Calling on Science: Making "Alternatives" the New Gold Standard

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Highlights of WC7 – part 1

Calling on Science:
Making "Alternatives" the New Gold Standard[1]

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Summary

All of life's great journeys start with a goal in mind! The 2007 NAS report, Toxicity Testing in the 21st Century – A Vision and A Strategy, has proposed a clear goal. This report envisions a not-so-distant future where all routine toxicity testing for environmental agents will be conducted in human cells in vitro evaluating perturbations of cellular responses in a suite of toxicity pathway assays. Dose response modeling would utilize computational systems biology models of the circuitry underlying each toxicity pathway; in vitro to in vivo extrapolations would use pharmacokinetic models, ideally physiologically based pharmacokinetic models, to predict human blood and tissue concentrations under specific exposure conditions. Results from these toxicity pathway assays and associated dose response modeling tools rather than those from high dose studies in animals would represent the new gold standard for chemical risk assessment. This talk focuses on some of the scientific challenges required to make this vision a reality, including characteristics of assay design, prospects for mapping and modeling toxicity pathways, assay validation, and biokinetic modeling. All of these tools necessary for this transformation of toxicity testing to an in vitro platform are either available or in advanced development. Science must lead the transformation. The scientific community, animal alternatives groups, regulatory agencies, and funding organizations will also have to muster the resolve to work together to make this vision a reality.

Keywords: gold standard, toxicity pathways, in vitro biology, computational systems biology, toxicity testing transformation

1 Introduction

This year marks the 50th anniversary of the publication of *The Principles of Humane Experimental Technique* by William Russell and Rex Burch. Their contribution focused attention on the 3Rs – replacement, reduction, and refinement. In toxicity testing, the primary initiative with the 3Rs in the intervening decades has arguably focused on reduction of animal usage while holding firm the belief that results from animal studies provide a "Gold Standard" for making decisions about possible human health risks of compounds. The very wording, "alternatives", has often been regarded by many in toxicology as those test methods that will reduce animal usage even though the result from the tests are not necessarily optimal for risk assessment decision-making. A second challenge in reduction of animal use through mechanistically-based testing arises from the idea of validating "alternatives". The process of validation with alternatives, in general, focuses on the ability of a test or a series of tests to give results consistent with those that would be obtained through testing in animals. In this context, all alternatives will fall short of the mark

of complete concordance with *in vivo* outcomes. Are all efforts to reduce animal use significantly doomed to disaster as they are dashed against the "gold standard" barrier?

The recommendations of a recent report (NRC, 2007) from the US National Academy of Sciences, *Toxicity Testing in the 21st Century: A Vision and A Strategy* argues that it is time to redefine the toxicity testing paradigm, moving away from high dose studies in animals to *in vitro* assays assessing perturbations of toxicity pathways by environmental agents. In essence, the report supports a sweeping redefinition of our "gold standard." The author of this present paper was a member of the NAS toxicity testing committee. Since the publication of the NAS report in June 2007, several of the NAS committee members have presented aspects of the report at more than 40 venues in North America and Europe. These presentations and the lively debate engendered on these occasions have sharpened ideas about the use of results from *in vitro* toxicity pathway assays in risk or safety assessments. The NAS report, although published in 2007, was essentially completed in fall 2006. Advances in several key technologies in the past three years – especially

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stem cell biology, computational systems biology, and pathway mapping and modeling – appear likely to be key catalysts for moving the vision forward. Finally, the transformation from current, traditional approaches to new *in vitro* methods based on human biology will not come easily. Who will step up to assist in the transformation to a new approach to testing and risk assessment? Several initiatives within the United States, both in federal government research organizations and in the private sector, look likely to accelerate implementation. These topics – (1) the recommendations from the NAS report, (2) the manner in which the *in vitro* toxicity pathway data can be organized for risk/safety assessments, (3) the call to the alternatives community to embrace 21st century computational and bioinformatics methodologies in designing and interpreting *in vitro* results, and (4) the institutional opportunities to accelerate implementation of the NAS vision – are discussed in turn in this current paper.

2 Toxicity Testing in the 21st Century: a Vision and a Strategy

The US Environmental Protection Agency and the US National Institute of Environmental Health Sciences asked the US National Research Council (NRC) to provide guidance on new directions in toxicity testing, incorporating emerging technologies such as genomics and computational systems biology into a new vision for toxicity testing. In 2004, the NRC convened a 22 person committee for this purpose (Tab. 1). The committee produced two reports. The committee's interim report (NRC, 2006) provided an overview of testing methods and approaches that could incrementally improve traditional toxicity testing. This report noted that health protection agencies and the public had experienced increasing frustration with the failure of current approaches to toxicity testing to provide timely, relevant information to support informed regulation of environmental agents. These toxicity testing strategies relied primarily on the observation of adverse health responses in laboratory animals treated with high doses of these agents. Estimating risks to human populations based on high dose animal studies require difficult extrapolations, first from high doses to environmental levels that are usually orders-of-magnitude lower than those used in the animal studies, and then from animals to humans. These traditional toxicity testing approaches and methods for their interpretation date back some 30 to 60 years, and were developed at a time when knowledge of biology – and of the manner in which chemical exposures perturbed biological processes – was primitive. While there have been steady, incremental improvements in toxicity testing over the years, there has been no comprehensive evaluation of the manner in which advances in cellular and molecular biology might improve toxicity testing practices.

The final report of the toxicity testing committee (NRC, 2007) outlined design criteria that needed to be considered in any revisions of practices for toxicity testing. In choosing among various toxicity testing options, four criteria are important: (1) achieving broad coverage of chemicals, chemical mixtures, outcomes, and life stages, (2) reducing the cost and time required for toxicity testing, (3) developing a better scientific basis for

assessing human health effects of environmental chemicals, including knowledge of modes of action, and (4) minimizing use of animals in testing. The consideration of how these criteria should guide a modern approach to toxicity testing led the committee to propose a new framework for toxicity testing that would entail a major overhaul of current practice.

