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Expediting toxicity testing with increased precision, predictive power, and clinical utility

Peter Pressman[1], A Wallace Hayes[2], and Roger Clemens[3]

Abstract

Federal Government management of health risks associated with the use of therapeutics and unintended environmental chemical exposures must be expedited to meet public health needs. Although US agencies initiated the Tox21 strategy over a decade ago to expedite toxicity testing and improve the reliability of risk assessments, recent status reports indicate that achieving its goals is still decades away. Emerging technologies create an opportunity to both expedite toxicity testing and improve its predictive power. The way forward may be an augmentation of the strategy aimed at enhancing the resolution and scope of Tox21 and exploring the adaptability of real-time chemical sensor, digital imaging, and other technologies to toxicity testing. Among the anticipated returns on the associated investment would likely be enhanced accuracy in prediction, reductions in the time needed to conduct hazard identifications and toxicity assessments, and an overall increase in the precision and reliability of the risk assessment process. This in turn expedites risk management decisions and reduces scientific uncertainty and the need to incorporate margins of safety that can add cost without necessarily returning improved health protection.

Keywords

Toxicity testing, Tox21, predictive toxicology, pathways, hazard, risk, chemical sensors, neurite outgrowth, nanotransfection, real time, imaging, biomarker

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Introduction

There are more uncharacterized chemicals in use today than there are chemicals for which the exposure risks have been assessed. On the food and nutrition front alone, the explosion of nutritional supplements, cannabinoids, botanicals, bioactives, and nanoactives has further increased the need for more efficient and accurate screening and characterization. This situation, and the imperatives to reduce both toxicity testing costs and the number of animals used, has been recognized as drivers for change.

Toward addressing these issues, the International Life Sciences Institute, the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research, and other national and international research organizations have implemented programs aimed at making toxicity assessment more efficiently. In the United States, the National Institute of Environmental Health Sciences created the National Center for Toxicogenomics way back in September of 2000. A principal scientific objective was to create a reference knowledge base of chemical profiles of omics (genomics, transcriptomics, proteomics, and metabolomics) and to demonstrate the utility of chemical signature profiling. The concept was to use the accumulated information to define the mechanisms of toxicity and to predict the potential toxicity of chemicals and drugs. The

[1] The Daedalus Foundation, Mt. Vernon, VA, USA
[2] T.H. Chan School of Public Health, Harvard University, Boston, MA, USA
[3] USC School of Pharmacy, University of Southern California, Los Angeles, CA, USA

Corresponding author:
Peter Pressman, The Daedalus Foundation, PO Box 96, Mt. Vernon, VA 22121, USA.
Email: drpressvm2@gmail.com

proof-of-principle approach was to phenotypically anchor the profiles to conventional parameters of toxicity. Important to the issues discussed herein, the goals also included the definitions of dose and time relationships that precede the development of toxicity.[1,2]

Although enormous amounts of data were generated by the high-throughput technologies and made available in public databases, attempts to validate the predictive power of computational toxicogenomics were mostly unsuccessful. With the exception of a small number of chemicals that act through chemical-specific receptor-binding mechanisms, the initial computational models failed to predict previously published toxicities revealed by conventional animal toxicity testing.[3]

Eduati et al.[4] reported the results from the community-based Dialogue on Reverse Engineering Assessment and Methods challenge to predict toxicities of environmental compounds with potential adverse health effects for human populations. This exercise served as a measure of the progress of the Tox21 initiative, with a particular focus on the Tox21 1000 Genomes Project. It is worth emphasizing that the primary public health mandate for the field of toxicology is achieving the capacity for prediction of a toxic response in a population. The natural extension of this mandate is to establish safe levels of exposure to new and legacy compounds including naturally derived compounds and to identify individuals at increased risk for adverse health outcomes. The clinical reality is that the current risk assessment paradigm neither fully accounts for individual genetic differences in chemical exposure response nor does it provide analytical illumination of the behavior of substance isoforms or of the pharmacokinetics of novel interventions such as nanomedicines. Moreover, routine safety evaluation is performed on a small fraction of existing environmental compounds and generally relies on animal models that are costly, time-consuming, and do not always reflect human safety profiles. In silico predictions of risks to safety provide efficient and cost-effective approaches but are one-dimensional tools for the identification of potential health risks to specific populations. Prediction algorithms have undoubtedly been limited by lack of data about population variability and difficulties in extrapolating from model organisms.

