

**FIFRA SCIENTIFIC ADVISORY PANEL
Consultation
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**Integrated Approaches to Testing and Assessment Strategy:
Use of New Computational and Molecular Tools**

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1. Integrated Approaches to Testing and Assessment Strategy: Use of New Computational and Molecular Tools

1.1 Purpose of SAP Consultation

EPA's Office of Pesticide Programs (OPP) is committed to improving and transforming its approaches to pesticide human health and ecological risk assessment and management. The goals are to increase the efficiency, effectiveness, and accuracy of the testing and assessment process by enhancing our abilities to target the effects of concern and to base decisions on the information most relevant to the assessment. The strategy to attain this goal is to enhance and advance our ability to use an integrated approach to testing and assessment (IATA) in a consistent manner across all pesticide chemicals (OECD 2008). OPP views this as a critical time to take advantage of rapidly advancing molecular science and emerging *in silico* and *in vitro* technologies.

IATA is a progressive, tiered-evaluation approach that logically starts with hazard-based hypotheses about the plausible toxicological potential of a pesticide or group of pesticides based on their physical-chemical properties. Existing exposure and toxicity information is then combined with computer modeling and 'new' diagnostic *in vitro* (non-animal) assays to target information needs specific for a chemical or group of chemicals.

The purpose of this consultation is to seek guidance from the FIFRA Scientific Advisory Panel (SAP) about OPP's vision, strategy, plans, and initial efforts to incorporate advances in molecular science and emerging *in silico* and *in vitro* technologies (*e.g.*, structure-activity relationships (SARs), quantitative SARs [(Q)SARs], mechanistically-based *in vitro* assays, high throughput screening (HTS) assays) as components of an enhanced IATA. OPP requests your input on whether we have sufficiently articulated a sequence of events including research activities and plans to utilize new advances and tools that demonstrate a logical progression that will ultimately achieve increased efficiencies and effectiveness in the risk assessment of pesticides. To some extent, OPP already uses *in vitro/in silico* approaches, (*e.g.*, SARs, read-across, HTS assays) in certain aspects of our risk assessments (*e.g.*, for evaluating the toxicity of inert ingredients¹, metabolites and/or environmental degradates). Thus, OPP plans to build and expand on an established foundation of using a variety of tools in a tiered testing and assessment framework by systematically adding new tools and methodologies that, in particular, advance the biological understanding of the linkages between various levels of biological organization along adverse outcome pathways (AOPs) (NRC 2007, Bradbury *et al.* 2004, Dellarco *et al.* 2010, Ankley *et al.* 2010). These linkages will better enable the Agency to understand the intermediate effects from a molecular initiating event to apical adverse effects at the whole organism and/or population level. Once these linkages are established, a range of integrative tools can be used to estimate effects on higher levels of

¹ An inert ingredient means any substance (or group of similar substances) other than an active ingredient that is intentionally included in a pesticide product. Called "inerts" by the law, the name does not mean non-toxic.

biological organization. A series of SAP reviews is anticipated over the next decade as OPP prepares to adopt newly available tools, methods, and information.

Two case studies illustrate the use of mechanistic data to support chemical assessments and the importance of AOP knowledge to IATA. The first case study illustrates the use of genomic information to assess an AOP using the fungicide propiconazole and its induction of hepatocarcinogenesis (Nesnow 2009). The second case study illustrates the use of AOP information both qualitatively and quantitatively to inform the risk assessment and to identify susceptible populations using the antimicrobial triclosan as an example. In both case studies, the focus is on the AOP as a conceptual construct that portrays existing knowledge concerning the linkage between key events and an adverse outcome at a biological level of organization relevant to risk assessment. It is important to note that we are not considering the risk assessment of these example compounds; rather, we are focusing on the application of new approaches to guide data generation to illustrate the utility of different data streams within a biological framework as a scientifically rigorous approach to risk assessment. In addition, although the case-studies focus on the use of IATA approaches as they relate specifically to use in human health risk assessment, the approaches have utility for informing ecological risk assessments across multiple taxa as well. OPP seeks the SAP's guidance regarding acceptance of the approaches illustrated in the case studies to a general application to evaluating hazard and risk.

1.2 Problem Statement

Before new pesticides may be used in the United States, OPP is responsible for registering these chemicals (active and inert ingredients) under several statutes including the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Federal Food, Drug, and Cosmetic Act (FFDCA), the Food Quality Protection Act (which in 1996 amended both FIFRA and FFDCA), and the Endangered Species Act (ESA). The Agency also reevaluates the safety of existing registered pesticides on a 15-year cycle to ensure they meet current standards. Under this Registration Review program, EPA initiates the reevaluation of about 70 existing pesticides per year (http://www.epa.gov/oppsrrd1/registration_review/highlights.htm). OPP is a high volume program; there are approximately 1,100 active ingredients (including conventional, antimicrobial and biochemical) and 2,500 inert ingredients, comprising approximately 19,000 registered products with a wide variety of use patterns (including agricultural chemicals and consumer products). Each year, OPP makes over 5,000 regulatory decisions including the registration of dozens of products with new active ingredients. Additionally, OPP registers hundreds of new uses of currently registered active ingredients. Approximately 10-20 new active ingredients and 75 inert ingredients are evaluated each year (http://www.epa.gov/pesticides/fees/2010annual_report/pria_annual_report_2010.pdf).

OPP is a unique regulatory program in the EPA in that it has a strong statutory authority to collect data. Regulations in 40 CFR part 158 specify the types and minimum amounts of data necessary for chemicals under consideration for registration and/or reevaluation. These data are intended to address potential risks associated with a pesticide's use and include for example, data on a pesticide's identity, composition, potential adverse effects, exposure and environmental fate. In the risk assessment, OPP evaluates potential routes and durations of exposure and determines the potential adverse human and environmental health effects posed by the chemical to different non-target species and populations. The current testing paradigm requires extensive toxicity testing followed by selection of endpoints relevant to the risk assessment. For example, *in vivo* animal data are generated for multiple outcomes on food use pesticide active ingredients to determine which adverse effects are relevant. As such, EPA requires pesticide registrants to generate data for a wide range of endpoints (*e.g.*, two-generation rat reproductive studies; cancer bioassays in mice and rats; avian reproduction tests; fish full life cycle studies, *etc.*). For a conventional agricultural pesticide, the battery of tests, on average, costs an applicant approximately \$6 million and it takes the government approximately 15 to 36 months to review the results, at a cost to the government of approximately \$1.25 million. Inert ingredients typically have a more limited toxicological and environmental database when compared to pesticide active ingredients. Inert ingredient risk assessments are based on publicly available data on the chemical of interest, data on similar surrogate chemicals or SARs.

Each year the Agency makes over 5,000 regulatory decisions, registers dozens of products with new active ingredients and hundreds of new uses of currently registered active ingredients. Approximately 10-20 new active ingredients and 75 inert ingredients are evaluated each year. Extensive testing for a wide range of endpoints is required to support a registration action.

Despite the amount of data that may be available on pesticides, complex science issues constantly arise in the course of pesticide evaluation. The potential risk associated with technological advances, such as in the development of nanomaterials; the concern for certain toxicities such as the disruption of endocrine function; the identification of susceptible/vulnerable populations; the risk resulting from cumulative exposure to multiple chemicals; and extrapolation uncertainties across species and to environmental levels of exposure exemplify the scientific challenges the Agency regularly confronts. In short, the Agency must manage and review large amounts of data and resolve difficult scientific problems while adhering to strict schedules to meet statutory deadlines. The time it takes to make decisions affects public health, environmental protection and access to the overall benefits provided by new pest control products.

The current testing and assessment paradigm has provided a strong basis for risk management decisions for many years. However, the current approach is time consuming and resource intensive in terms of dollars and animal usage. In addition, the current approach is not easily adaptable when new issues arise (*e.g.*, novel toxicities associated with a particular subpopulation, endocrine disruption, cumulative risk). Thus, there is a need for a new agile approach that provides broad coverage of outcomes, life stages, and taxa and is readily augmented and changed as our knowledge advances. The end result is

an ability to evaluate safety with increased efficiency and effectiveness and focused on the information most relevant to an assessment of human and environmental health. The long-term solution to meeting emerging issues will not be the generation of more data faster, but rather, the determination of what specific effects data, for which chemicals, which exposures, and which populations, are essential to assess risks. In this context, the Agency would require sufficient, targeted, credible information from which to make a decision, rather than an overwhelming amount of information from which only a subset may ultimately be utilized in the decision-making process. Consistent with this view is the consideration of time and cost efficiencies associated with the generation and interpretation of toxicity and exposure data and the sound and responsible use of animals in testing. With this perspective, OPP's vision to advance an evolution in the risk assessment paradigm requires a strong foundation of the best available science.

1.3 Vision and Strategy for a New Direction

In the future, OPP will evaluate pesticides more efficiently and with greater accuracy by enhancing our abilities to target the effects of concern and to focus decision-making more quickly on the information most relevant to the assessment. This will reduce the amount of required *in vivo* testing, which will reduce the costs associated with bringing new pest management tools to the market both in terms of dollars and animals and in turn, will reduce the costs associated with evaluating that data. Concomitant to reducing costs, our increased abilities to quickly adapt the testing program to respond to new issues by, for example, examining a broader range of effects, will provide greater insight on population vulnerabilities, low dose effects and effects from mixtures. To that end, OPP envisions a better way to utilize information associated with the real-world use of pesticides such as monitoring data and incident reports in order to inform faster risk management adjustments and to provide a feedback mechanism that will improve the methods and tools that better enable more effective and comprehensive risk assessments. By achieving these outcomes, OPP will ensure that society's resources are focused on those chemicals and potential adverse effects of greatest likelihood and concern.

OPP's vision:
Evaluate pesticides more efficiently and with greater accuracy by enhancing our abilities to target the effects of concern and focus on the information most relevant to the assessment.

Technological advances in predicting chemical toxicity and exposure are absolutely essential for the Agency to be able to more efficiently and thoroughly review and assess pesticides toward anticipating and managing potential risk. EPA and research organizations around the world are developing and evaluating evolving technologies in molecular, cellular and computational sciences to supplement or replace *in vivo* testing. Research is underway to refine exposure models and develop improved methods for integrating monitoring and surveillance data into exposure assessments. EPA is committed to incorporating the use of these new techniques into regulatory decision-making as the science evolves (US EPA 2009, http://www.epa.gov/spc/toxicitytesting/docs/toxtest_strategy_032309.pdf and <http://www.epa.gov/pesticides/science/testing-assessment.html>).

This strategy is consistent with the ambitious vision founded on 21st Century tools and methodologies that the National Academy of Sciences (NAS) laid out in its 2007 report “Toxicity Testing in the 21st Century: A Vision and Strategy” (NRC 2007). The report envisions that risk assessment would shift toward the avoidance of significant perturbations of normal cellular pathways in exposed organisms by using subcellular- and cell-based assays to measure these perturbations. This includes dose-response modeling organized around computational systems biology models of the “circuitry” underlying each toxicity pathway, and *in vitro* to *in vivo* extrapolations based on pharmacokinetic models to predict tissue concentrations under specific exposure conditions. As such, information from multiple levels of biological organization represented through *in silico*, *in vitro* and *in vivo* assays would be integrated to characterize AOPs.