Toxicity testing and targeted in life studies

The NAS committee vision consisted of several key technology areas (Fig. 1). While also including *in silico* methods for assessing structure activity relationships and population assessments, the transformative parts of their new toxicity testing paradigm was the types of toxicity testing and the manner in which results from these tests could be organized to support human health risk assessment. This vision centers on defining dose-response relationships for toxicity pathway perturbations that would be expected to lead to adverse health outcomes if the perturbations were maintained *in vivo* at a sufficient level of intensity and for a sufficient duration of exposure. The key component of the vision is assaying perturbations of toxicity pathways, which are simply normal biological signalling pathways that may be perturbed by chemical exposures. Toxicity pathway testing would require a suite of *in vitro* tests that could identify the range of significant perturbations of human pathways that might occur as

Tab. 1: The Roster of the NRC Toxicity Testing Committee

Daniel Krewski (*Chair*), University of Ottawa, Ottawa, ON

Daniel Acosta, Jr., University of Cincinnati, Cincinnati, OH

Melvin Andersen, The Hamner Institutes for Health Sciences, Research Triangle Park, NC

Henry Anderson, Wisconsin Division of Public Health, Madison, WI

John Bailar III, University of Chicago, Chicago, IL

Kim Boekelheide, Brown University, Providence, RI

Robert Brent, Thomas Jefferson University, Wilmington, DE

Gail Charnley, HealthRisk Strategies, Washington, DC

Vivian Cheung, University of Pennsylvania, Philadelphia, PA

Sidney Green, Howard University, Washington, DC

Karl Kelsey, Harvard University, Boston, MA

Nancy Kerkvliet, Oregon State University, Corvallis, OR

Abby Li, Exponent, Inc., San Francisco, CA

Lawrence McCray, Massachusetts Institute of Technology, Cambridge MA

Otto Meyer, Danish Institute for Food and Veterinary Research, Søborg, Denmark

D. Reid Patterson, Reid Patterson Consulting, Inc., Grayslake, IL

William Pennie, Pfizer, Inc., Groton, CT

Robert Scala, Exxon Biomedical Sciences (Ret.), Tucson, AZ

Gina Solomon, Natural Resources Defense Council, San Francisco, CA

Martin Stephens, The Humane Society of the United States, Washington, DC

James Yager, Jr., Johns Hopkins University, Baltimore, MD

Lauren Zeise, California Environmental Protection Agency, Oakland, CA
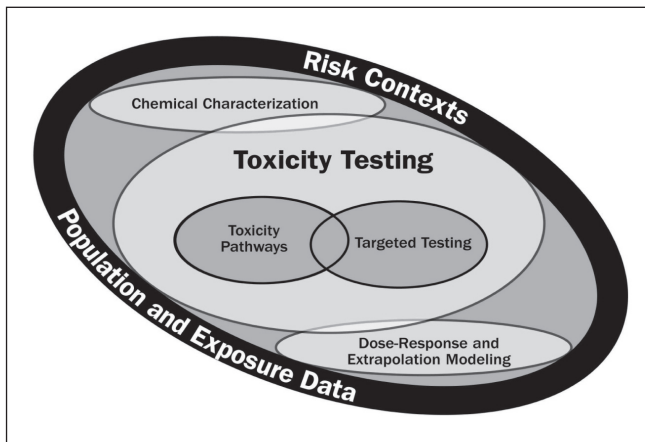
Fig. 1: Components of the vision for Toxicity Testing in the 21st Century (NRC, 2007).
The key elements in this proposal are related to Toxicity Testing, which includes the types of *in vitro* tests and short term *in vivo* tests to evaluate perturbations on toxicity pathways, and Dose-Response and Extrapolation Modeling, which provides the requisite tools for interpreting toxicity testing results for assessing human health risk assessment. Reproduced from the NRC report (NRC, 2007) with permission.

a result of chemical exposure (Fig. 2). Biologic responses are viewed as results of an intersection of exposure and biologic function. The intersection results in perturbation of biologic pathways. The circuitry affected by the chemical is expected to determine shapes of dose response relationships for these perturbations. Ideally, these assays would be conducted in human cells, cell lines or in engineered human tissues. The committee believed that the use of a comprehensive array of *in vitro* tests

with human cells would markedly reduce the need for whole animal testing, and provide much stronger, mechanistically-based tools for human health safety assessment. It was recognized that the conversion to an *in vitro* basis had challenges and the committee also suggested that targeted *in vitro* testing was also likely to continue for some time where such studies could provide information about metabolism, possible metabolite toxicity, toxicity pathways, etc. Metabolism has been recognized as a particular challenge for developing *in vitro* testing alternatives (Coecke et al., 2006).

