evaluation of a series of apical endpoints as key events for the ultimate apical endpoint of transitional cell tumors of the urinary bladder in rats. These apical endpoints included transitional cell death, transitional cell proliferation, and transitional cell hyperplasia. A series of experiments were performed to characterize the effects in the target cell – the transitional cell of the urinary bladder – using transcriptional profiling (Sen et al., 2005, 2007). These studies were designed to identify key molecular changes that are associated with exposure to DMA and to better characterize the pathways or key events leading to the various apical outcomes associated with DMA exposure. In brief, the authors identified transcriptomic changes at doses below which one sees the apical adverse endpoint of transitional cell death in the target
epithelium from exposed rats as well as in vitro (Sen et al., 2005, 2007). The authors suggested that the toxicity observed at the higher doses may add to precursor effects present at the lower doses to drive the development of the apical outcome of a tumor (Sen et al., 2005). However, while the changes in gene expression indicated potential RPTCs, there was not enough knowledge of these pathways to quantitatively describe the key events in development of the tumor, nor were there sufficient species-specific descriptions of these pathways that could determine if the rat and human would have different or similar responses. Therefore, the response did not qualify as a RRR.

In the context of the present discussion on adaptation and adversity, it may be that the cellular response to the presence of DMA sets up a series of activated genes which allow the transitional cells to survive insults at the lower doses. The adaptive response may impart both protection and enhanced susceptibility to the toxic effects. As one increases the dose of DMA to the target cell, a series of increasingly severe responses occur (US EPA, 2005b). At the lowest doses tested in these studies, only altered gene expression was identified (Sen et al., 2005, 2007). At intermediate doses, the apical endpoint of cell death and increased cell proliferation occurred but resolved over time, suggesting an additional adaptive response to the continued exposure (Sen et al., 2005; US EPA, 2005b). After treatment for extended durations with the highest doses of DMA, irreversible tissue responses occurred resulting in the apical endpoints of cellular hyperplasia or tumors (US EPA, 2005b). This example illustrates the combined significance of context, amount of exposure, and duration of exposure. The biological significance of the various exposure-related effects identified and the determination of whether they were adverse depended on the establishment of a relationship among the several key events described for this mode of action. Figure 2 illustrates the hypothetical dose-response relationship for putative RPTCs and how this information might be used to determine adverse
effect levels. This example also illustrates the need to gain a more detailed, quantitative knowledge of molecular initiating events, toxicity pathways, and their interactions in order to improve the understanding of where transition points occur between adaptive changes and adverse effects. This will also aid species extrapolation of effects and decrease uncertainty in risk assessments, potentially reducing reliance on uncertainty factors. A similar case can be made for data on hepatotoxicity of acetaminophen (Powell et al., 2006; Heinloth et al., 2004; Bushel et al., 2007; Fanin et al., 2010) but this will not be discussed in detail here.

Differentiating Adaptive Changes from Adverse Effects

In addition to characterization of new endpoints and markers that emanate from emerging tools and testing approaches, the interpretation of these changes demand attention and continued discussion. The data generated must be interpreted with identification of the most relevant responses considered to be early biomarkers of an exposure that would lead to an adverse effect. Given the upstream nature of many new genomic and other endpoints that are being reported, it is important to determine where such changes lie along the continuum of biological response within a living system and how the changes detected are connected to other levels of biological organization. Additionally, one would need to understand and characterize the normal background and variability of responses of the biomarkers, and whether these biomarkers of toxicity or exposure can be appropriately extrapolated to humans. Although the concept is feasible, pathways have not been sufficiently elucidated for many modes of action already known to lead to an adverse outcome, such as genetic alterations leading to cancer (Boekelheide and Andersen, 2010). A significant investment in research describing toxicity pathways and adverse outcome pathways, with identification of biomarkers of these pathways associated with adversity, is needed.
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With the development of new toxicity testing approaches and identification of biomarkers and signatures of potential toxicological change comes the need for a new framework for how these data could be used in risk assessment to inform regulatory decision-making. Iteration, revision, and rigorous validation of new approaches will be needed to ensure that new tools and endpoints are sensitive, accurate, and reflective of biological change and are relevant to human biology. Similarly, development of target-specific screening assays, including toxicogenomics data, should be founded on proper context of rigorous, validated, and standardized in vitro and in silico data that have an established relevance to human biology.