Hierarchical consideration of exposure and effect information in a more tailored manner in the context of the information needed for risk management is also consistent with the 2009 National Research Council (NRC) report “Advancing Risk Assessment: Science and Decisions” (NRC 2009). This report recommended that the utility of a risk assessment would be increased by dialogue between risk assessors and risk managers early in the planning process, *i.e.*, problem formulation. Furthermore, the 2009 report recommended inclusion of other stakeholders in that dialogue to ensure that the technical analyses within the risk assessment would align more closely with the risk management options and provide a more transparent analysis.

OPP’s critical path focuses on fully utilizing these 21st century tools in an integrated approach to testing and assessment (IATA). The long-term goal is to move from a paradigm requiring *in vivo* testing for “every possible adverse outcome” toward a hypothesis-driven paradigm where *in vivo* testing is targeted to the most likely hazards and risks of concern across taxa. This is a progressive, tiered-testing approach that starts with hazard-based hypotheses about the plausible toxicological and fate potential of a pesticide or group of pesticides based on their physical-chemical properties (*e.g.*, using read-across, bioavailability, bioaccumulation, and SARs to examine exposure and toxicological potential). Existing exposure and toxicity information is then combined with refined exposure models, computational toxicological models (*e.g.*, (Q)SARs), and diagnostic *in vitro* assays to narrow requirements for *in vivo* testing (US EPA 2009a, Bradbury *et al.*, 2004; van Leeuwen *et al.* 2009, Schulz 2010). This necessitates an improved understanding of chemically-induced AOPs, *i.e.*, the linkages between a molecular initiating event and an adverse outcome at the individual or population level (Ankley *et al.* 2010). Figure 1 illustrates the current activities that will enable future risk assessments based on targeted *in vivo* testing and *in vitro/in silico* based predictions. A significant foundation of information and new tools is necessary to enhance OPP’s IATA. The critical components of this foundation (highlighted in yellow in Figure 1) – knowledge bases and databases of existing information, predictive tools (*e.g.*, (Q)SAR, *in-vitro* methods, HTS assays), and AOP libraries – are currently under development. As OPP considers adopting new tools they will be evaluated using existing criteria and methods and through an iterative process of case studies and expert review. Ultimately, predictions and targeted testing will provide the basis for future risk assessments.

OPP’s strategy: Adopt 21 st century tools and apply them through IATA.
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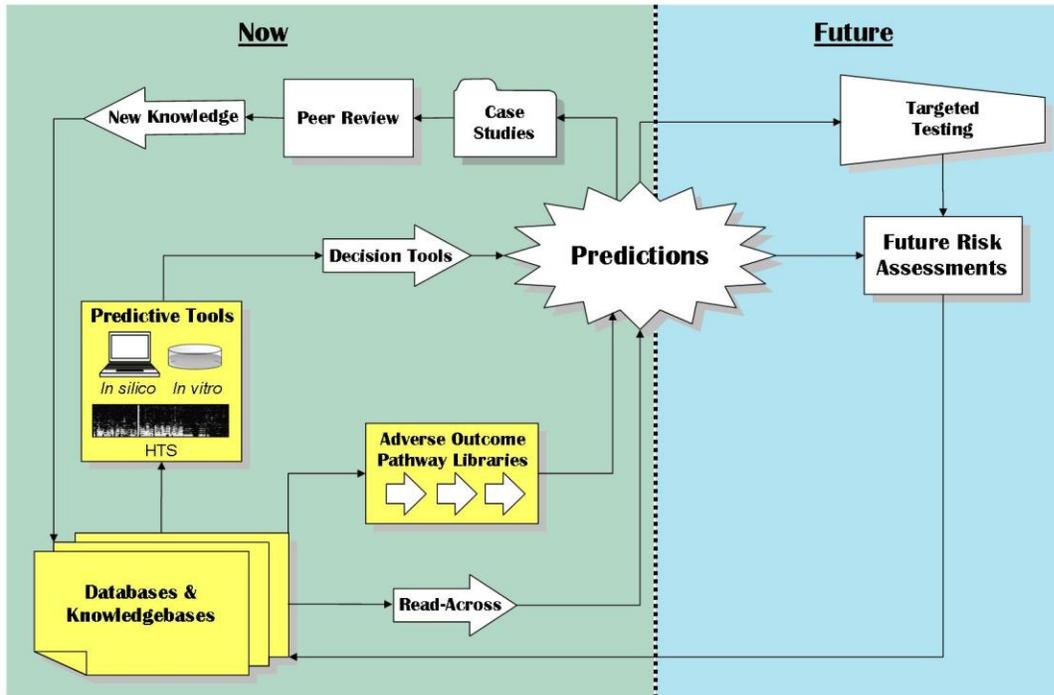


Figure 1. Critical path to an enhanced integrated approach to testing and assessment

The goal is to require only those *in vivo* tests that provide the specific data needed to address remaining uncertainties for human health and ecological risk assessments given a chemical's toxicological potential at a relevant expected environmental exposure. Moving away from a paradigm that requires extensive hazard testing will be both an evolution and a revolution. This process will take time, but as it occurs, it will result in a fundamental shift in the information we use to make decisions about pesticides.

Development and implementation of this more integrated approach to the testing and assessment of pesticides will be a challenge and will take a long time. This approach cannot be adopted and implemented overnight. Scientific tools and knowledge must be advanced and public understanding and confidence must be developed. As science advances, our regulatory structure also needs to evolve. Any changes we make in our risk assessment approaches and regulatory practice must reliably and credibly meet our safety standards. Initially, new tools will be used on a case-by-case and pilot program basis depending on the quality of the underlying science. Ultimately, OPP will explore and make changes to policies, regulations and test guidelines as appropriate. Our overarching goal is to improve the quality of environmental and public health protection through timely and effective analyses.

1.3.1 Integrated Approach to Testing and Assessment

The challenge is to develop the means to move in a scientifically credible and transparent manner from a paradigm that requires extensive *in vivo* hazard testing in which some data prove to be of limited utility (given what may already be known about the chemical or class of chemicals or that certain information is ultimately not central to the assessment), to a paradigm that provides the means to use a risk-based, hypothesis-driven approach to identify the specific *in vivo* information most relevant to the assessment. OPP's goal is to have pesticides tested in animals only for those most relevant endpoints. This will require a systematic process to evaluate the potential of each chemical to initiate molecular interactions that are the basis for causing adverse effects. To achieve this goal, a more comprehensive understanding of pathways leading to toxicity from molecular initiating events to adverse outcomes at the whole organism and/or population is required.

An AOP represents existing knowledge concerning the linkages between a molecular initiating event and an adverse outcome at the individual or population level (Ankley *et al.* 2010). As such, AOPs, by definition, span multiple levels of biological organization. Figure 2 illustrates the relationship between chemical structure and the initial molecular interaction (*e.g.*, receptor binding) and the sequent cellular responses (*e.g.*, gene activation and protein expression), organ level responses, and ultimately an adverse outcome (*e.g.*, impaired reproduction). The different data streams that inform this pathway include, for example, (Q)SARs reflective of the types of chemicals that can initiate a pathway, *in vitro* assays that measure the chemical-biological interactions, *in vitro* assays that confirm the sequent cellular responses (*e.g.*, gene expression), and ultimately *in vivo* tests that measure endpoints that are directly relevant to the adverse outcomes (*e.g.*, impaired growth, reproduction and/or survival) that drive regulatory decision making. By understanding the likelihood of effects (*i.e.*, initiation of a toxicity pathway) at lower levels of biological organization (*e.g.*, from SARs and *in vitro* assays) one could efficiently determine if more expensive and time-consuming testing at higher levels of biological organization (*i.e.*, *in vivo* assays) are needed. For a particular pesticide, *in vivo* tests could be eliminated for those outcomes that are not likely to occur because the chemical or its metabolites do not interact with the biomolecules identified in the AOP's molecular initiating event at environmentally relevant concentrations.

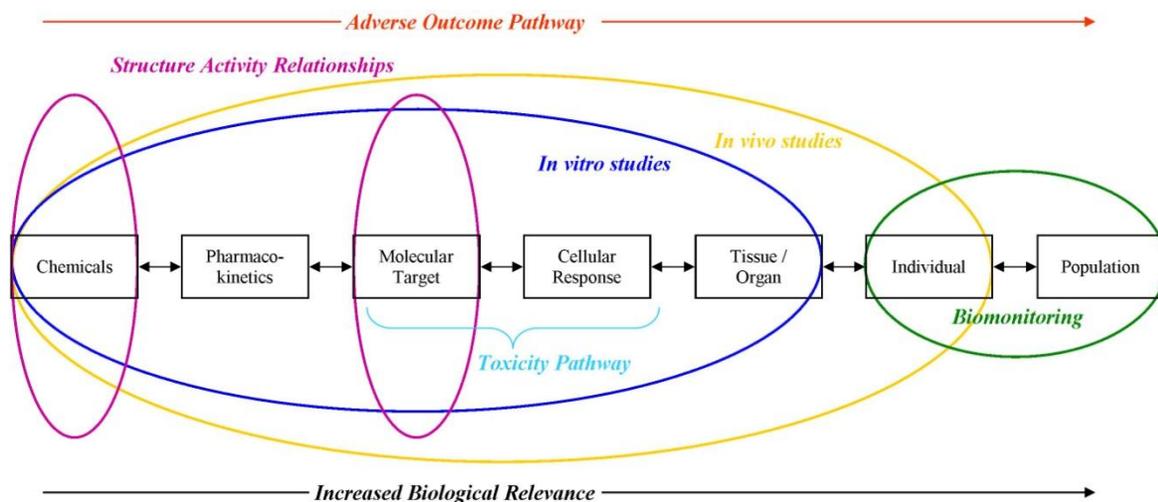


Figure 2. Adverse outcome pathway and the data streams that inform the pathway

AOP and mode of action (MoA) are similar in concept. MoA is defined as the sequence of key events and cellular and biochemical events (measurable parameters), starting with the interaction of an agent with the target cell, through functional and anatomical changes, resulting in cancer or other adverse health effects (USEPA 2005; Boobis *et al.* 2008). When available, OPP incorporates MoA information into risk assessment so as to more accurately characterize what effects may be biologically plausible in humans and the environment to inform which susceptible lifestyles or subpopulations may be more impacted by chemical exposure. For example, OPP has used information on metabolism and key events to identify reproductive and neurodevelopmental adverse outcome pathways to better characterize potentially susceptible populations (e.g., USEPA, 2008, USEPA, 2010a, 2010b).

To bring structure, rigor and transparency to the evaluation of MoA data, a framework approach is used that was developed and adopted by both EPA (USEPA, 2005) and by the International Programme for Chemical Safety (IPCS) (Sonich-Mullin *et al.*, 2001; Boobis *et al.*, 2006 also see <http://www.who.int/ipcs/methods/harmonization/en/>). This MoA framework provides a weight-of-evidence approach that is based on considerations for causality as originally articulated by Bradford Hill in epidemiologic studies, and includes considerations of dose response and temporal concordance, consistency, specificity, biological plausibility and coherence (Hill *et al.*, 1965). The MoA framework provides an important tool to promote and formalize the use of mode of action data in risk assessment regardless of whether the information comes from traditional approaches or evolving molecular and *in vitro* technologies (Seed *et al.*, 2005).