Dose-response and extrapolation modeling
How will results from a comprehensive suite of toxicity pathways inform quantitative risk/safety assessments for environmental agents? In this new toxicity testing strategy, *in vitro* concentration response curve would cover multiple orders of magnitude (Inglese et al., 2006, 2007) and evaluate responses in cells/tissues from humans, the species of primary interest. The broad range of concentrations permit the definition of dose ranges resulting, or not resulting, in significant alterations of normal biological function. While low dose and interspecies extrapolations are not as problematic, new challenges arise in understanding the mechanistic bases for dose-response behaviors of the toxicity pathway assays, in calibrating expected blood/tissue concentrations in humans against the vitro concentrations used in the toxicity pathway assays, and in understanding the linkages of early perturbations to adverse responses expected in exposed people. The report identified key technologies that will assist dose response and *in vitro-in vivo* extrapolations, including (1) empirical dose-response models based on results from the *in vitro*, mechanistically based toxicity pathway assays, (2) mechanistic dose-response models based on knowledge of toxicity pathway circuitry and dynamics of pathway function, and
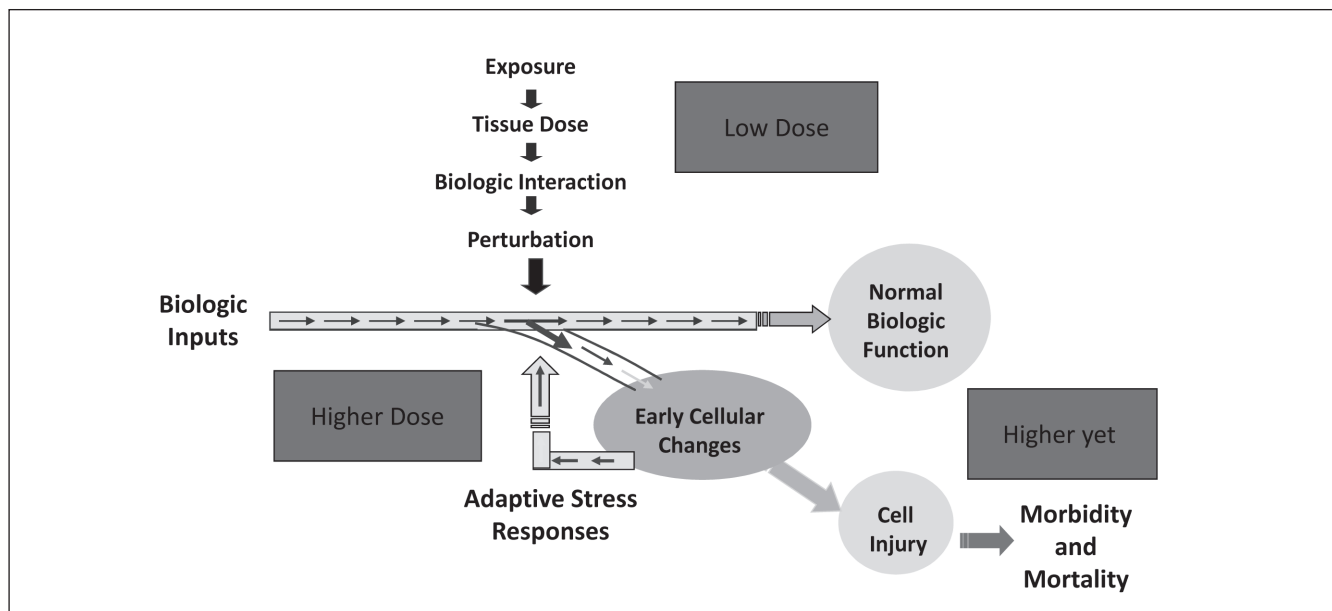
Fig. 1: Number of animals used for experiments in Germany (until 2000) and for animal experiments and other scientific purposes (since 2000).
tg = transgenic, WT = wild type. (Courtesy of Prof. Dr. Rainer Nobiling)

(3) physiologically based pharmacokinetic (PBPK) models to equate tissue-media concentrations with tissue dose in exposed people. Two recent perspectives on the NAS report provide good overviews of the report and directions for implementation (Krewski et al., 2009; Andersen and Krewski, 2009).
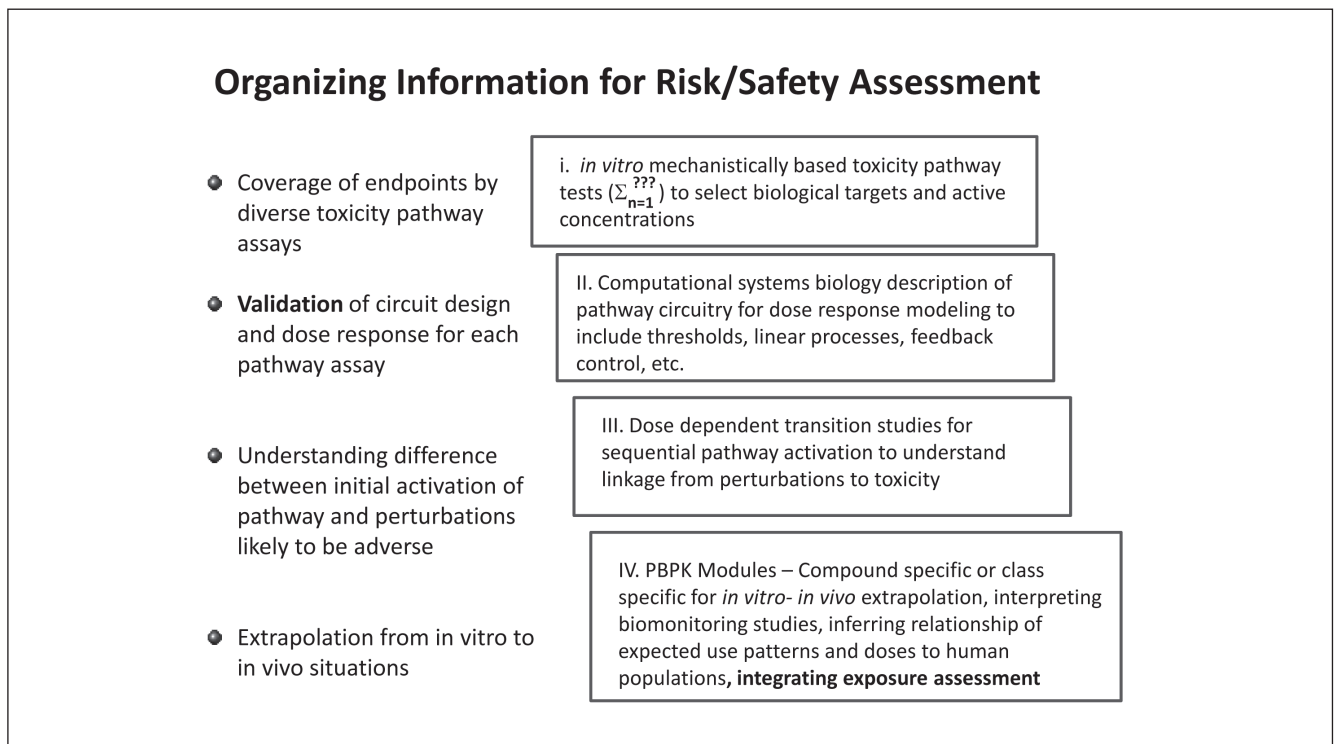
3 The new gold standard in practice

Over the past three years, there has been continuing discussions about the NAS report with diverse stakeholder audiences. During these discussions, many questions were directed at the manner in which the pieces of the new test paradigm would integrate together to provide quantitative approaches for risk or safety assessment. The NAS report did outline two hypothetical cases of "assessments" that might arise from a battery of *in vitro* test using examples of a reactive gas and of a compound with estrogenic activity. These examples were cursorily developed, but indicated how various parts of the testing and analysis would likely contribute to health assessments. It is possible today to provide a more complete picture of how these pieces might be integrated (Fig. 3).