Once data are available from screening assays, mechanistic studies, pathway analyses, and other in vitro and in vivo methods, decisions will need to be made on appropriate use of the data. At the May 2011 workshop, the question was asked, "What criteria should be used to decide if there is sufficient information to identify an effect as adverse?" While all data should be considered, a weight-of-evidence (WoE) approach allows for an analysis of the strengths and weaknesses of the data and the relative importance of the data elements for animals or humans.

Our present understanding of adversity is linked to the apical effect, which is normally a phenotypic response. Therefore, phenotypic anchoring of changes in gene, protein, or metabolite expression, or other in vitro endpoints, is critical to understanding if changes in a system are adverse or not. As more experience is gained with RPTCs, key transition points in pathways, and other details of biology, the need for phenotypic anchoring with a specific chemical should decrease. How long this paradigm switch will take will be dependent on the quality of data produced to support hypotheses. The use of prototypical, data-rich chemicals to develop the experience and understanding of biology is critical, and studies will need to be designed for this specific purpose.
Adaptive responses to toxicant exposure are likely to be characterized by reversibility (upon withdrawal of treatment or exposure). Furthermore, adaptive changes can be distinguished as early homeostatic adjustments, such as benign metabolism or gene expression / transcriptomic changes (Goetz et al., 2011). These modulations are typically not considered to be precursors of functional impairment, but rather a metabolic response that would return to a homeostatic condition (i.e., a return from hormone level variation(s) / cycling, or blood sugar fluctuation(s), or blood pressure increase owing to stress). In some situations, a minor change may be sustained resulting in a "new normal" state where the cell/tissue/organism has adapted without adverse consequences. These types of adaptive changes, in a different context, may be indicators of a potentially adverse outcome. For example, short-term decrements of circulating thyroid hormone may result in an adaptive response in an adult, non-pregnant female, but the same change in early gestation could be an indicator of a potential adverse outcome on fetal brain development. Alternatively, very high doses, as often used in traditional toxicity studies, can induce secondary effects that are inappropriate for human risk assessment (Counts and Goodman, 1995).

A WoE evaluation for risk assessment also needs to consider variables such as exposure, which will require the use of dosimetry, reverse dosimetry, and biomonitoring. This issue is beyond the scope of this paper, but is an important element of the risk assessment process. The context of exposure as part of the WoE evaluation is a critical factor in differentiating an adaptive change from an adverse effect. Consideration of proper context needs to be incorporated when designing studies, as well as during evaluation, to properly interpret the data for informing regulatory decisions. For example, age-related biological differences can impact interpretation of results from exposure to a drug or chemical compound at various life stages (in utero or early life-exposures, or for juvenile and adult later life-exposures). Toxicological responses resulting from exposure across life stages must be considered along with the time- and dose-response continuum.
Potential Usefulness of a Framework for Making Decisions on Adversity

During the workshop, the participants discussed the usefulness of a formalized framework or a consistent series of questions to be answered for deciding if effects are adverse or not. This framework was loosely based on previously published decision trees (Dorato and Engelhardt, 2005; Lewis et al., 2002), and considered factors such as change in tissue or cellular function, reversibility, transition points in pathways, context of exposure, and species differences. Such an approach to interrogating data could prove useful both in the design of additional studies, as well as the assessment of potential risk. It could substitute for currently used tiered testing schemes which are designed to cover all possibilities rather than to develop targeted perspectives based on knowledge of modes of action of chemicals. Ultimately, the participants agreed that while such an approach for interrogating data would be useful, there is currently not sufficient knowledge to establish a specific framework for decision-making. Nonetheless, efforts should be made to characterize RPTCs and critical nodes in these pathways. As more data become available on the key pathways, the development of a framework may become more feasible, thus leading to better characterization of the RRR for a specific chemical and exposure scenario.

Conclusions and Recommendations

The major conclusions of the May 2011 workshop can be summarized as follows:

- Workshop participants agreed that a primary goal for the future is to leverage in vitro and in silico data to accurately predict later-occurring apical endpoints from kinetically earlier dose transitions in RPTCs. Therefore, a dose transition considered to be a relevant response for
regulation may not correlate temporally with an observable apical endpoint, but the two should be linked through the pathways in a way that is biologically meaningful.