The development of AOPs builds in part on one of the key concepts of the MoA framework developed by EPA and IPCS. Once a MoA has been established, the key events can be used for read across to other chemicals, both qualitatively and quantitatively. If a new compound triggers the key events, it will potentially trigger the adverse effect identified in the MoA. The likelihood of an adverse outcome will then simply depend on a significant perturbation of key events (*i.e.*, dose response). This allows the use of existing

knowledge and concepts while grounding the results of new methods in toxicologically relevant outcomes.

Development of the toxicological understanding across a range of AOPs, from their causal molecular initiating event and subsequent key events to the adverse outcome will establish the necessary knowledge to streamline the pesticide testing and assessment to more thoroughly integrate, in a tiered fashion, existing data, computer modeling, and *in vitro* models diagnostic of AOPs to target *in vivo* testing. Thus, establishing AOPs will reduce animal testing, since, at each level of biological organization (starting with the molecular characteristics of the compound), as the potential for a chemical (or a group of chemicals) to elicit toxicological effects is refined in the context of likely human or environmental species exposure, fewer tests at higher levels of biological organization are needed. Therefore, instead of every chemical being tested for every possible endpoint in an *in vivo* testing battery, the only *in vivo* tests required are those that are essential for understanding remaining uncertainties regarding a chemical's toxicological potential at a relevant expected environmental exposure. Furthermore, once an AOP is established the information can be used to develop new quantitative predictive tools and qualitative read-across methods. Thus, AOPs enable a shift from a chemical by chemical approach to a category based approach. Given that there could be hundreds of AOPs and that varying levels of AOP information are needed depending on the decision being made (*i.e.*, the tier of the risk assessment), the Agency is seeking the Panel's guidance on principles for scientific acceptance of AOPs and the use of AOPs to move away from chemical by chemical approaches.

IATA approaches have utility for informing ecological risk assessments across multiple taxa. For example, an understanding of the biology behind an AOP in mammals could be useful for understanding AOPs across a variety of other taxa, and vice-versa. If the key molecular event(s) within an AOP are phylogenetically conserved across mammals and other taxa, then a mechanistic-based understanding of an AOP in mammals may be extrapolated to those other taxa. At a minimum, an understanding of an AOP in one species could be a useful starting point for understanding and possibly identifying AOPs in other taxa (*e.g.*, birds, fish, invertebrates, and plants) particularly where there is sufficient information regarding specific deviations in highly conserved pathways. Successfully integrating emerging technologies such as *in vitro* high through-put assays, *in silico* predictive tools such as (Q)SARs, and read-across, along with existing *in vivo* data across multiple taxa will improve our mechanistic-based understanding of the underlying biology. Conversely, an understanding of the biology of these various taxa will also enable the use of these emerging technologies to establish linkages between various levels of biological organization that will allow increased use of data from lower levels of biological organization to estimate risk at the whole organism and population level with reduced reliance on *in vivo* testing. The Agency is seeking comment from the Panel on the use of highly conserved processes to extrapolate AOP linkages across taxa and to identify methodologies to develop AOPs.

Most known AOPs derive from a long history of focused, hypothesis-driven experimentation (as described in the conazole case study). Such reductionist

experimentation, *i.e.*, understanding the nature of adverse outcomes by reducing them to the interactions of their parts (key events along an AOP), is critical, particularly for defining the cause-effect relationships that underlie the connections between key events at different levels of biological organization. However, given that there are potentially hundreds of AOPs relevant to regulatory risk assessment decisions; there is a critical need to accelerate the pace of AOP discovery and development. Once AOPs have been defined, there is a need to evaluate and build confidence in their predictive utility and determine their domains of applicability in both chemical and biological (*i.e.*, taxonomic) space.

EPA envisions that 21st century approaches including omics ², and HTS can help meet these needs. Specifically, omics can be applied to more efficiently survey the breadth of molecular/cellular effects elicited (*in vivo* or *in vitro*) by specific chemicals. Functional interpretation of omic data is generally dependent on annotations derived from existing knowledge, thereby placing some limits on the scope of novel discovery. However, as omic datasets grow sufficiently large and diverse, network inference and “reverse engineering” approaches may facilitate discovery of some novel interactions of toxicological significance. Overall, application of omics can lead to novel hypotheses regarding the linkage between molecular perturbations and specific chemical exposures or adverse outcomes, which can subsequently be tested through focused experimentation, thereby accelerating the discovery of novel AOPs.

High-throughput screening can be similarly effective for increasing the efficiency with which a wide breadth of molecular/cellular responses can be surveyed, at least *in vitro*. However, unlike omics, HTS can dramatically enhance the efficiency with which chemical space can be surveyed. Thus, HTS is well positioned to identify new/novel chemicals that would be predicted to initiate specific AOPs via interaction with specific molecular targets (molecular initiating events) or perturbation of cellular response pathways. Identification of chemicals associated with molecular initiating events can facilitate the development of QSARs. It may also accelerate the pace of AOP evaluation by providing rapid identification of chemicals that would be hypothesized to cause specific outcomes, based on their AOPs. These hypotheses can then be tested to assess the accuracy of AOP-based predictions and the supporting extrapolation tools that would be required to make the predictions quantitative.

The current AOP framework employs a simplified depiction of a linear progression of events across levels of biological organization that translate a molecular perturbation to an adverse outcome (assuming the perturbation is sufficiently severe). However, it is well recognized that AOPs actually operate as networks within a systems biology context and that various AOPs can share key events and interact with one another in a variety of ways. Reductionist experimentation has generally been characterized by a more linear mode of thinking. In contrast, because omics and HTS (viewed as a large suite or battery of assays implemented in a high-throughput manner) can efficiently cover greater breadth of molecular initiating events and/or key events and associate them with a given chemical,

² Omics is used to represent the broad group of toxicogenomics, proteomics, metabolomics, transcriptomics, *etc.*

they are, perhaps, better equipped for promoting a network-view. Ultimately, understanding of responses to stressors and effectively predicting toxicity, in a systems biology context that operates across multiple levels of biological organization, will require the kind of network-thinking that HTS and omics promote. Consequently, effective integration of data from hypothesis-driven, focused, testing, omics, and HTS represents an important foundation for an enhanced IATA. The Agency is seeking input from the Panel on how the Agency can take advantage of this foundation to speed the discovery, development, and acceptance of AOPs both qualitatively and quantitatively in risk assessments.

1.3.2 Exposure

Although this consultation is focused on toxicity, exposure is a critical consideration in fully advancing an IATA for pesticides. For the purposes of context, this section provides a brief summary of OPP activities and future plans with respect to 21st century science tools for exposure assessment.

Limitations in exposure data and models and issues associated with the use of available biomonitoring and population surveillance data confound OPP's ability to refine testing needs. Human biomonitoring data can provide information about the contribution of certain chemicals to body burden which informs potential associations with human disease. Thus, further advances in exposure science are critical to an integrated approach to testing and assessment. Exposure data are central to selecting doses for *in vitro* and targeted *in vivo* testing, interpreting and extrapolating from *in vitro* screening assay results, and identifying adverse outcome pathways. Exposure data provide an understanding of the linkage between source pathways and the resulting biological doses and adverse effects. In its 2007 report, NAS envisioned that understanding the toxicity pathways for a select group of health/environmental effects or molecular mechanisms would guide research to identify biomarkers associated with the toxicity-pathway perturbations that could be used for biomonitoring surveys and population surveillance. The information gathered through biomonitoring surveys and surveillance programs, in turn, would be critical to interpreting toxicity data and for evaluating the effectiveness of the new predictive tests and models. For example, population surveillance may indicate a human health risk that was not detected in toxicity tests. In addition, comparison of human exposure data from biomonitoring surveys with concentrations that perturb pathways in screening assays can be used to identify potentially important exposures.

Probabilistic modeling is an example of current work in the context of human exposures. The Food Quality Protection Act of 1996 (FQPA) mandated that EPA consider aggregate (single-chemical, multi-route/pathway) and cumulative (multi-chemical, multi-route/pathway) human exposure, particularly for infants and children, when making pesticide regulatory decisions. Implementation of FQPA necessitated developing new methodologies to assess human exposures resulting from food and residential use of pesticides. Probabilistic models have been recommended by the National Research Council because they allow better quantification of exposure at different percentiles of a population of interest, as well as the improved characterization of the uncertainty associated with the exposure estimates at those percentiles. OPP scientists have worked closely with ORD

scientists to develop SHEDS-Multimedia (Stochastic Human Exposure and Dose Simulation, see http://www.epa.gov/heasd/products/sheds_multimedia/sheds_mm.html). SHEDS-Multimedia is a physically-based, probabilistic model that simulates individual exposures to chemicals in food and drinking water and through residential exposures over different time periods (*e.g.*, daily, weekly, yearly). In addition, ORD scientists are working to link daily SHEDS exposure outputs to a sophisticated Bayesian-based PBPK/PD (Physiologically-Based Pharmacokinetic/Pharmacodynamic) model which will use the output from SHEDS to predict toxicant concentrations as the target organ on a temporal basis.

In the context of ecological risks, OPP typically uses monitoring studies (*e.g.*, groundwater and surface water) and incident (*i.e.*, adverse effect) data to ground truth estimated exposures and effects. OPP continues to seek out exposure data and to develop modeling approaches that allow us to describe ecological risks in both spatially and temporally explicit contexts, to support not only pesticide registrations but also endangered species assessments. Concurrently, OPP envisions extending the current 'field-scale' (*i.e.*, small-scale land model) exposure assessment methods to a 'watershed scale' in both agricultural and residential/urban settings. With these improved tools, risk management decisions can effectively target specific regions of the country and times and methods of application that would yield the greatest reductions in risk while maintaining needed pest management tools.

In order to advance 21st century science tools for exposure assessment, EPA has commissioned a second NAS committee to provide the Agency with specific feedback and advice on critical research and the science and public policy issues associated with evaluating pesticide exposure. The NAS will prepare a report with its recommendations in 2012 (<http://dels.nas.edu/Study-In-Progress/Human-Environmental-Exposure-Science/DELS-BEST-09-02>). OPP's vision for exposure science is to develop better environmental measures of exposure and enhance our ability to use tiered processes that integrate new predictive models across various classes and categories of chemicals so as to permit refined and characterized ecological and human health risk projections at varying biological, spatial, and temporal scales for risk management decisions.

1.3.3 Benefits

A paradigm shift to a more integrated approach to testing and assessment will significantly improve EPA's ability to carry out its mission of protecting public health and the environment. It will focus on the most likely hazards of concern and determine what specific data for each chemical and exposure situation are essential to assess and manage risks appropriately. As a result, the approach will lower the costs for the government and tax payers because the Agency could avoid reviewing unnecessary tests. For the pesticide-producing industry, this approach will reduce and may eventually eliminate complex and expensive *in vivo* testing. It will also refine and reduce the use of animals in testing. This approach also has the potential to increase the feasibility of assessing the risks posed by mixtures. In the long run, the approach will provide opportunities for improved diagnostic biomonitoring and surveillance methods to detect chemical exposures and identify causes

of toxic effects. Finally, it will improve health and environmental protection and increase efficiency by focusing the regulated community, government, and interested parties on chemicals and endpoints of greatest concern. OPP will be able to evaluate more chemicals across a broader range of potential effects in a shorter time frame, thereby enhancing the quality and efficiency of risk assessment and risk management decisions. In short, by focusing resources where they are most needed, this paradigm shift will better meet the needs of EPA risk managers, those in the broader community of stakeholders engaged in risk assessment and risk management, and – most importantly – the public.