The core component of the testing will be the suite of toxicity pathway assays (Fig. 3; section i). These assays would be developed for human cells, human cells in culture, or human three-dimensional tissue surrogates. The toxicity test assays themselves need to be capable of evaluating the progression from initial

activation of the pathway on through degrees of perturbation that would be considered sufficiently large to be associated with likely toxicity if maintained over a period of time in an intact organism. For most, if not all assays, concentrations are expected to range from sub-threshold through those causing initial pathway activation, on to regions of adaptation, and finally to those causing adverse cellular consequences. To cover these various degrees of response, each assay would likely provide different levels of biological readout as a function of concentration and duration of treatment.

Each pathway assay is expected to have specific dose response characteristics depending on the organization of the circuitry that determines the action of compounds on the toxicity pathway. The dose response behaviors should arise from the underlying biology of the circuitry. These core signaling processes include the initial signal recognition and then the larger scale network through which the initial perturbation progresses to generate toxicity in the test system. Computational systems biology (Alon, 2006, 2007) provides the tools for describing these circuits and the differential behavior of the circuits with increasing degrees of perturbation.

The process of validating toxicity pathway assays would be to study its behavior for positive control compounds and to extract the network structure and network dynamics that determine dose response. The sequential passage from sub-threshold, to adaptive, to toxic conditions represents dose-dependent transitions in modes of action in an *in vitro* system. Dose-dependent

Organizing Information for Risk/Safety Assessment

- Coverage of endpoints by diverse toxicity pathway assays

- **Validation** of circuit design and dose response for each pathway assay

- Understanding difference between initial activation of pathway and perturbations likely to be adverse

- Extrapolation from in vitro to in vivo situations

i. *in vitro* mechanistically based toxicity pathway tests ($\Sigma_{n=1}^{???}$) to select biological targets and active concentrations

II. Computational systems biology description of pathway circuitry for dose response modeling to include thresholds, linear processes, feedback control, etc.

III. Dose dependent transition studies for sequential pathway activation to understand linkage from perturbations to toxicity

IV. PBPK Modules – Compound specific or class specific for *in vitro- in vivo* extrapolation, interpreting biomonitoring studies, inferring relationship of expected use patterns and doses to human populations, **integrating exposure assessment**

Fig. 3: Representation of the application of *in vitro* toxicity testing results for risk assessment.
Four components will contribute. (1) The suite of assays to provide broad coverage of possible targets and mechanisms of toxicity.
(2) The mapping and modeling tools to validate pathway architecture and provide dose-response models for risk assessment.
(3) The flexibility in assay output to evaluate networks that incrementally control target interactions, adaptive stress responses and overt toxicity. Lastly, (4) the use of PBPK and biokinetic tools to relate *in vitro* concentrations with *in vivo* exposures.

transitions in *in vivo* toxicology studies are well known (Slikker et al., 2004a, 2004b). This type of response cascade has been described *in vitro* by Xiao et al. (2003) and by Nel et al. (2006) in work on hierarchical oxidative stress. Feedback process control and dose response have also been examined more theoretically for anti-stress gene regulatory networks (Zhang and Andersen, 2007). Alon (2007) has provided a good overview of network motifs in signaling pathways and in what were termed "developmental" networks. In practice, toxicity pathway characterization would optimally include standard operating procedures (SOPs) for preparing cells, conducting specific assays, generating read-outs, and the detailed process by which the pathway structure, circuit, and dynamics had been evaluated to support dose-response modeling. The detailed pathway characterization (essentially the process of validation of the pathway behavior) would be the mainstay of dose response analyses. For the safety assessment, primary attention would focus on pathways affected at the lowest concentration (Fig. 3; sections ii and iii).

How do we relate concentrations affecting cells *in vitro* with exposures in human populations likely to cause similar responses in an intact individual? Human biomonitoring for chemicals in blood and excreta is becoming more widespread. In some instances, concentrations of exogenous compounds in humans may be available. Comparisons could be made between those concentrations seen in exposed populations and those affecting cells in the toxicity pathways assays in order to estimate a "margin of safety" or "margin of exposure." This comparison is unlikely to be possible with very many compounds. A more general methodology would be development of biokinetic models (DeJongh et al., 1999) to determine the human exposure situations expected to give cell and tissue concentrations similar to those affecting the human cells in the *in vitro* pathway assay test (Fig. 3; iv). These approaches are extensions of the physiologically based pharmacokinetic types of models that have been of interest both with toxic substances and pharmaceuticals (Reddy et al., 2005; Bouvier d'Yvoire et al., 2007). A coordinated effort is required to develop a larger suite of PBPK models and to enhance efforts in reverse dosimetry, i.e., estimating the exposure levels in a human population that produce specific blood/tissue concentrations (Clewell et al., 2008). Current efforts to improve dosimetry methods are also advancing *in vitro-in vivo* extrapolation tools (Gulden and Seibert, 2003; Heringa et al., 2004).

The risk assessment process would entail running the suite of assays for a compound to see the pattern of activation of pathways and the concentrations at which effects were noted in various pathway assays. The most sensitive hits from the suite of assays would then be organized to support both dose response modeling and *in vitro-in vivo* extrapolation. The pattern of activity across the suite of assays could also provide signatures to indicate the types of toxic endpoints that might be observed *in vivo* (Dix et al., 2007). For example, specific signatures might indicate a high likelihood of reproductive toxicity or of hepatic toxicity in a qualitative manner.