- All toxicological responses should be viewed and considered within a time- and dose-response continuum. A recurring theme of discussion during the workshop was the lack of a qualitative distinction between the toxicogenomic profile (and other *in vitro* or *in silico* biomarkers) associated with early or low-dose exposure (not linked to an adverse apical endpoint) and later or higher-dose exposure (potentially or more often linked to an adverse apical endpoint). Because of this, the exact point at which a transition to adversity occurs can appear to be ambiguous or even arbitrary, and the value of the response for predicting significant biological impact on an exposed individual may appear to be questionable. This continuum of response presents a significant challenge in the regulatory environment.

- Two important concepts that emerged from the workshop were Relevant Pathways of Toxicological Concern (RPTCs) and Relevant Response for Regulation (RRR). These two concepts are fundamentally different. RPTCs refer to discrete biological mechanisms that are indicators of a toxico-pathological response in human cells or organ systems. It is anticipated that a finite number of RPTCs exist and will be identified. RRR, on the other hand, is a prescribed effect on which regulatory action, which is designed to protect individuals from unacceptable risk of a specific toxicological outcome, is based. For example, genomic or epigenomic changes that modulate a critical pathway of toxicant metabolism may define a RPTC. If, however, these changes are exceedingly rare in the human population, or if they occur exclusively in experimental models, they might not qualify as a RRR.
• A systematic effort to define and characterize RPTCs is critical. Because the intent is to predict rather than evaluate toxicity, the number and identity of relevant pathways and the identity of the most commonly affected RPTCs should be a research priority. An attempt should be made to prioritize the level of concern associated with perturbation of each pathway. For each pathway, it is important to characterize dose transitions, identify the critical nodes, and determine the presence or absence of threshold effects. For each pathway, it is also important to describe specific RRRs. The relationship between dose-response changes in the pathways and dose-response changes in apical endpoints, such as histology, will be a key to having confidence in this approach. A paradigm needs to be developed and refined that provides an understanding of RPTCs and critical nodes. In addition to characterizing the pathways, links between the pathways into networks must be investigated to understand the dynamics and kinetics of how an organism adapts or proceeds to an adverse effect (Figure 3). Model compounds should be used to provide detailed examples of perturbations in the pathways and networks to develop the way the information is used.

• Scientifically-informed decision-making is of high value. This suggests that the emerging risk assessment framework should ultimately promote effective use of rigorous, validated, and standardized in vitro and/or in silico data that have established relevance to human biology.

• Consideration of context (at the level of organism, tissue, and cell) is critical for determining the point of concern or point of departure. The significance of an in vitro or in silico response to a putative toxicant can only be determined through a careful and thorough consideration of biological context and a realistic estimate of a relevant exposure to the putative toxicant.
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The HESI workshop was held in response to the rapid development of screening assays and high-density data that is outpacing the ability to use the data in an effective manner for risk assessment. Caution should be used when interpreting screening assay data for anything other than ranking or prioritizing chemicals. One needs to be extremely cautious in using these types of data for determining all or part of a mode of action. It is hoped that the present effort has helped to focus the scientific community's attention on this important area of research in understanding the spectrum of adaptation and adversity as it applies to risk assessment.

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References


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Figure 1. Present and future testing paradigms for understanding mechanisms of toxicity. In the present case, modes of action are postulated followed by determination of intermediate networks and pathways, culminating in screening assays to detect compounds that present this mode of action. In the future, screening assays will be used to postulate modes of action by prior understanding of the links between the screen, targets, pathways, networks and modes of action.
Figure 2. Dose transitions for adverse toxicant response with four differentially-susceptible nodes. A hypothetical toxicant has functional effects on four network nodes (N1 to N4), inducing four distinct dose transitions detectable at increasing toxicant doses. At the highest cumulative dose, toxicant exposure produces high incidence of an apical endpoint (i.e., cell or organism death). *In silico* studies can link adverse effects to exposure to lower doses of the toxicant.
Figure 3. Future state of toxicity testing based on knowledge of key toxicity pathways and the critical nodes in the pathways. Boxes in red indicate the areas for research where the most emphasis is needed to allow use of this paradigm.