1.4 Building the Components of IATA: Key Research Activities

OPP is closely collaborating with the EPA Office of Research and Development (ORD) and other Agency regulatory programs to plan the development and evaluation of components of IATA. ORD's Chemical Safety and Sustainability (CSS) research program ([http://yosemite.epa.gov/sab/sabproduct.nsf/BC3D06D94442465C8525784700782CE3/\\$File/Kavlock CSS SAB public.pdf](http://yosemite.epa.gov/sab/sabproduct.nsf/BC3D06D94442465C8525784700782CE3/$File/Kavlock%20CSS%20SAB%20public.pdf)) provides the research direction for the Agency to support transformative approaches to further improve both the quality and nature of information used in managing chemical risks. Regulatory program offices need to be engaged early and often in the development of the science to ensure that the new methods will meet the needs of chemical risk management. As such, OPP has partnered with ORD on a number of activities including the continued development of new tools and methodologies including new *in vitro*, molecular, and computational science approaches to evaluate the inherent chemical and biological properties of pesticides as well as providing data to support the development of more reliable predictive tools such as (Q)SARs. As highlighted in Figure 1, the following critical components are necessary to enhance OPP's IATA:

- Databases and knowledge bases to capture existing data (*e.g.*, toxicity, metabolism);
- AOP and MoA libraries; and
- Predictive tools (*e.g.*, (Q)SAR, *in vitro* methods, HTS assays).

Some significant examples of ongoing Agency research activities on each of these components are highlighted in the subsections below. OPP is seeking feedback on whether we have sufficiently articulated a sequence of events including research activities and plans to implement new advances and tools that demonstrate a logical progression that will ultimately achieve increased efficiencies and effectiveness of the program.

1.4.1 Developing Databases and Knowledge Bases to Capture Existing Data

An important component of IATA is capturing, in a searchable format, what we already know, *i.e.*, legacy data. The development, maintenance, and use of data warehouses and “knowledge” bases are critical to achieve this goal. These databases in turn serve as a means to develop predictive tools for estimating similar information for data-limited chemicals. Thus, their utility will rely on establishing

Knowledge bases are more than databases (storage systems built for simple queries) because they are designed to help analyze information in a relational integrated fashion.

databases with training sets that are sufficiently populated with pesticide data by chemical class and mode of action.

OPP makes extensive use of ECOTOX, a comprehensive web-based database developed and maintained by ORD's National Health and Environmental Effects Research Laboratory (NHEERL). It contains data on effects of single chemicals to aquatic and terrestrial organism. The data primarily come from studies published in the open literature. More details can be found at the following websites:

http://www.epa.gov/med/Prods_Pubs/ecotox.htm and
<http://cfpub.epa.gov/ecotox/help.cfm?sub=about>.

Several efforts are underway at EPA to build knowledge bases and expert systems; these include decision-support tools developed by ORD's National Center for Computational Toxicology (NCCT) that guide users through chemical structural alerts, fate, hazard and risk information. Many of these tools are publicly available. For example, ACToR (Aggregated Computational Toxicology Resource) is a knowledge base available online (<http://www.epa.gov/actor/>). ACToR is made up of 500 public data sources on over 500,000 chemicals and it provides information on chemical exposure, hazard and potential risks to human health and the environment (Judson *et al.* 2008). It can be used to query a specific chemical and find all available public hazard, exposure and risk assessment data as well as previously unpublished studies related to cancer, reproductive and developmental toxicity.

ACToR is linked to the Toxicity Reference Database (ToxRefDB <http://actor.epa.gov/toxrefdb>), which allows Agency scientists and the public to search and download thousands of mammalian toxicity test results on hundreds of chemicals. The database captures 30 years of animal *in vivo* testing data that were previously found only in paper documents. The animal toxicity data contained in ToxRefDB, when combined with other sources of information such as exposure and metabolism data, provide the basic elements for estimating risk.

ACToR is also linked to the Distributed Structure-Searchable Toxicity (DSSTox) database network (<http://www.epa.gov/ncct/dsstox/index.html>). DSSTox provides a public forum for publishing downloadable, structure-searchable, standardized chemical structure files associated with toxicity data developed from available structure-viewing freeware and open-source programming tools. It delivers a simple, easy-to-use structure-searching capability through the chemical inventory of published DSSTox Data Files (Richard *et al.* 2002, Richard *et al.* 2008).

The metabolism pathways system (METAPATH) is another important knowledge base that is being developed by ORD's NHEERL and the National Exposure Research Laboratory (NERL). METAPATH is a software system for collection and analysis of metabolism pathway data. Using the METAPATH platform, a database of mammalian liver metabolism of pesticide chemicals was created. The database currently contains > 400 metabolic pathways (maps) for > 200 pesticides. The metabolic pathways were observed in various experimental systems, primarily for studies using rats. A wide range of additional

supporting information to aid interpretation was also coded – test species details, experimental treatment conditions, biological compartments sampled and processing methods, dosing levels and duration, etc. The METAPATH platform provides very powerful and flexible data analysis and search capabilities. Search queries can be defined and subsequently combined in complex logical AND, OR, NOT clauses. The search queries can be based on experimental treatment group information (e.g., dose level, gender, biological sample matrix, etc), chemical structure, or substructure. The system can calculate the similarity between maps with regard to a selected parameter of interest, for instance, how similar are the metabolites found for two different species exposed to the same chemical, or males vs females. Efforts are underway to further populate this knowledge base with livestock and plant metabolites as well as environmental degradate data.

As a means of accurately and quickly populating METAPATH, electronic Data Evaluation Record (DER) Composers have been developed based on North American Free Trade Agreement (NAFTA) harmonized study review templates for rat metabolism studies and studies characterizing the nature of the residue in livestock. The DER Composers serve as external stand-alone applications allowing direct input of summary information from studies that can be easily imported into METAPATH or exported as text files for risk assessor use in reviewing submitted study information. Populating METAPATH directly using the DER Composers allows for more efficient and accurate transfer of data. OPP has worked closely with ORD to ensure data accuracy and the development of data verification procedures. Making greater use of DER composers to populate other databases will further ensure the appropriate level of quality assurance for databases.

METAPATH also provides critical data for the development of metabolic simulators, which are being developed to simulate the metabolism of xenobiotic chemicals in biological systems(http://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=216644&fed_org_id=770&sitype=pr&timstype=presentation&showcriteria=0&address=nerl&view=citation&sortby=pubdate&count=100&datebeginpublishedpresented=10/01/2009). Reliable forecasting of chemical metabolism is a critical factor in estimating a chemical's toxic potential. A major challenge for scientists and regulators is accounting for the metabolic activation of chemicals, which may lead to increased toxicity. Research is underway to develop computer modeling approaches to predict chemical metabolism as a component of efforts to screen and categorize chemical risks. The quality of these metabolic predictions depends on the amount and quality of data used to train programs to simulate observed metabolic transformations. The METAPATH databases provide a source of metabolism data consistently collected under OPP guidelines.

1.4.2 Defining Adverse Outcome Pathways and Building Predictive Systems

One conceptual approach the Agency is taking to build the scientific foundation for expert predictive systems, starts with the description of the chemically-initiated perturbation (molecular initiating event) which leads to an adverse effect in the form of an AOP. The Estrogen Receptor (ER)-mediated reproductive impairment adverse outcome pathway (Schmieder *et al.*, 2004), used as a basis for development of an estrogen receptor

(ER) binding Expert System for chemical prioritization to target required testing, which was reviewed in 2009 both by the FIFRA SAP (<http://www.epa.gov/scipoly/sap/meetings/2009/082509meeting.html>) and by the Organization for Economic Cooperation and Development (OECD; OECD, 2009), is an illustration of this use of the AOP approach. As shown in Figure 3, this particular AOP describes the linkage between the event that initiates the pathway (*i.e.*, chemical binding to the ER) and the series of events that occur at successively higher and more complex levels of biological organization. The key events in this pathway include:

- Initiation of events by a chemical binding the ER as a result of sufficient chemical uptake into the organism and partitioning to a target tissue with ER-containing cells;
- Cell and tissue level gene transcription and translation, *e.g.*, activation/transcription of ER responsive genes indicated by vitellogenin mRNA induction (Vtg; an egg-yolk pre-cursor glycolipoprotein)
- Protein production in fish liver;
- Organ effects (*e.g.*, appearance of ova in male fish testicular tissue); and
- Adverse reproductive and developmental outcome(s) observed in the individual (*e.g.*, change in secondary sex characteristics (feminization of males);
- Cessation of spawning in females; complete sex reversal (*i.e.*, genetic males with fully developed and functioning ovaries).

The boxes in Figure 3 describe responses that may be observed at the various levels of biological organization. Binding and activation of the ER pathway was measured in an *in vitro* binding assay. A rainbow trout (*Oncorhynchus mykiss*) liver slice assay was then used to confirm ER-mediated gene activation (production of vitellogenin) within a metabolically-competent tissue. By using *in vivo* data, this pathway could be extended to potential consequences to a fish population as represented in the “Population Responses” box in Figure 3 where, for instance, sex reversal in individuals could result in skewed sex ratios in the population, or where cessation or decrease in spawning in individuals may potentially result in reduction in a year class in the population.

ER-mediated Reproductive Impairment AOP

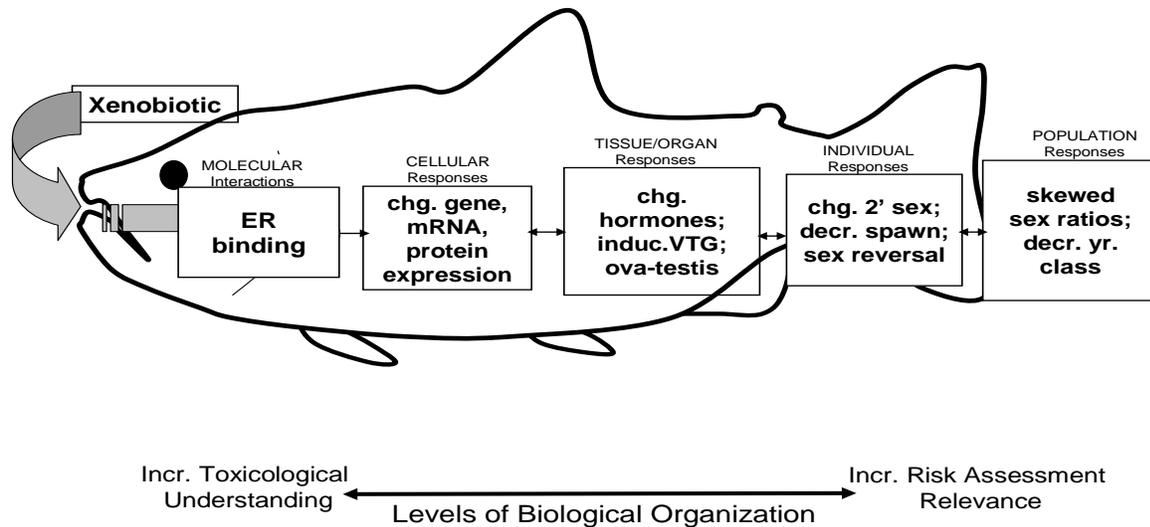


Figure 3. An ER-mediated AOP in Fish (modified from Schmieder et al., 2004)

It should be emphasized that the use of the ER-mediated reproductive impairment AOP in this case study was not to predict *in vivo* potency of ER binding chemicals which is a more complex and different problem than chemical prioritization. The ER-mediated AOP in fish is used as the scientific basis for focusing on chemical binding to the ER as the parameter to prioritize chemicals for *in vivo* testing, providing plausibility of the binding interaction resulting in an adverse outcome. The results of subsequent *in vivo* tests conducted on the priority list of chemicals will then be used to answer the higher tier risk assessment question of whether or not reproductive effects are likely to occur upon environmental exposure to these chemicals.