Risk assessments completed based on results from these toxicity pathway assays are likely to be quite different from those arising from current approaches. Today, we see effects in animals, usually at fairly high doses, and estimate the likely inci-

dence of response at lower doses in exposed populations. For cancer, we might try to estimate the expected concentration estimated to give a 1/1,000,000 level of population risk. This process has two less than desirable outcomes – first, labeling compounds based on high dose hazard studies and (2) providing a false sense of precision regarding our ability to extrapolate across doses and species. For instance, if Compound A causes cancer, at a maximally tolerated dose, it becomes labeled as carcinogen regardless of considerations of exposure levels. Secondly, the public is led to believe that the estimates of the low dose extrapolations are scientifically valid without any appreciation of the uncertainties about these estimates. In contrast, the assessments based on the *in vitro* toxicity pathway assays would be more directed at safety assessment, estimating regions of exposure where no appreciable perturbations are expected in human cells or human tissues in culture.

4 Calling on 21st century science

The NAS committee discussed a variety of key technology areas for toxicity testing in the 21st century. While the broad suite of new tools are likely to influence many areas of toxicology research and to greatly improve understanding of cell signaling pathways, it is important to ask more narrowly how specific technologies and advances will contribute to the four components noted in Fig. 3. The three areas most likely to benefit immediately are in assay design, using stem cell technology, pathway mapping and modeling, and computational systems biology for assessing expected dose response behaviors.

Assay design
A frequently voiced concern after publication of the report was the difficulty in obtaining and working with primary human cells and the caveats associated with use of human cell lines. The past few years have provided optimism in the ability to obtain tissue-specific human and rodent stem cells from which more mature cell types can be generated (Alonso and Fuchs, 2003). The stem cells can be stored and grown as needed for assays and will likely become available for a wider and wider suite of tissues (Reya and Clever, 2005; Gaudio et al., 2009). Embryonic and fetal amniotic fluid stem cells can be used and differentiated through frequently tedious, multi-step processes to multiple cell types (DeCoppi et al., 2007). With tissue-specific stem cells, the route to mature cells is shorter and requires less manipulation (Wang et al., 2009).

In addition to availability of tissue specific stem cells, other advances bringing biomedical and small-scale manufacturing processes offer opportunities to utilize human 3-dimensional tissue in higher throughput contexts. For instance, Khetani and Bhatia (2008) discuss the application of semiconductor manufacturing microtechnology for fabrication of microscale tissues. A miniaturized, multiwall culture system for human liver cells with optimized microscale architecture maintained phenotypic functions or several weeks. These organotypic cultures could be useful in insuring better correspondence between *in vitro* tests and expected behaviors *in vivo*.

A major emphasis is required to produce appropriate assays with the right level of detail and an ability to provide appropriate read-outs across different responses levels. For risk/safety assessments with a single compound, rapid, *in vitro* testing for the suite of pathways is essential. High throughput and high data content methods were emphasized in the NAS report. In this usage, high throughput assays allow evaluation of hundreds or thousands of compounds across multi-point dose response in a period of just a few days. Some assays such as the organotypic liver assay above may not be amenable to high throughput. For toxicity testing, it is useful to distinguish the need for high throughput methods for testing large numbers of compounds from efficient *in vitro* tests that can be done over the course of days but may not be easily scalable to the ultra-high throughput. For evaluating the chemical space active for a particular pathway, high throughput permits evaluation pathway perturbations for large compound libraries, leading to better *in silico* modeling of structure activity relationships.

Mapping and modeling toxicity pathways
Assay outputs can be diverse as clearly evident from the US EPA ToxCast group of assays (Dix et al., 2007). Nonetheless, the area where the diverse array of new technologies has the greatest possible for contribution is in mapping and modeling the underlying signaling networks for specific toxicity pathways. The vast majority of perturbations are associated with networks that affect transcriptional control. Such a conclusion is obvious for so-called receptor-mediated toxicants, such as dioxin and the aryl hydrocarbon receptor, but is equally valid for stress response pathways. Antioxidant response signaling starts with oxidants reacting with cellular sensors – primarily Keap1. The modification of Keap1 leads to its dissociation from a complex with Nrf2, allowing Nrf2 and other partnering proteins to form a promotional complex altering expression of genes controlling cellular anti-oxidants (Motohashi and Yamamoto, 2004).

As toxicity pathway circuitry becomes better understood over time, it will be possible to create computational systems biology models for expected dose-response relationships for each of the assays used for toxicity testing following similar principles. Over the past decade, tools for mapping and modeling have blossomed. In a recent paper, Bromberg et al. (2008) described the network by which cannabinoid receptor (CB1R) controls neurite outgrowth. Activation of several hundred transcription factors within the nucleus after cell stimulation was measured to understand the logic of the signaling network. Bioinformatic methodologies connected CB1R to 23 activated transcription factors. Experiments with pharmacological inhibitors of kinases revealed a network organization of partial "OR" gates regulating kinases stacked above AND gates that control transcription factors. As in most instances of current research in systems pharmacology and network modeling, the goal of these studies was not dose-response as would be a primary interest for toxicity pathway analyses. This example provided a glimpse of the structure of the network without attempting a quantitative computational model. The epidermal growth factor (EGF)

signaling network is particularly well studied. Amit and colleagues (2007) used a suite of experimental and bioinformatic tools to determine the forward signaling and feedback processes controlling the EGF network. The network was dissected by transcriptional profiling coupled with reverse phase protein lysate assays that assessed phosphorylation states of proteins within the EGF pathway. The analysis provided the structure of the logic of the circuitry for the early, immediate and later stage portions of the network.