The development of automated, high-throughput, target/pathway-specific, in vitro toxicity studies using human-derived cell (e.g. lymphoblastic) models has facilitated the characterization of phenotypically distinct populations in response to environmental chemical or drug exposure. This approach, theoretically, enables rapid, systematic, and economically desirable toxicity screening of a wide range of compounds in human cell lines in an effort to predict population-level responses and also to examine variation in risk profiles across individuals. As noted above, thus far, these technologies and the associated algorithms are able to predict cytotoxicity traits based on genetic profiles and chemical structure with only higher than random accuracy, that is, with very modest or "suboptimal" success.

Tice et al.[5] comprehensively inventoried the obstacles to successful prediction:

> "Perfect" assays do not exist. Coverage of all chemicals of interest is incomplete. A high throughput system for measuring the free concentration of a compound in vitro is not yet available. Xenobiotic metabolism is lacking in virtually all in vitro assays. Interactions between cells are poorly captured. Distinguishing between statistical and biological significance is difficult. Extrapolating from in vitro concentration to in vivo dose or blood levels is not straightforward. Assessing the effects of chronic exposure conditions in vitro is not possible. Identifying when a perturbation to a gene or pathway would lead to an adverse effect in animals or humans remains a challenge. Achieving routine regulatory acceptance of the developed prediction models is years away.

FDAs predictive toxicology roadmap

In December of 2017, the US Food and Drug Administration (FDA) published a document entitled, "FDA Predictive Toxicology Roadmap (https://www.fda.gov/downloads/ScienceResearch/SpecialTopics/RegulatoryScience/UCM587831.pdf)."

While it mentions the Tox21 initiative, and notes that it "relied heavily" on recommendations derived from Tox21 progress, there are no other specific references to any apparent progress that Tox21 has made. The FDA report goes on to call for "enhanced FDA engagement" and an invigoration and strengthening of methods and new technologies for the "better prediction of responses to the wide range of substances relevant to FDA's regulatory mission." The report then lays out a very general inventory of pragmatic goals that are clearly seen as yet unmet. It also identifies and calls for application of "promising new technologies" such as microphysiological systems, that is, "organs on a chip."

We believe that the very existence of this FDA mandate is a remarkable and implicit reaction to the rather slow and disappointing rate of progress of the ambitious Tox21 programs. What follows are our recommendations in support of the FDA mandate.

Neurite outgrowth assay

So, how do we facilitate the odds of clearing the obstacles to achieving clinically relevant toxicology? To begin with, we would assert that certain assays that perhaps have not been viewed as readily automated also must be perfected, standardized, and somehow coherently integrated with the developing scheme. An important example is neurite outgrowth (well-described by Ryan et al.[6]). To date, there are no routinely performed assays to evaluate chemical-specific effects on the nervous system. Ongoing efforts

have focused on an outgrowth assay as a possible screen to explore the ability of compounds to adversely affect the developing nervous system. This assay was selected due to its reflection of a critical process in nervous system development in which neurons extend their neurites to form complete networks. Disruption of this process can lead to adverse effects in humans and rodents, and studies suggest that immature, developing, and mature neurites are targets of chemical toxicity (reviewed by Krug et al.[7]). The assay can also be used to evaluate neurodegeneration as measured by neurite retraction. Finally, we would concur that this assay is adaptable and scalable to high-throughput, which would be required for screening the large numbers of compounds in commerce with little or no preexisting toxicological data.[8] In the Ryan study, neurons differentiated from human-derived induced pluripotent stem cells were used since they are more directly relevant to the human nervous system and, hence, are rapidly gaining impetus as a screening tool in drug discovery and safety assessment for central nervous system disorders.

Tissue nanotransfection

Another emerging technology that we believe should be considered for incorporation into an evolving Tox21 is that of tissue nanotransfection (TNT). This topical, nonviral in vivo approach allows direct cytosolic delivery of reprogramming factors by applying a highly intense and focused electric field through arrayed nanochannels which nanoporates cell membranes and electrophoretically drives reprogramming factors into the cells.[9] A chip loaded with selected nucleic acids is placed on a specific patient's own cells, and a small electrical current is applied, which creates channels through which the genetic material may be injected. Epithelial cells can thus be readily reprogrammed and transformed into endothelial cells. The implications for broadening toxicologic sensitivity and predictive precision seem very significant.

Translational research and standardized measurement precision

Fundamental to the traditional toxicity testing paradigm is the concept that animals, and what we understand about animal physiology and pathophysiology, can serve as models for human disease processes. This concept acknowledges the significant differences between hominids and lower animals but recognizes that many human gene sequences have been conserved among mammals since the beginning of time. A core Tox21 strategy attempts to address the limitation of traditional toxicity testing by generating new knowledge of cross-species shared toxicity pathways. However, the growing body of evidence, generated from research on omics, regulatory RNAs, epigenetics, and systems biology, challenges the ability of the putative common pathway approach to meet the outstanding need.