OPP and other offices in EPA are faced with large numbers (hundreds to thousands) of chemicals that need to be assessed for their potential to cause endocrine disruption. Time and resources dictate that all chemicals cannot be evaluated at once. A challenge is to determine which chemicals should be tested first. The chemicals that most need to be prioritized and screened are typically those with the least amount of data. The Endocrine Disruptor Screening and Testing Advisory Committee (<http://www.epa.gov/scipoly/oscp/endo/pubs/edspoverview/finalrpt.htm>), the combined Science Advisory Board, and the FIFRA SAP recommended the development of a prioritization scheme that includes both an effect and an exposure component (http://www.epa.gov/endo/pubs/sab_sap_report.pdf). Ideally, EPA would be able to put into use a hypothesis-based approach that focuses on the potential for adverse effects, and prioritizes for testing the chemicals most likely to cause an effect. A predictive system built on the ER-mediated reproductive impairment AOP provides a hypothesis-based strategy to chemical prioritization. One example for how this could be put into use is the ER binding affinity Expert System which was developed to predict which of the regulatory food use

inert pesticide ingredients and antimicrobial active ingredients, *i.e.*, the chemical domain and structures of interest, could initiate the ER binding AOP. It is known that interference with hormone receptors (*e.g.*, the estrogen receptor) can adversely affect reproduction, and, further, that diverse chemical structures can bind to the ER. Thus, this model will be used to help establish which inert ingredients and antimicrobial active ingredients (grouped in chemical categories) should be given higher priority for testing in EPA's Endocrine Screening Program (<http://www.epa.gov/endo/pubs/edspoverview/primer.htm>).

The ORD NHEERL lab that developed the ER binding Expert System is taking a similar approach to build an (Q)SAR system to predict which chemicals are potential thyroid hormone disruptors. As shown in Figure 4, this AOP would include:

- The initial molecular event, which is inhibition of thyroid peroxidase (TPO) enzyme,
- Followed by a decrease in thyroid hormone synthesis,
- A consequent reduction in serum thyroid hormone and increase in thyroid stimulating hormone (TSH),
- Which leads to arrested metamorphosis in amphibians, and, more generally, to alterations in thyroid-driven normal development.

TPO inhibition is measured *in vitro* and confirmation of activation of this pathway is with a metabolically-competent thyroid gland culture assay. However, though the AOP has been elucidated (linking molecular, cellular, tissue effects to *in vivo* developmental delay), this work is not as far along as the ER-Binding expert model. So far, *in vitro* assays have been optimized for a range of industrial-type chemicals (*e.g.*, pesticide inerts and antimicrobials). An initial set of chemicals have been tested sufficiently to hypothesize possible chemical structural features associated with activity.

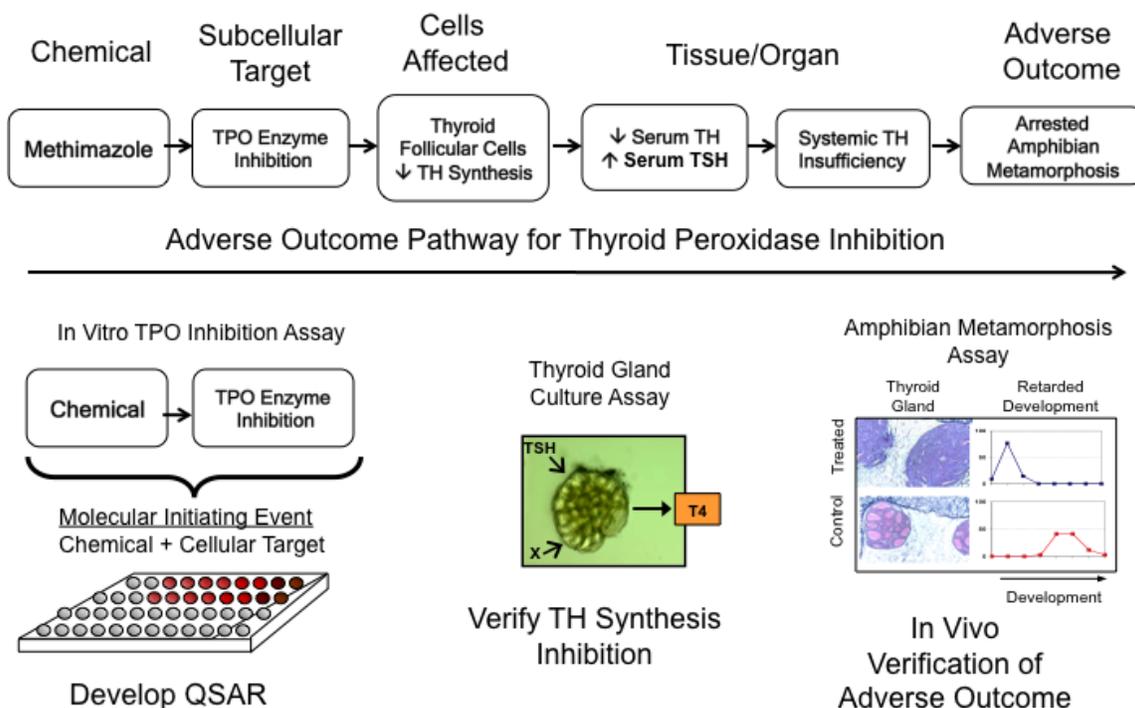


Figure 4. (Q)SAR-based approach to predict thyroid disruption based on the adverse outcome pathway for thyroid peroxidase (TPO) inhibition (M. Hornung, personal communication).

As described, knowledge of AOPs allows the development of refined (Q)SAR/expert systems. In addition to these highly specific (Q)SAR tools, which are being developed to predict individual pathways of effects, the Agency is conducting biological profiling using high-throughput *in vitro* assay systems. HTS assays are being investigated as a means to efficiently evaluate the ability of chemicals to initiate molecular interactions that are the basis for causing adverse effects. Through its ToxCast™ program (<http://www.epa.gov/ncct/toxcast>), the Agency uses a battery of relatively inexpensive *in vitro*, HTS assays on a relatively large and diverse chemical space to develop methods to predict potential human toxicity of environmental chemicals at a fraction of the cost of full-scale animal testing (Dix *et al.* 2007; Judson *et al.*, 2010). ToxCast™ uses advanced science tools to help efficiently understand biological processes impacted by chemicals that may lead to adverse health effects. ToxCast™ currently includes 500 fast, automated chemical screening tests. The major goals of ToxCast™ are to: (1) identify *in vitro* assays that can reliably indicate alterations in biological processes of relevance to *in vivo* mammalian toxicity; (2) develop signatures or prediction models based on multiple assays, along with computed or available chemical properties, that can achieve higher predictive power than single assays or chemical structure alone; and (3) use these combined computed and *in vitro* assay-based signatures to screen large numbers of previously untested environmental chemicals. Results from ToxCast™ are available via ACToR.

1.5 Building the Components of IATA: Key Partnership Activities

Achieving our goal of increasing the efficiency and accuracy of chemical risk assessment and risk management by implementing a strategy to use enhanced integrative approaches to testing and assessment that incorporate emerging technologies will require coordinated efforts and acceptance across the Agency's programs and across regulatory authorities globally.

The Agency is involved in a number of international fora to advance 21st Century science approaches in IATA for regulatory purposes (*e.g.* OECD, NAFTA, World Health Organizations IPCS). For example, in 2007, the Agency hosted an OECD "Workshop on Integrated Approaches to Testing and Assessment" which provided an international forum to exchange information and views on applying the various components of IATA including *in vivo* and *in vitro* testing, (Q)SAR models, chemical category and read-across assessment methodologies, toxicogenomics, and exposure considerations to different kinds of chemicals and in different regulatory frameworks (<http://www.oecd.org/dataoecd/45/52/40705314.pdf>).

The Agency is also partnering with the Pest Management Regulatory Agency (PMRA) of Health Canada on the "21st Century Toxicology: Integrated Approaches to Testing and Assessment" project approved under the auspices of NAFTA. The objective of this project is to "supplement, replace and reduce traditional animal toxicity testing methods in risk assessment by using a variety of tools and approaches in combination" and through making more efficient use of existing information.

The intent of this section is not to provide an exhaustive list of all of the international activities but to highlight a few pertinent activities related to the advancement of IATA. These activities are complimentary to the Agency's research program in developing essential IATA components:

- Databases and knowledge bases to capture existing data (*e.g.*, toxicity, metabolism);
- AOP and MoA libraries; and
- Predictive tools (*e.g.*, (Q)SAR, *in vitro* methods, HTS assays).

Current partnership efforts to put these components of IATA into place are summarized in the following subsections.

1.5.1 Capturing Existing Data and Building Libraries of Adverse Outcome Pathways

In 2010, EPA proposed a project that was accepted by the OECD Working Group on Pesticides to initiate a multi-national collaborative venture to develop and populate a comprehensive and searchable metabolism database that stores metabolism data on laboratory animal, livestock, plant and environmental transformation products. This database, METAPATH (discussed above), facilitates the development of new predictive

structure-based methods, and allow for a more efficient comparison of metabolic profiles across chemicals, chemical classes, and species.

In 2010, the Agency hosted an OECD Workshop entitled "Using Mechanistic Information in Forming Chemical Categories to Fill Data Gaps for Regulatory Purposes". The objective of this workshop was to solicit scientific input to further the development of AOPs. The workshop focused on how AOP can be of use in forming toxicologically meaningful chemical categories for filling data gaps using existing knowledge (*e.g.*, read-across and SARs). Participants also provided feedback on how scientific information can be gathered and generated to aid in developing AOPs and establishing linkages between data collected at multiple levels of biological organization, and the best platform for information exchange in AOP development.

1.5.2 Developing Predictive Tools

The Agency is committed to ensuring a transparent process for the objective determination of the reliability of (Q)SAR models in order to further enhance the accuracy and regulatory acceptance of (Q)SAR models. To that end, the Agency is engaged in a number of partnership activities related to the development and evaluation of predictive tools.