Computational systems biology
It appears likely that a major contribution of 21st century science will be the application of an array of technologies to elucidation, mapping and modeling the behavior of the test systems for assessing toxicity pathway dynamics. The tools would include mRNA, transcription factor and phospho-protein time course profiling, coupled with bioinformatic technologies to extract network structure. The outcome would provide dynamics of the signaling networks and the dose-and time dependence of expected consequences of perturbations by test compounds, including positive controls for each of the pathways. Dynamic behavior of signaling networks have been described quantitatively using computational systems approaches focusing on models of transcriptional control (Alon, 2007; Aldridge et al., 2006). Theoretical descriptions of networks leading to better understanding of modular design elements in biological circuits have refined our vocabulary – concepts of ultrasensitivity, bistability, network gain, feedback and feed forward motifs, noise, stochasticity, and sequential levels of early, mid-term and late gene expression – to allow discussion of network behaviors with some commonality of terminology. These concepts are more extensively elaborated in a course text on "Computational Systems Biology and Dose Response" available at the Hamner Institutes web-site (http://www.thehamner.org/education-and-training/drm_workshop.html).

5 Creating the transformational mindset

In a Figure (5-1) in Chapter 5, the NAS report discussed a strategy for implementation, including ballpark estimates of the time (1 to 2 decades) and costs ($ 1-2 billion) for transitioning from current animal intensive toxicity testing to a toxicity pathway based approach. The report stressed the need for an organization to have the lead responsibility for overseeing the technology development to support the transition – a role that could eventually be played by an appropriate laboratory within the US National Institutes of Health. The overall timeline was shown in the report in a linear fashion leading to a transition to new approaches after completion of technology development for assays and achieving some confidence that the suite of assays would provide adequate coverage of possible pathway perturbations. In the current global economic climate and with a variety of competing interests for biomedical research, is it reasonable to expect federal agencies or the private sector to support such a long-term, expensive initiative?

Some aspects of the NAS vision are embedded in other programs. Three federal US agencies with responsibilities for health-related research – the Environmental Protection Agency (EPA), the National Institute of Environmental Health Sciences' National Toxicology Program (NTP), and the National Institutes of Health Chemical Genomics Center (NCGC) – have a memorandum of understanding to conduct research necessary to advance the NRC committee vision for the future of toxicity testing. Collaboration among these organizations in the US will be essential in establishing a national commitment to develop the scientific foundation of the vision. This collaboration (Collins et al., 2008) focuses on research (1) to develop high throughput test methods, (2) to identify toxicity pathways, (3) to pursue targeted testing in short-term *in vitro* tests, and (4) to develop dose-response and extrapolation models. New approaches for *in vitro* toxicity testing and toxicity profiling are key parts of several federal programs in the US (Dix et al., 2007; National Toxicology Program, 2004). The US EPA ToxCast program (Dix et al., 2007) is using a variety of high throughput tests and computational methods to enhance prioritization of compounds for targeted testing in animals. A professed goal of the new interagency collaboration is predicting high dose results in animals. Prioritization and predicting high dose results are not part of the NAS vision. Nonetheless, the tools and approaches being developed in this collaboration will be important for achieving the long-term vision for transforming toxicity testing. Other tools will mature from efforts that are today primarily focused on animal alternatives (e.g., Spielmann et al., 2000).

In the past year, the Humane Society of the United States (HSUS) and its affiliates, the Humane Society Legislative Fund (HSLF) and Humane Society International (HIS) have taken steps to enlist partners to a stakeholder consortium – The Human Toxicology Project Consortium. The goal of this group is to facilitate the global shift to a cell response pathway paradigm for chemical safety assessments. This shift, in the words of the consortium, holds great promise for more rapid predictions of human health outcomes while superseding traditional animal testing for environmental agents and pharmaceuticals. The goals of this consortium is to (1) promote dialogue, information sharing and establishment of a research and development roadmap, (2) lobby for, coordinate and provide resources to support transatlantic efforts necessary to fulfill NAS vision, (3) engage in collaborative outreach to legislative, regulatory, corporate, academic and public interest audiences, and (4) to urgently develop a targeted research program to jump-start the transformation.

This targeted research plan, focusing on proof of concept efforts, would first focus on prototype compounds and provide examples of the application of results from toxicity pathway assays for risk/safety assessments sequentially rather than waiting 10 to 20 years to bring a totally new risk assessment paradigm on line. The proposed research over a 5 to 10 year period would provide examples with ten to fifteen pathway assays and generate opportunities for diverse stakeholders to gain experience in collecting and using these results for safety assessments. The outline of steps for this more targeted research program includes several components.

– Select about 10 prototypes compounds/pathway. These compounds would be chosen based on the breadth of information about animal toxicity and of the expected toxicity pathway targets, serving as a test bed for examining relationships between *in vitro* toxicity test assays and historical information regarding *in vivo* results.

– Design appropriate cell-based toxicity assays. For these prototype compounds, test assay systems would focus on both rodent and a human assay, preferentially using stem cells or mature cells derived from these stem cells. With one or more of the prototypes, 3-dimensional tissue systems could be used for the assays.

– Develop the next generation quantitative risk assessment tools. These assays would be subjected to mapping and modeling analysis to uncover pathway circuitry, the dynamics of pathway responses to positive controls, and the dose response behaviors expected from different levels of perturbation.

– Examine relationships between perturbations and toxicity for prototypes. The assay design would require consideration of cascades that contain initial target activation, adaptive responses, and adverse responses with prolonged levels of perturbation.

– Integrate results from studies to provide representative health risk/safety assessments. The outcome of each of the prototypes would be risk/safety assessments that would be compared to more conventional approaches from animal toxicity data sets.

– Within the first 3-5 years expand from the first 10-prototypes to a larger suite of pathways/compounds. This transition should also allow some mid-course correction in the strategy, stemming from a continuing evaluation of successes and challenges in applying the new science in assisting human health safety assessment.

– With success in getting the program jump-started through the consortium, other partners, including toxicity testing organizations, regulatory agencies, and federal research organizations, could be enlisted as partners in moving forward with the transformation.