Homeostatic versus adverse outcome pathways: A dynamic balance

In the absence of standardized measurement precision needed to elucidate species and organ-specific pathway dynamics, the need to expedite and improve the reliability of hazard identification and predictive toxicology remains outstanding. We must be able to distinguish the specific dose and time at which pathways that maintain cellular homeostasis become saturated, blocked, degraded, or otherwise dysfunctional. We must elucidate the dynamics of molecular events that begin to favor pathways that lead to adverse outcomes, that is, toxicity. The U-shaped dose–response curve of essential nutrients is illustrative of this concept.

Consumption of nutrients below the recommended daily allowance (RDA) evokes a risk of an adverse effect due to deficiency. At doses above the RDA and below the tolerable upper intake limit (UL), the molecular pathways are in a dynamic balance that favors homeostasis. As the dose increases beyond the UL, the balance favors pathways leading to toxicity and significant adverse events.

The common analgesic, acetaminophen, is just one of many compounds that can be used to further illustrate the challenge of defining pathway dynamics (Figure 1). At therapeutic dose rates, acetaminophen is conjugated in the liver with glucuronic acid and sulfate and then excreted in the urine.[11] A minor pathway is oxidation by cytochromes P450 to a reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI).[12,13] NAPQI usually undergoes subsequent conjugation with glutathione (GSH) to cysteine and mercapturate and the conjugate is excreted in the feces. At toxic dose rates, acetaminophen causes potentially fatal necrosis of the centrilobular region of the liver as hepatic GSH becomes depleted. As a result, the pathway dynamics favor covalent binding of the reactive NAPQI to cysteine groups forming protein adducts.[14,15] Formation of these adducts is correlated with the development of toxicity.

Among the challenges enumerated by Tice et al.,[5] the most critical step to the protection of public health is "coverage of all chemicals of interest is incomplete." We suggest that a critical weakness of contemporary strategies is the lack of sufficient precision to distinguish the balance between potential pathways that maintain homeostasis, such as the acetaminophen conjugation and elimination pathway and the adverse outcome pathway involving metabolism to NAPQI.

The precision of the time measurement: It's the dose rate that makes the poison

As the acetaminophen example illustrates the toxicological significance of pathway dynamics, so too it illustrates the toxicologically significance of dose rate. Just as street maps cannot predict traffic flow, the definition of toxicity pathways cannot predict toxicity in the absence of knowledge of