OPP has partnered with the U. S. Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition (CFSAN) in sharing publically available toxicity data and experience on the application of (Q)SAR in the regulatory risk assessment decision-making process. This partnership was formalized in a Memorandum of Agreement signed in 2009 and since then has resulted in a number of (Q)SAR training seminars for OPP science staff members and information sharing meetings between the two agencies.

The Agency is partnering with PMRA of Health Canada to test the predictive performance of selected (Q)SAR models for pesticides and develop guidance for the application of (Q)SAR models to pesticide risk assessments.

EPA has also contributed to several OECD reports and guidance documents on practical applications of (Q)SARs in specific regulatory contexts by governments and industry and has participated in OECD Expert Consultation to evaluate the application of the OECD (Q)SAR principles for the use of (Q)SAR expert systems in regulatory assessment of chemical safety (<http://www.oecd.org/dataoecd/33/37/37849783.pdf> and [http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono\(2004\)24&doclanguage=en](http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2004)24&doclanguage=en)). The Agency continues to aid in the development of the OECD (Q)SAR Application Toolbox through contribution of new tools as well as participation in the QSAR management group (http://www.oecd.org/document/23/0,3343,en_2649_34365_33957015_1_1_1_1,00.html)

Over 20 years ago, OECD member countries endorsed the Mutual Acceptance of Data (MAD) agreement (OECD, 1981) (http://www.oecd.org/document/41/0,3746,en_2649_34365_1890473_1_1_1_1,00.html),

which commits member countries to accepting test data that have been produced with tests conducted in accordance with the OECD Test Guidelines (TGs) and Principles of Good Laboratory Practice. However, it is not realistic or efficient to develop TGs for every possible HTS or *in vitro* system. The Agency is involved in efforts through OECD to work toward “performance-based test guidelines” for *in vitro* systems under the Validation Management Group for Non-Animal Testing (VMG-NA) of the OECD Task Force for Endocrine Disrupting Testing and Assessment (EDTA).

To improve chemical risk assessment, there needs to be harmonized principles and approaches and agreement that the newer methods deliver protection of human and environmental health within a framework of sustainability³. Achieving a fully harmonized approach is critical to the implementation of IATA as it will ensure consistency in how information is used in regulatory decision making while providing incentives for directing the appropriate level of resources to ensure these tools are properly developed, vetted, and integrated into the regulatory decision-making process. International cooperation and coordination is also critical to making the best use of all available resources, avoiding duplicative efforts, and ensuring global public health protection based on the best available information.

1.6 Building the Components of IATA: Key Activities Toward Regulatory Adoption

Working in partnership with ORD and others, OPP has already begun to harness components of IATA. Over the next several years, depending on the readiness of the science, OPP plans to operationalize new *in vitro* and *in silico* predictive methods for priority setting and to enhance its integrated approach to testing and assessment to better target what toxicity data are needed to further refine risk assessments for chemicals that do not have extensive toxicity information (*e.g.*, inert ingredients and metabolites and degradates of pesticide active ingredients). Over the long-term, as we gain experience with new *in vitro* and *in silico* approaches and as knowledge of AOPs increase, OPP’s view is that reliance on animal testing will be reduced and highly targeted. Thus, we are actively engaged in developing hypothesis-based AOP approaches to phase in these new technologies and to phase out the less efficient animal intensive approaches over time. IATA provides OPP with the means to systematically link the various tools we currently have at our disposal with those that will be developed in the future in an iterative fashion to efficiently and effectively evaluate hazard and exposure and to assess risk. OPP has a long and proven track record of implementing new test requirements and risk assessment approaches to ensure the safety of pesticides when used according to the label. Thus, OPP already has a number of “tools” in its “toolbox” to accomplish this. OPP will move from the application of specific tools to an integrated approach to testing and assessment by expanding and integrating that toolbox over time as our knowledge evolves.

³ Sustainability is being used in the context of developing approaches or policies that integrate environmental, economic, and social values in decision making.

1.6.1 Components of IATA in Current Use

OPP is already gaining improvement and efficiencies in pesticide risk assessment by using searchable databases and predictive systems. Furthermore, in the near term, the Pesticide Program plans to adopt new components of IATA in a stepwise fashion (Sec 1.6.2).

Figure 5 illustrates the use of IATA in the pesticide program now versus the future.



Figure 5. Current and future use of IATA in the pesticide program.

An example of a knowledge base currently used by OPP is METAPATH (Sec 1.4.1). The system METAPATH, in conjunction with other information, assists OPP risk assessors to determine 1) major metabolites of a pesticide that have toxic potential and 2) which metabolites to include in the risk assessment and tolerance expression of the pesticide active ingredient. The information stored in METAPATH on pesticide metabolism allows comparisons across maps to identify common metabolites with potential toxicity concerns and include these in risk assessment. This information, in conjunction with QSAR predictions, is used to judge testing needs for metabolites and degradates of pesticide active ingredients.

In certain contexts, OPP has used existing data from similar compounds and computational models to evaluate pesticides for a number of years. For example, OPP's ecological assessment process includes extensive use of read-across and QSAR tools as well as a tiered testing regime. Tools used by OPP include:

- Ecological Structure Activity Relationships (ECOSAR): a component on the Estimation Program Interface Suite (EPI Suite), which estimates the aquatic toxicity of industrial organic chemicals and pesticides. (<http://www.epa.gov/oppt/newchems/tools/21ecosar.htm>)
- ECOTOX (Sec 1.4.1): chemical toxicity data for aquatic and terrestrial plants and animals (<http://cfpub.epa.gov/ecotox/help.cfm?sub=about>)

- Assessment Tools for the Evaluation of Risk (ASTER): used for hazard ranking and the development of comprehensive environmental risk assessments for aquatic organisms and wildlife. Provides high quality data for discrete chemicals in the associated databases (*i.e.*, ECOTOX and EcoChem) and QSAR-based estimates when data are lacking. (http://www.epa.gov/med/Prods_Pubs/aster.htm).

As with the Agency's assessment of industrial chemicals, pesticide inert ingredients are frequently evaluated with QSARs and read-across methods to determine whether *in vivo* testing is necessary to make a safety determination. Tools include,

- EPI Suite™: estimates physical / chemical properties and environmental fate (<http://www.epa.gov/opptintr/exposure/pubs/episuite.htm>)
- Screening-level tools for exposure (<http://www.epa.gov/opptintr/exposure/pubs/screen.htm>).
- Read-across (<http://www.epa.gov/hpv/pubs/workshop/wkshebi.htm>)

OPP's assessment program is based on the concept of tiered-testing and targeted data needs informed by existing knowledge of the chemical or class of chemicals, including an understanding of the mechanism of biological activity, and the proposed use of the chemical. When available, OPP incorporates MoA information into its risk assessments so as to more accurately characterize what effects may be biologically plausible in humans and what susceptible populations may be more likely impacted by chemical exposure.

1.6.2 Anticipated Near-Term Activities

Over the next five years, the Pesticide Program will adopt new components of IATA in a stepwise fashion. In collaboration with ORD and other partners, OPP will evaluate new tools as they are developed using available criteria and methods such as the OECD principles for (Q)SAR expert systems (<http://www.oecd.org/dataoecd/33/37/37849783.pdf> and [http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono\(2007\)2&doclanguage=en](http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2007)2&doclanguage=en)) and the EPA-IPCS mode of action framework (Sonich-Mullin *et al.*, 2001; Boobis *et al.*, 2006). As OPP considers adopting new tools they will be evaluated through an iterative process of case studies and expert peer review (see Figure 1). Case studies will examine questions such as: for a set of chemicals, does the new tool provide predictions comparable to available *in vivo* studies? Each situation will require criteria and a determination of acceptable level of uncertainty. Once a tool has been sufficiently evaluated by the scientific community, OPP may set up a pilot program to refine the assessment and regulatory process associated with the use of the new tool. For example, in 2009 OPP established a voluntary pilot program for using non-animal methods for testing the likelihood and degree of certain antimicrobial pesticides to cause eye irritation (<http://www.epa.gov/oppad001/eye-irritation.pdf>). Application will be context specific, for example, a new tool may first be used to support non-food uses. OPP anticipates a series of SAP reviews as new tools become available. For example, the recent SAP on structure activity relationships of estrogen binding affinity to support prioritization of pesticide inert ingredients and antimicrobial pesticides for screening and testing

(<http://www.epa.gov/scipoly/sap/meetings/2009/082509meeting.html>). The Agency is seeking input from the Panel on criteria and methods the Agency can use to reliably integrate predictive tools into the process without delay.

Near-term developments could include, for example, an analysis of HTS signaling profile alerts as part of the evaluation of pesticide inert ingredients. Also, the Agency is expanding the library of QSAR tools with knowledge databases sufficiently relevant to pesticide structures to enable their use in an improved evaluation of metabolites and degradates of pesticide active ingredients. In the context of EPA's Endocrine Disruptor Screening Program it is envisioned that *in vitro* and *in silico* methodologies will also be helpful in screening pesticide actives for potential impacts on endocrine-mediated processes. EPA plans to use the schedule for re-evaluating registered pesticides in the Registration Review program to determine testing schedules for pesticide actives in the EDSP. For pesticide inert ingredients, the Agency also plans to employ predictive HTS *in vitro* and *in silico* tools in the EDSP prioritization process (<http://www.epa.gov/endo/>). It is possible that computational and HTS tools could eventually even serve as a substitute, or partial substitute, for screening, which currently consists of primarily (6 of 11) *in vivo* studies.

The upcoming 158W antimicrobial rule is another milestone towards OPP's long-term vision (<http://www.federalregister.gov/articles/2008/10/08/E8-23127/data-requirements-for-antimicrobial-pesticides>). Antimicrobial pesticides provide an appropriate opportunity for advancing the paradigm shift. Many antimicrobials have both pesticidal and non-pesticidal uses and many antimicrobial products are regulated under multiple jurisdictions. Thus, many antimicrobial chemicals have been assessed by other regulatory programs and agencies. The ready availability of published literature and publicly-available assessments offers a unique opportunity for the pesticide applicant to use the available information as a starting point for fulfilling data requirements, and, when appropriate, using computer modeling and *in-vitro* data to supplement or fulfill data requirements. As described in the preamble to the proposed rule, the Agency encourages submission of alternative studies *e.g.* (Q)SAR data.

Consistent with the long-term goals of the NAS vision, OPP is committed to advancing the use of biomonitoring data in exposure assessment. Currently, these data are used in trend analyses of exposure. For example, cancellation of a pesticide should correspond with decreases in urinary concentration of the pesticide in the general population. OPP is also beginning to use biomonitoring data such as the Center of Disease Control's National Health and Nutrition Examination Survey (CDC NHANES) data to provide more accurate estimates of exposure than modeled estimates. OPP plans to continue to incorporate biomonitoring data into assessments when sufficient appropriate data are available.