Regardless of which organization seizes leadership for the efforts to create the technology base for shifting to a new "Gold Standard," the central question is whether such an initiative is a good public health investment. From the point-of-view of sparing animal use and a more humane infrastructure for testing the answer has to be yes. Is it also likely to be a good investment in terms of its likely scientific value? The answer here is also a resounding yes! Our primary investment in toxicity testing today is simply box-checking, becoming a bit more mechanistically oriented for high value chemicals that show responses in animal toxicity tests. The *in vitro*, human biology approach, elaborated here and arising from the NAS vision, has a much reduced emphasis on rote testing and much increased emphasis on generating detailed understanding of the signaling pathways affected by chemicals and how perturbations/modulations in these pathways affect biological outcome. These tools and ap-
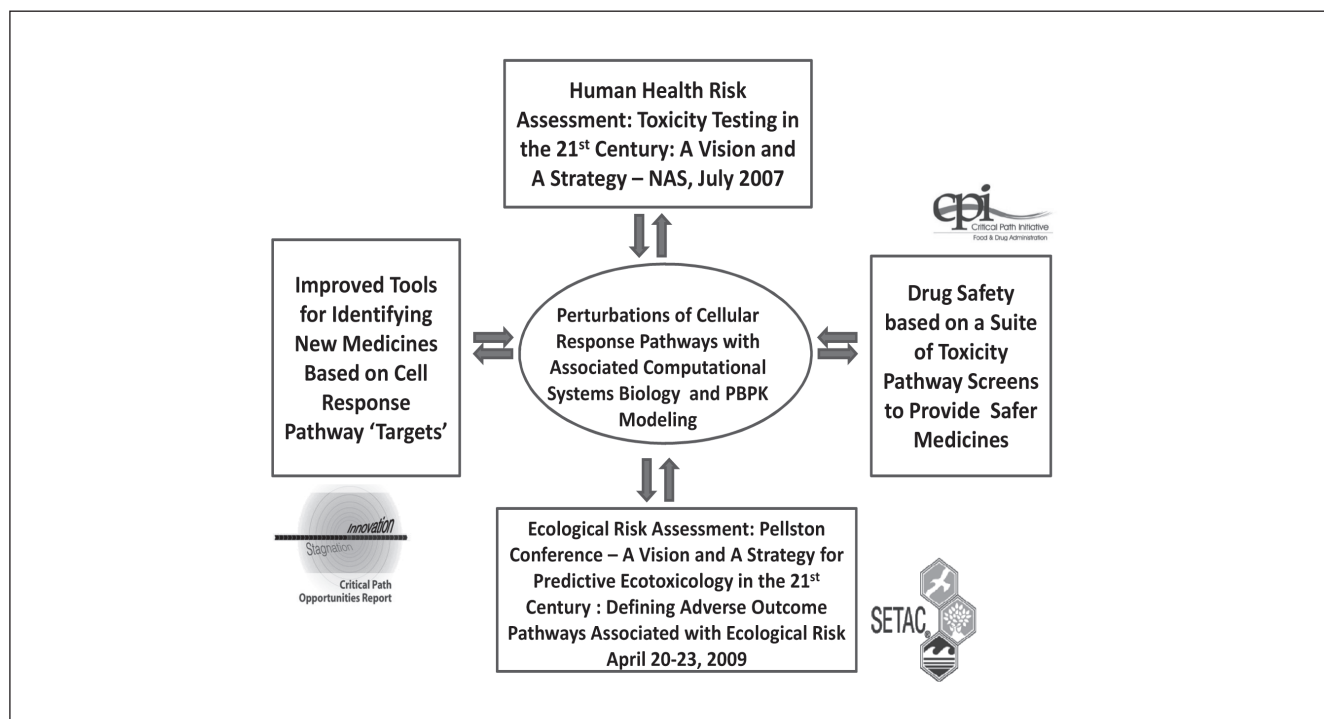
Fig. 4: Commonality of systems approaches to examining perturbations/modulations of normal biology for safety testing with environmental agents, pharmaceuticals, consumer products, and foods and for drug development. SETAC: Society for Environmental Toxicology and Chemistry.

proaches will be just as valuable in drug safety/drug development, in evaluation of safety of food and consumer products, and in ecotoxicology (Watanabe et al., in press). In addition to the broader applicability for human health outcomes for modulation of these pathways (Fig. 4), the organization of information on pathway structure and function is a natural post-genome program that could provide a better understanding of health, disease and susceptibility within the human population – a much preferred investment compared to today's approach of *in vivo* testing and the cataloging of testing results.

6 Conclusions

Toxicity testing and much of the discipline of toxicology have reached a tipping point. Old practices focusing primarily on high dose studies to evaluate end-organ toxicity in animals are giving way to modern practices that assess how chemicals are likely to affect human biology and the concentrations under which these effects might be expected in exposed humans. This change will not occur easily. Even though the current toxicity testing is far from optimal, it is difficult to move away from entrenched traditional practices to a new footing. Change of this magnitude is discomfiting for most everyone. There are, of course, serious challenges to consider in such a transformation. They should not be dismissed or diminished. Chemical toxicity may relate to metabolism. How will the *in vitro* tests adequately assess metabolites with new compounds undergoing *in vitro* screening? Which of the observed perturbations will be considered

appropriate for the safety assessment – will it be target activation, adaptive responses, or only some clear definition of overt toxicity in the cells? Will it be possible to describe circuitry for most toxicity pathways in enough detail to be confident in expected dose response behaviors? Finally, where will we find the scientists and regulators with the training and background to be comfortable with new practices? These issues are all important, legitimate questions that need to be considered. Yet, they should not divert us from the goal – to move toward a redefinition of the toxicity testing gold standard that focuses on human biology and perturbations of human toxicity pathways *in vitro*. We must bear in mind all the challenges, but push relentlessly toward the goal of a modern approach to human safety assessment.

References

Aldridge, B. B., Burke, J. M., Lauffenburger, D. A. and Sorger, P. K. (2006). Physicochemical modelling of cell signalling pathways. *Nat. Cell. Biol. 8*, 1195-1203.

Alon, U. (2007). Network motifs: theory and experimental approaches. *Nature Reviews Genetics 8*, 450-461.

Alon, U. (2006). *An Introduction to Systems Biology: Design of Biological Circuits*. Boca Raton, FL.: CRC Press.

Alonso, L. and Fuchs, E. (2003). Stem cells of the skin epithelium. *PNAS 100*, 11830-11835.