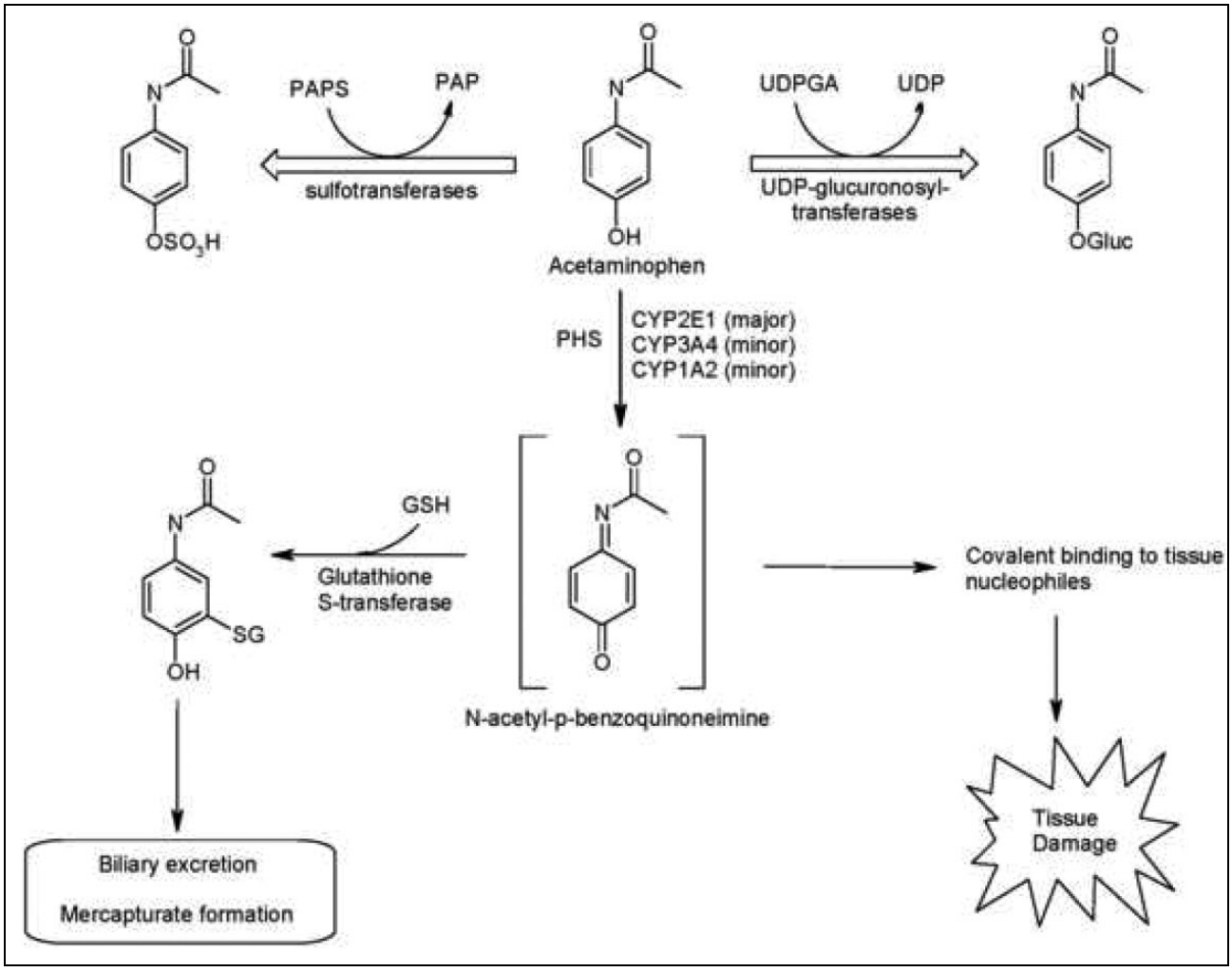

Figure 1. The dynamic equilibrium between pathways that detoxify acetaminophen (open arrows) and pathways that lead to toxicity is shown. The toxicity pathway starts with acetaminophen being oxidized to NAPQI, with subsequent covalent binding to tissue nucleophiles and tissue damage. *Source*: Adapted with permission from Wallace Hayes.[10] NAPQI: *N*-acetyl-p-benzoquinoneimine.

the molecular dynamics. This knowledge can only be gained by increasing the precision with which dose, time, and location are measured.

The rate constants of toxicokinetic and toxicodynamic parameters used to model each molecular component of the pathways are measured in seconds. In this context, neither traditional toxicity testing nor the Tox21 approach has the measurement precision required to reveal in vivo pathway dynamics. In traditional animal testing methods, dose rate is measured in terms of milligrams per kilogram per day (i.e. the weight of the test chemical per kilogram weight of the test animal per day). In the in vitro assays being implemented by the Tox21 consortium, dose is commonly measured in terms of concentration only. These are fundamental weaknesses in both approaches to modeling of stressor-induced human pathology and are captured in the Tice et al.'s[5] inventory of research constraints: "a high throughput system for measuring the free concentration of a compound *in vitro* is not yet available" and "extrapolating form *in vitro* concentration to *in vivo* dose or blood levels is not straightforward."

The inability to measure dose and response on a time scale that is capable of revealing pathway dynamics is the single largest source of uncertainty that reduces the reliability of our health risk assessment paradigm. Perhaps, this weakness has been overlooked/tolerated because, up until recently, the technologies needed to capture real time and precise dose–response measurements have not been available. With the emergence of real-time sensor and real-time imaging technologies, this is no longer the case.

Discussion and recommendations

The strategy of the systems toxicology approach is to describe the toxicological interactions that occur within a living system under stress and use the omics profiles generated in one species to help predict the toxicity pathways of similar stressors in other species. The overarching risk

management goal is to use newly generated knowledge of early pathway events to predict downstream toxic effects and intercede either pharmacologically or by reducing exposure.

While we recognize value in the Tox21 approach to identifying adverse outcome pathways, we also recognize that it is not meeting an ever-growing need. The hazard identification and toxicity assessment processes must be expanded, expedited, and made more reliably predictive. In the face of the outstanding need, and in addition to expansion of meaningful assays that could provide convergent validity, we recommend an extension of the current strategy. First, we must expedite toxicity testing by returning the focus on molecular biomarkers of adverse effects as the response-to-be-measured. Second, we should investigate the potential to both expedite the processes and reduce the inherent scientific uncertainties in agencies' toxicity testing methodologies by improving the spatiotemporal precision with which dose and response are measured. This can be done by applying real-time imaging and sensor technologies to capture the relevant molecular dynamics. The strategy is to label and image the molecular components of the homeostatic and adverse outcome pathways, for example, the biomarkers of exposure, biomarkers of molecular mechanisms, and biomarkers of pathology. It certainly seems possible to measure the molecular dynamics, if not concurrently, perhaps in sequence.

