TOXICOLOGICAL HIGHLIGHT

Hypoxia Response: A Model Toxicity Pathway for High-Throughput Screening

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The identification of toxicity pathways associated with known adverse health effects combined with engineered cellular assays that measure perturbations of these pathways is the keystone to the successful implementation of the recently formulated National Research Council (2007) report, Toxicity Testing in the 21st Century: A Vision and a Strategy. While the toxicology community probes biological space to define the edges of what may be confidently considered "toxicity pathways," some well-characterized cellular signaling pathways will clearly fall within any reasonable definition of the term and represent first-tier candidates for immediate testing. Hypoxia response, like several of the toxicity pathway examples cited in the NRC report such as DNA damage response and Nrf2-mediated antioxidant response, is a key cellular stress response pathway that enables a cell to combat a variety of stressors. Although, there is a long-standing debate as to whether adaptive response such as hypoxia can be considered "adverse effects," and while a imminent resolution is unlikely, these cell autonomous adaptive responses should be regarded as reflecting "perturbation" brought on by toxicant exposure that may portend dose-related adverse effects (reviewed by Simmons et al., in press). It is in this vein that a more judicious use of assays that measure adaptive biology can and should be included in strategies to identify and characterize toxic chemicals as exemplified in the paper by Xia et al. in this issue of Toxicological Sciences.

Eukaryotic cells depend on a constant supply of oxygen to generate energy-rich adenosine triphosphate (ATP) molecules needed for nearly every aspect of normal cellular function (Serkova et al., 2008). Depletion of oxygen availability to cells, hypoxia, is a component of many pathological conditions including cardiovascular disease, stroke, and cancer. While often times hypoxia is secondary to other pathological changes, for example, atherosclerosis or stroke, environmental agents such as metals and carbon monoxide can directly induce hypoxia with consequent adverse effects on health (Maxwell and Salnikow, 2004; Bani Hashemi et al., 2008). Simonides et al. (2008) demonstrated hypoxia induced by cobalt and other inducers stimulated expression of type 3 deiodinase (D3), which led to a localized reduction in active thyroid hormone T3, an important regulator of metabolic rate. Hypoxia-inducing metals such as cobalt and nickel can induce the expression of angiogenic gene products that may facilitate the angiogenesis required by neoplasias for tumor expansion (Maxwell and Salnikow, 2004). Thus, the identification and characterization of hypoxia-inducing chemicals is vital to protecting human health and the environment. To this end, the study presented by Xia et al. describes a quantitative high-throughput approach to screen a relatively large and diverse chemical library (Inglese et al., 2006; Xia et al., 2008, 2009) with well-characterized toxicities, to identify novel compounds that stimulate the hypoxia cellular response pathway.

Intracellular levels of available oxygen are constantly monitored by a family of oxygen-requiring enzymes known as prolyl hydroxylases (PHDs; Lee et al., 2007). Under normal cellular conditions, PHD destabilizes the hypoxia pathway’s key transcription factor, hypoxia-inducible factor 1 alpha (HIF-1α). Hypoxia impairs PHD enzymatic activity, thus stabilizing HIF-1α which leads to the formation of transcriptionally active HIF. Activated HIF binds DNA via the hypoxia response elements (HREs) within target gene promoters triggering the expression of genes that enable the cell to adapt to and overcome conditions of decreased oxygen by increasing oxygen transport, stimulating angiogenesis, and regulating glucose uptake (Wenger et al., 2005).

The primary screening approach adopted by Xia et al. relies upon the nodal event in hypoxia signaling, namely the binding

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of activated HIF to the HRE to stimulate the expression of a reporter gene amenable to high-throughput detection. Since HIF-1α is highly inducible and its activation is largely specific to hypoxic conditions, it is ideally suited as an assay target for such a screening strategy. The exploitation of such “screenable” targets to *voir dire* chemical libraries will undoubtedly become a common feature of toxicity testing in the future, as envisioned by the recent NRC report. This study provides an apt example of how quantitative high-throughput testing methods can and will be implemented to identify and characterize chemicals that trigger key events in well-established cellular toxicity pathways such as hypoxia.

Because promoter reporter genes, no matter how carefully conceived, can be activated by nonspecific mechanisms, Xia *et al.* devised and executed a well-conceived strategy to identify which of the HRE-stimulating chemicals tabbed in the primary screen signaled through HIF-1α activation. The authors measured the secretion of a known HIF target gene product, vascular endothelial growth factor (VEGF), from wild-type and *Hif1a* nullizygous mouse embryo fibroblasts treated with the compounds identified in the primary screen. In contrast to the HRE reporter gene, which is engineered to respond to activated HIF, expression of VEGF relies upon stimulation of the VEGF promoter containing native HREs as well as other control elements. This follow-up study ensures that the HIF activation detected in the primary screening experiment is physiologically relevant in the context of the cell-based model.

A second novel follow-up study employed by Xia *et al.* tested the newly identified HIF-1α inducers using a battery of reporter genes driven by 36 known or suspected hypoxia-responsive gene promoters to establish biological response profiles for each of the identified compounds. Three of the chemicals identified in the primary screen produced reporter gene expression profiles nearly identical to that of 1% O2, the standard hypoxic condition used in cell-based studies. Other compounds had profiles distinct from that of 1% O2, suggesting that these chemicals may stimulate HIF-1α through a mechanism other than oxygen depletion. The use of biological response profiling combined with hierarchical clustering techniques as a means to segregate chemicals that operate through a similar mode or mechanism of action is a relatively new approach to generate hypotheses about potentially novel modes or mechanisms (Huang *et al.*, 2008). Although the panel of reporter genes used for this experiment were selected based on a suspected mechanism (hypoxia), this larger strategy of grouping chemicals together based on their response profiles across a panel of biological assays is attractive because it does not require any *a priori* knowledge of a toxicant’s mechanism or mode of action.

Lastly, the work of Xia *et al.* highlights important issues that will require thoughtful contemplation moving forward, particularly those related to transitioning a suite of technologies originally developed to expedite lead identification in drug discovery to a platform that identifies chemicals of concern to toxicology. While drug discovery works diligently to avoid false positives that waste time and resources in development, toxicologists must, in addition, work to minimize false negatives, ever mindful of the impact on human health and the environment (Inglese *et al.*, 2006). This key difference between the mandates of pharmacology and toxicology should translate into different “rules” for HTS testing. Screening of small-molecule libraries for drug discovery is often conducted at a single concentration (usually 10μM). Concentration-response information such as lowest observable effect level and relative efficacies are critical and obtainable data that can be generated using a quantitative HTS approach in support of toxicity screening. Pharmacological screening usually employs relatively conservative “hit criteria” that minimize the chance of randomly generating a false positive to less than 1%. Such stringent criteria which maximize the rate of false negatives may be inappropriate for identifying potential toxicants.

Long-term trends point to the increased reliance on the use of cell-based approaches for toxicity studies. Persistent challenges with in vitro models cast a long shadow on their biological relevance, namely issues such as limited metabolic capability, kinetics and dynamics extrapolation, the lack of relevant tissue organization, and abnormal growth states (Holsapple *et al.*, 2009). Understandably, these limitations translate to reluctance on the part of risk assessors to use data generated from in vitro models to inform decisions. Consequently, much work is underway to address these issues and generate more biologically relevant models for in vitro studies. Any success will depend heavily on constructive collaborations between the developers of alternative approaches and the risk assessment community to design testing strategies that can ultimately refine or replace current testing methods in a way that does not undermine the imperative to protect human health and the environment.

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**REFERENCES**