1.6.3 Screening using “Threshold of Toxicological Concern” Values

Combined with simple or more refined exposure scenarios, “Threshold of Toxicological Concern” (TTC) values can form the basis of an integrated approach in a tiered risk-assessment scheme. The TTC approach is widely acknowledged as a useful (Q)SAR based concept for addressing information gaps and has a long history of being used in different regulatory contexts for risk assessments of chemicals found at low exposure levels including chemical residues in food packaging, flavoring agents, cosmetic ingredients, and genotoxic contaminants in pharmaceuticals. (Blackburn, *et al.*, 2005; Felter, *et al.*, 2009; Kroes, *et al.* 2004, Kroes *et al.* 2005, Kroes *et al.* 2007; Munro, 1996; Munro, *et al.*, 1996; Munro, *et al.*, 2008). TTC values represent an exposure threshold for chemicals below which no significant risk of adverse toxicological effects would be expected. The approach is based on extrapolation of toxicity data from an available database to a specific chemical for which the structure is known, but for which limited toxicity data are available. Thus, the utility of TTC in safety evaluation is dependent on the quality, quantity and relevance of the underlying toxicity database (e.g., the chemical domain of applicability) and the availability of a reliable and relevant estimation of the exposure for the durations and routes of interest.

The Agency is currently participating in an International Life Sciences Institute (ILSI) Research Foundation project to develop a TTC-based approach for the evaluation of antimicrobial pesticide active ingredients (http://www.ilsilife.org/Europe/Pages/TF_ThresholdToxicological.aspx). The TTC concept is being proposed as a means for waiving testing requirements based on knowledge of exposure limits and known toxicities of chemical classes in order to focus finite resources and animal testing on the evaluation of chemicals with a greater potential to pose risks. This concept is very similar to that of setting reference doses (RfD) and margins of exposure (MOE), however, it expands on the concept by extending it from an individual chemical to a class or grouping of chemicals. The ILSI project is being coordinated with the European Food Safety Authority’s (EFSA) initiative to investigate broadening the TTC concept across a range of programs including its application to metabolites, degradation products, and reaction products of active substances of plant protection products. In the ILSI project, the TTC approach is being investigated in relation to structural information and the toxicological data of antimicrobial chemicals. The development of structural categories and corresponding TTC values would be considered for an array of human endpoints relevant to the evaluation of these active ingredients including systemic, reproductive, and developmental toxicities, and carcinogenicity. The chemical domain for antimicrobials has been defined and the existing toxicity data has been housed in a searchable database (ToxRefDB) to evaluate whether the chemical structures for antimicrobials are adequately represented to derive the exposure thresholds to support use of TTC toward chemical testing prioritization and targeted testing strategies, and eventually in the risk assessment of the antimicrobial chemicals. Additionally, because TTC values are largely based on oral toxicity data, an approach is being developed to adjust the oral TTC values to account for dermal exposure. In sum, the TTC approach is a potential near-term methodology that could considerably advance OPP’s goals of increasing efficiency and reducing animal testing.

1.6.4 Maximizing Efficiency of Animal Testing

Another short-term activity is to make the existing animal testing paradigm more efficient, reliable, and responsive to risk assessment and management needs. OPP supports the development of increasingly effective laboratory animal tests that are designed to maximize the information generated about the nature of the effects being studied. For example, the ILSI Health and Environmental Sciences Institute's tiered-testing proposal on Agricultural Chemical Safety Assessment (ACSA) (<http://www.24d.org/scientific/Carmichael2006.pdf>), published in 2006 in *Critical Reviews in Toxicology* (Carmichael *et al.* 2006), is consistent with EPA's vision of a more efficient and reliable science-based paradigm. Toward this end, EPA has partnered with other countries through the OECD to develop the extended one-generation rat toxicity test as a replacement for the two-generation reproductive test. Without compromising the quality of information obtained from these studies, adoption of the alternative protocol will substantially reduce the number of animals used. Furthermore, modules can be included to assess developmental immunotoxicity and developmental neurotoxicity. The alternative methodology was adopted in November 2010 at the OECD's Joint Meeting of the Working Party on Chemicals, Pesticides and Biotechnology and the Chemicals Committee approved a draft version of a test guideline for the Extended One-Generation Reproductive Toxicity Study (EOGRTS). Consistent with OPP's commitment to advancing its vision without delay, OPP has already begun to accept the use of EOGRTS when appropriate, in place of the combination of the two-generation reproductive assay and the developmental neurotoxicology (DNT) assay which are resource intensive studies in terms of their conduct, analysis and use of animals.

In conclusion, OPP has made considerable progress in developing the components essential to IATA. OPP seeks guidance on whether we have sufficiently articulated a sequence of events including research activities and the early use of new tools and methodologies that demonstrates a logical progression toward an enhanced IATA.

1.7 Stakeholder Engagement

Achieving OPP's vision requires active, collaborative partnerships with stakeholders. In order to construct a new paradigm in which stakeholders have confidence, it is important that OPP understands the issues that are critical to address from the perspectives of a broad range of stakeholders. It is also incumbent upon the Agency to ensure that stakeholders understand new tools, methodologies, and processes as they are adopted. Communicating and engaging with stakeholders now in a clear, open, and transparent manner will enable OPP to take advantage of advances as soon as they are available.

To inform and engage stakeholders early in the Agency's efforts to implement the NRC recommendations, the Pesticide Program Dialogue Committee (PPDC) work group on "Integrated Testing Strategies/21st Century Toxicology" was established in 2008 (<http://www.epa.gov/pesticides/ppdc/testing/index.html>). The workgroup consists of

members from multiple stakeholder groups, including pesticide manufacturer and user organizations, animal rights advocates, local, state, Federal, and international agencies, and public health, worker, and environmental advocates. The key objective of this workgroup is to advise OPP on communication and transition issues as we move forward to adopt components of IATA. The workgroup is concerned with both short- and long-term implications of the transition and has focused on identifying critical issues, developing potential metrics of success, and examining the biomarkers component of the NRC strategy.

In December 2010, OPP held a stakeholder workshop on “21st Century Science and Integrated Testing and Assessment Strategies: Transitioning Research to Regulatory Practice” to broaden the involvement of stakeholders beyond the workgroup. The objectives of the workshop were as follows:

- To communicate the strategic vision for integrated approaches to testing and assessment through the application of case studies.
- To increase the common understanding of emerging 21st Century science tools and how they might be applied;
- To further OPP’s understanding of stakeholder perspectives, priorities, and expectations.
- To build an effective, transparent communication strategy.

In general, stakeholders have responded favorably to the overall direction that OPP is taking. There is broad support for expected outcomes such as regulatory efficiencies, an increased ability to examine a range of adverse effect endpoints, and reduced animal testing. However, stakeholders have expressed concerns and raised issues regarding potential implications of the transition. Furthermore, stakeholders have provided recommendations to the Agency on ways to address some of these concerns and issues.

Some of the more common issues and concerns expressed include:

- How will EPA ensure that the current level of protection of human health and the environment is maintained or enhanced?
- Will moving away from the use of a whole animal and towards the use of a limited set of *in silico* and *in vitro* assays and predictive tools miss important biological endpoints?
- What are the expected impacts on the reliability of and the uncertainties associated with human health and environmental risk assessments and risk management decisions?
- Will new testing approaches be able to account for differences between individuals in human and ecological populations?
- Will the use of molecular tools increase our ability to characterize mixtures?
- How will the new toxicity-testing paradigm streamline the pesticide regulation process?
- What barriers stand in the way of using newer tools and methods and what can be done to remove these barriers?

- Will new tools and methodologies be incorporated into the regulatory process in a timely manner?
- How might these changes affect EPA's efforts to harmonize pesticide testing and regulation guidelines with other international regulatory agencies?

Recommendations to the Agency on ways to address certain issues and concerns include:

- Use existing data to develop a detailed case study of how future testing, assessment, and decision processes would work.
- Assess new testing approaches by comparing results to animal testing results. Conduct prospective and retrospective checks.
- Build feedback mechanisms and safeguards into the system. For example, incident reporting and surveillance programs would provide a check to ensure effects are not being missed. In turn, results from surveillance programs will provide feedback to develop improved tools.
- Establish a clear process for risk assessment and management.

As we move forward, it will be important for OPP to remain mindful of the issues and concerns raised by stakeholders because they will guide us in understanding essential process considerations and in developing strategies for evaluating emerging tools and methodologies. In light of these stakeholder issues and recommendations, OPP seeks advice on potential gaps in our strategy or factors we should consider as we begin to develop metrics (Sec 1.8).

1.8 Metrics

To ensure progress toward and successful implementation of a transition to an integrative approach for testing and assessment, OPP must develop and employ a set of metrics, each specific to a critical factor of the vision. Metrics are useful internally as well as being a valuable communications tool. However, OPP is only at the beginning stages of determining how best to do this. OPP seeks guidance on the concepts and attributes we should consider as we start to develop metrics.

Two types of metrics have been recommended to the Agency by the PPDC workgroup: metrics of progress and of success. Metrics of progress track milestones along the way to the goal; examples are new test guidelines, new testing or toxicity evaluation tools for use by industry and regulators, and identification and acceptance of adverse outcome pathways. Metrics of success involve comparing the use of new tools for testing and assessment to the existing paradigm, or current activities. Examples are: enhancing the quality of assessments by efficiently considering a broader range of endpoints, quantifying the development of AOPs and their qualitative and quantitative application, reducing testing costs, and reducing the number of animals used in testing.

OPP is at the beginning stages of contemplating metrics to demonstrate success in achieving our vision. OPP is looking for metrics that are easy to define, can be practically measured, are understandable, and easily communicated. In addition, as mentioned above, OPP would like to consider issues identified by stakeholders to define concepts for potential measures. OPP is interested in the Panel's initial thinking regarding the concepts, considerations, and factors we should take into account as we start to build metrics.

2. Use of "Omic" Technology to Inform the Risk Assessment A Case Study: Propiconazole

2.1 Background

This propiconazole case study document and its two appendices were developed to supplement the FIFRA Scientific Advisory Panel (SAP) background paper entitled "Integrated Approach to Testing and Assessment Strategy: Use of New Computational and Molecular Tools". This case study describes how EPA's Office of Pesticide Programs (OPP) and the Office of Research and Development (ORD) have expanded their research by linking key events along a specific adverse outcome pathway (AOP), *i.e.*, the progression of cancer in rodents, to define a mode of action (MOA) as illustrated below for propiconazole and discussed in detail in the subsequent pages of this document.