While the elucidation of molecular pathways is essential knowledge for drug development and future nutrient safety assessments, let us also suggest that the knowledge most relevant to predictive toxicology remain the downstream apical molecular pathologies. It may be justified to argue that it seems reasonable to return the focus to downstream pathologies. This may be interpreted as giving up the strategic advantage of being able to predict the downstream adverse effects from the upstream pathway events. However, we suggest that (a) the growing need to expedite toxicity testing and (b) the technical challenges of identifying and elucidating the dynamics of pathways drive us to refocus on apical end points.

Yes, it is the dose that makes the poison, but more precisely, it is the dose rate and duration that actually makes the poison. Traditional animal toxicity testing adjusts experimental dose by increasing the dose per unit time, that is, milligrams per kilogram per day. We suggest that hazard identification and toxicity assessment can be both expedited and made more precise by embracing new time dimensions for experimental dosing. This clinical reality-driven approach would include a systematic reduction in the time over which the selected doses are delivered. The goal is to capture the response in terms of dose rates that more closely approximate cellular toxicokinetics and toxicodynamics. The technologies that are enabling this approach are miniature chemical sensors that are capable of returning laboratory-quality data in real time. Insertion of such electronics into biological systems has been limited by persistent irritation and engineering difficulties. More recently, however, wireless cellular-scale opticoelectronic devices injected into brains of mice were well-tolerated and functional for months. The investigators were able to induce and measure gene expression from the free-moving animals.[16]

Further emerging light-emitting diode technologies are capable of delivering improved signal-to-noise ratios by exciting chemical-specific fluorescence in a spectral region, the deep ultra violet range, where there is no natural background fluorescence.[17] It is noteworthy that the applicability of sensor technology to toxicity testing was recognized by the authors of National Research Council's 2012 report titled, "Exposure Science in the 21st Century: A Vision and A Strategy."[18] However, to capture the full potential benefits, digital imaging technologies need to be applied alongside the real-time chemical sensors described in the previous paragraph.

Just as innovative sensors are delivering extraordinarily precise spatiotemporal dose measurement, clinical imaging technologies are enabling unprecedented spatiotemporal measurement of disease processes. We suggest that Tice et al.'s[5] constraints, "interaction between cells are poorly captured," "distinguishing between statistical and biological significance is difficult," and "assessing the effect of chronic exposure conditions *in vitro* is not possible," can be addressed by applying chemical sensor and digital imaging technologies in whole animal studies such as those of the National Toxicology Program. We envision that these technologies, in combination, will transform the familiar dose/response curve to a dose–rate/response/time topography. We also anticipate that growing precision in measuring (1) dose, (2) time, and (3) location will eventually become refined enough to reveal organ-specific dynamic interactions between homeostatic, adaptive, and adverse outcome pathways. In this context, these technologies are essential tools for meeting the goal stated in the 2007 "Toxicity Testing in the 21st Century: A Vision and A Strategy," that is, development of a systems' approach to describe the fundamental biologic events involved in toxicity pathways and definition of cellular circuits and their perturbations by drugs and other chemicals.

Adapting real-time chemical sensors and digital imaging to toxicity testing requires the coordinated efforts of individuals from diverse fields within the health sciences, bioinformatics, and the optics/electronics engineering communities. Although a dedicated cross-disciplinary workforce does not exist, the underlying ethics of Tox21 certainly defines a fusion of traditional toxicology and multidisciplinary high-tech. It is tempting to speculate and call for a vehicle that includes (a) an interdisciplinary training grant program and (b) a new technology transfer program aimed specifically at exploring the potential to expedite and improve the precision of toxicity testing by applying emerging technologies.

Methods such as the neurite outgrowth assay and adaptation of microphysiological systems such as TNT and tissues on a chip can and must be readily adapted for cost-effective and routine use, perhaps in clinical toxicology modules. A very pragmatic, clinical utility approach similar to that which gave rise to the modern clinical microbiology laboratory may bring this recommendation close to realization.

We recognize the conundrum of current fiscal realities in which federal agencies must do more with less. Aggressively pursuing partnerships with private industry and the academy may offer an opportunity to more fully realize the potentials of the Tox21 program. We encourage the federal agencies' research program managers to placing the highest value on fully operationalizing Tox21 and genuinely expediting hazard identification and toxicity assessments. We suggest the potential health and economic benefits to be achieved merit federal spending on the scale of that invested in the human genome project.

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