Amit, I., Citri, A., Shay, T. et al. (2007). A module of negative feedback regulators defines growth factor signaling. *Nature Genetics 39*, 503-512.

Andersen, M. E. and Krewski, D. B. (2009). Toxicity testing in

the 21st century: Bringing the vision to life. *Toxicol*. *Sci*. *107*, 324-330.

Andersen, M. E., Dennison, J. E., Thomas, R. S. and Conolly., R. B. (2005). New directions in incidence-dose modeling. *Trends Biotechnol*. *23*, 122-127.

Bouvier d'Yvoire, M., Prieto, P., Blaauboer, B. J. et al. (2007). Physiologically-based Kinetic Modelling (PBK Modelling): meeting the 3Rs agenda. The report and recommendations of ECVAM Workshop 63. *ATLA 35*, 661-671.

Bromberg, K. D., Ma'ayan, A., Neves, S. and Iyengar, R. (2008). Design logic of a cannabinoid receptor signaling network that triggers neurite outgrowth. *Science 320*, 903-909.

Clewell, H. J., III, Tan, Y. M., Campbell, J. L. and Andersen, M. E. (2008). Quantitative Interpretation of Human Biomonitoring Data. *Toxicol*. *Appl*. *Pharmacol*. *231*, 122-133.

Coecke, S., Ahr, H., Blaauboer, B. J. et al. (2006). Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. *ATLA 34*, 49-84.

Collins, F. S., Gray, G. M. and Bucher, J. R. (2008). Toxicology. Transforming environmental health protection. *Science 319*, 906-907.

DeCoppi, P., Bartsch, G., Jr., Siddiqui, M. M. et al. (2007). Isolation of amniotic stem s-cell lines with potential for therapy. *Nat*. *Biotechnol*. *25*, 100-106.

DeJongh, J., Forsby, A., Houston, J. B. et al. (1999). An integrated approach to the prediction of systemic toxicity using computer-based biokinetic models and biological in vitro test methods: overview of a prevalidation study based on the ECITTS. *Toxicol*. *In Vitro 3*, 549-554.

Dix, D. J., Houck, K. A., Martin, M. T. et al. (2007). The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol*. *Sci*. *95*, 5-12.

Gaudio, E., Carpino, G., Cardinale, V. et al. (2009). New insights into liver stem cells. *Digestive and Liver Disease 41*, 455-462.

Gulden, M. and Seibert, H. (2003). In vitro-in vivo extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro. *Toxicology 189*, 211-222.

Heringa, M. B., Schreurs, R. H. M. M., Busser, F. et al. (2004). Toward more useful in vitro toxicity data with measured free concentrations. *Environ*. *Sci*. *Technol*. *38*, 6263-6270.

Inglese, J., Johnson, R. L., Simeonov, A. et al. (2007). High-throughput screening assays for the identification of chemical probes. *Nat*. *Chem*. *Biol*. *3*, 466-479.

Inglese, J., Auld, D. S., Jadhav, A. et al. (2006). Quantitative high-throughput screening: A titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc*. *Natl*. *Acad*. *Sci*. *USA 103*, 11473-11478.

Khetani, S. R. and Bhatia, S. N. (2008). Microscale culture of human livers cells for drug development. *Nat*. *Biotechnol*. *26*, 120-126.

Krewski, D., Andersen, M. E., Mantus, E., and Zeise, L. (2009). Toxicity testing in the 21st century. *Risk Analysis 29*, 474-479.

Motohashi, H. and Yamamoto, M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol*. *Med*. *10*, 549-557.

National Research Council (NRC) (2007). *Toxicity testing in the 21st century: a vision and a strategy*. Washington, DC: National Academy Press.

National Research Council (NRC) (2006). *Toxicity testing for assessment of environmental agents: interim-report*. Washington, DC: National Academy Press.

National Toxicology Program (NTP) (2004). *A national toxicology program for the 21st century: A roadmap for the future*. NTP, NIEHS, Research Triangle Park, NC. (http://ntp.niehs.nih.gov/vision).

Nel, A., Xia, T., Madler, L. and Li, N. (2006). Toxic potential of materials at the nanolevel. *Science 311*, 622-627.

Reddy, M. B., Yang, R. S. H., Clewell III, H. J. and Andersen, M. E. (eds). (2005). *Physiologically Based Pharmacokinetics: Science and Applications*. Hoboken, NJ: John Wiley & Sons, Inc.

Reya, T. and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature 434*, 843-850.

Slikker, W. Jr., Andersen, M. E., Bogdanffy, M. S. et al. (2004a). Dose-dependent transitions in mechanisms of toxicity. *Toxicol*. *Appl*. *Pharmacol*. *201*, 203-225.

Slikker, W. Jr., Andersen, M. E., Bogdanffy, M. S. et al. (2004b). Dose-dependent transitions in mechanisms of toxicity: Case studies. *Toxicol*. *Appl*. *Pharmacol*. *201*, 226-294.

Spielmann, H., Müller, L., Averbeck, D. et al. (2000). European Centre for Validation of Alternative Methods. *ATLA 28*, 777-814.

Wang, Y., Yao, H. L., Barbier, C. et al. (2009). Matrix signals replacing lineage-dependent mesenchymal reeders for growth versus differentiation of hepatic stem cells to adult fates. *J*. *Experimental Med*., in review.

Watanabe, K. H., Andersen, M. E., Basu, N. et al. Development of computational models of known adverse outcome pathways: domoic acid and neuronal signaling as a case study. *Environmental Toxicology and Chemistry*, in press.

Xiao, G. G., M. Wang, N. Li, J.A. et al. (2003). Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. *J*. *Biol*. *Chem*. *278*, 50781-50790.

Zhang, L., Theise, N., Chua, M. and Reid, L. M. (2008). The Stem Cell Niche of Human Livers; Symmetry between Development and Regeneration. *Hepatoloy 48*, 1598-1607.

Zhang, Q. and Andersen, M. E. (2007). Dose response relationship in anti-stress gene regulatory networks. *PLoS Comput Biol*. *3(3):e24*. Epub 2006 Dec 22.

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