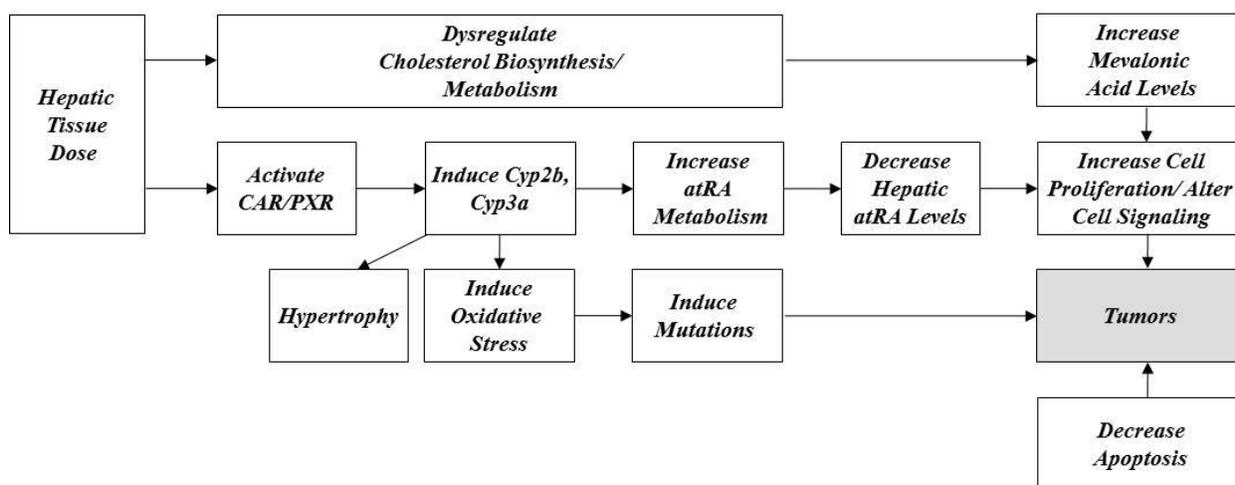


Figure 6. Proposed Mode of Action for Propiconazole

Additionally, the case study shows how this approach may increase efficiency and reliability of assessment relative to traditional approaches that are heavily reliant on resource intensive *in vivo* studies. EPA hopes to apply this approach more broadly over time and, as described in the background papers, the Agency is seeking the SAP's advice and recommendations about this proposal. It is important to note that the purpose of this background paper is to illustrate an approach and is not intended to assess the potential risks of propiconazole. Key terms used throughout this document are listed and defined as follows:

Definitions of Key Terms as Used in This Document

- **Adverse outcome pathways (AOP)** represent existing knowledge concerning the linkage between the molecular initiating event, intermediate key events, and an adverse outcome at the individual organism or population level.
- **Mode of action** is defined by the USEPA as the sequence of key events and cellular and biochemical events (measurable parameters), starting with the interaction of an agent with the target cell, through functional and anatomical changes, resulting in cancer or other adverse health effects (USEPA 2005).
- **Key events** are empirically observable, necessary steps in a disease (*e.g.*, cancer) process.

- **Apical endpoints** are tissue- or organism-based alterations observed *in vivo* and are often the first evidence that is observed after treatment-related chemical exposure; these can include but are not limited to tumors, loss of body weight, altered urinary proteins, and blood markers.
- **Toxicogenomics** is a field of science that examines apical endpoints from classical toxicology studies and combines them with the collection, interpretation, and storage of information about gene, protein, and endogenous metabolite levels within particular cells or tissues of an organism in response to a toxic substance. These data provide the molecular underpinnings for many of the adverse outcomes and offer novel insights into the adverse events associated with chemical exposure.
- **“Omics”** is a general term that is applied to the data generated from genomics (global analysis of genes expression), proteomics (global analysis of protein expression) and metabolomics (global analysis of endogenous metabolites).
- **Toxicity pathways** are subcellular and cellular response pathways that can lead to adverse outcomes (*e.g.*, health effects) when sufficiently perturbed.
- **Molecular initiating event** is the initial point of chemical-biological interaction within the organism that starts, *i.e.*, initiates, the AOP. Additional key events further along the pathway that lead to, and are experimentally or toxicologically associated with the adverse outcome, are referred to as “key events”.
- **Cytochrome P450:** Mixed function oxidases (MFO) also known as “CYPs”, are a host of enzymes, referred to as Phase I enzymes that metabolize chemicals, often as part of the body’s defenses to aid in the excretion of potentially harmful substances (*e.g.*, pesticides and other toxins).
- **AROD:** Alkoxyresorufin O-dealkylase is one of the MFO enzymes used to assay for up- or down-regulation of the enzymatic activities of individual and /or groups of CYPs.
- **CAR/PXR:** Constitutive androstane receptor/pregnane X receptor, is both part of a nuclear receptor subfamily that function as a sensor of xenobiotic substances and in response up-regulates the expression of target genes (CAR, *e.g.*, *Cyp2b10*; PXR *e.g.*, *Cyp3a11*) to produce the gene product (*e.g.*, Cyp2b, Cyp3a), which are responsible for the metabolism of toxic chemicals. Activation of CAR is a key necessary step in the hepatocarcinogenic MOA for certain chemicals.
- **Pathways** are fixed and confined groups of genes (or proteins) associated with a specific biological function (*e.g.*, retinol metabolism or CAR/PXR activation). Two types of software have been used in this analysis: Ingenuity Pathway Analysis (IPA) and GeneGo MetaCore software.
- **Networks** are groups of genes (or proteins) that are linked together in both simple and/or complex arrangements. The linking of one gene (or protein) and another gene (or protein) could be one of a series of functions such as binding or activating (*e.g.*, phosphorylation). Ingenuity creates networks from the list of genes submitted after they are mapped to the knowledge database of relationships. Multiple networks are possible from the same groups of genes because of the multiple functions of each gene.
- **IPA Tox List:** Functional gene groupings based on critical biological processes and key toxicological responses.
- **IPA Canonical Pathway:** Well-characterized metabolic and cell signaling pathways that have been curated by expert scientists.
- **High-throughput screens:** *In vitro* biochemical or cellular assays that can be run efficiently on a large number of compounds to determine their activity on different biological targets such as ion channels, receptors, enzymes, proteins, and signaling pathways Typically involves batch testing of chemicals for toxicological endpoints using automated liquid handling, detectors, and data acquisition methods.

2.1.1 General Trends with Toxicogenomic Information

The traditional risk assessment paradigm is based on exposure – dose – response without necessarily considering the underlying mechanism of action. In this model, the individual organism or particular tissue is exposed to chemicals or other stressors at some dose/concentration and a response in the organism or tissue is elicited. Thus, conventional

rodent toxicity studies, which are used to detect such responses, characterize adverse effects of a chemical primarily on apical endpoints such as clinical signs or pathological states. Data on precursor events such as enzyme induction or target cell proliferation are often available and could be used as indicators of the response in place of apical endpoints if a sufficient association of these events to the apical outcomes is provided. For many decades, the findings and interpretation of frank effects (*i.e.*, tumors) from rodent cancer bioassays have formed the basis for human health risk assessments and regulatory decisions about carcinogens and noncarcinogens. With the advent of toxicogenomics, however, significant advances have been made in the assessment of human/environmental health risks. In this case study, we explain how toxicogenomics can provide a more efficient and effective means of evaluating the potential toxicity of a chemical by taking advantage of knowledge about the chemical's mode of action (MOA) (NAS, 2007). Given the increasing number of chemicals requiring evaluation [more than 80,000 chemicals on the market, approximately 700 new chemicals added annually, (GAO, 2005)] and the need to provide more timely evaluations to insure appropriate regulation of chemicals deemed to pose high risks to human and/or environmental health, there is a need to streamline the testing scheme toward a more hypothesis-based, efficient and less resource intensive process based on adverse outcome pathways (AOPs).

A case in point is carcinogenesis, a complex process involving multiple levels of biological organization including genetic alterations and altered cellular events, progressing toward tumor formation, an apical endpoint and adverse outcome. The quickly evolving field of toxicogenomics has enabled a more comprehensive understanding of this progression starting with a molecular initiating event to intermediate key events at various levels of biological organization and culminating in tumor formation. The power of toxicogenomics and other molecular profiling technologies (collective referred to as "omics") such as transcriptomics, proteomics, and metabolomics to understand the steps leading to cancer is amplified when combined with conservative analytic techniques such as biochemical analysis and histopathology (Leighton, 2005; Reynolds, 2005). Transcriptomics is the global analysis of mRNA expression; proteomics is the global analysis of protein expression; and metabolomics is the global analysis of endogenous metabolites. The combination of new and standard practices in toxicology enhances the ability to inform mode of action analyses of chemical-induced toxicities such as cancer because toxicogenomic investigations have shown the ability to discriminate between mutagenic and nonmutagenic MOAs (Fielden *et al.*, 2007). Hence, pathway-associated gene, protein, and metabolite networks derived from the use of "omics" data may prove to be a robust approach in predicting mechanisms of toxicity and classifying chemical-specific MOAs. Our goal was to use "omics" data in the MOA framework to provide an improved scientific basis for determining potential cancer risk. Accordingly, the following documents were prepared: 1) Case study, 2) Data supporting the case study (Appendix A), and 3) Tables and Figures representing the data (Appendix B).

2.1.2 EPA Cancer Guidelines and Mode of Action (MOA) Framework

An organized approach to risk assessment and research in support of risk assessment was presented by the U.S. National Research Council (NRC) that describes how

the biologically effective dose is related to the precursor biological response that is ultimately related to the adverse health consequence from exposure to the stressor of concern (NRC 1994). More recent cancer risk assessments have been developed through the process of describing an MOA (USEPA 2005), which is the identification of the key (but not all) events associated with the development of an adverse effect. In contrast to MOA, mechanism of action implies a more detailed understanding and description of events, often at the biochemical and molecular level, than is meant by MOA (US EPA 2005).

Since the prediction of human health risks relies heavily on data generated from laboratory animals (generally rodents), it is unlikely that complete knowledge at the molecular level, (*i.e.*, the mechanism of action, of how an agent causes an adverse effect such as cancer) could be determined. As described earlier, this level of understanding is not necessary for establishing health protective risk assessments as knowledge of the MOA may be sufficient for this purpose. With respect to the potential risk of cancer from a chemical, once the animal MOA is established, qualitative and quantitative comparisons of each key event between the experimental animal and humans enable a conclusion of the likely relevance of the MOA to human risk. Data from the chronic toxicity/carcinogenicity studies conducted in laboratory rodents should not be interpreted in isolation. There are significant differences between rodents and humans in tumor prevalence, anatomy, or tumor histology. This awareness has influenced the U. S. EPA Cancer Guidelines emphasis on the use of MOA for carcinogenicity to interpret and quantify the potential cancer risk to humans. This awareness has influenced the U. S. EPA *Cancer Guidelines* emphasis on the use of MOA for carcinogenicity to interpret and quantify the potential cancer risk to humans. MOA analysis is central to any cancer risk assessment, and toxicogenomics can allow new research to be brought to bear particularly in comprehending the carcinogenic processes (USEPA, 2005; Cohen et al., 2003; Boobis et al., 2006). Consequently, the creation of an MOA to determine how the underlying biology translates across species, life stages, and guides the shape of the low dose region of the extrapolation curve has been adopted by EPA. The extension of this concept goes far beyond the scope of a cancer risk assessment and has been recognized by international bodies such as the International Life Science Institute (ILSI) and the International Program on Chemical Safety (IPCS), which have fashioned guidance for the use of the MOA framework to elucidate MOAs for both cancer and non-cancer endpoints (Cohen et al., 2003; Boobis et al., 2006; 2008).

2.1.3 Toxicogenomic Data in Risk Assessments

Since the EPA 2005 guidelines were promulgated, MOA data have been used, when available, for chemicals whose cancer risk assessment is being evaluated. Typically, an MOA analysis incorporates data from required toxicology studies and supplemental mechanistic data. Recently, toxicogenomic data have been considered as part of the weight-of-the-evidence (WOE) to test the application of these data to MOA analysis. At this time, the U.S. EPA has no guidance for incorporating genomic data into risk assessments, but the U.S. EPA Science Policy Council (SPC) has developed a draft interim guidance on various aspects of genomic data (USEPA, 2007). This report is presented to illustrate how the new technology can be used to enhance the efficiency and accuracy of our risk assessments. In keeping with these objectives, EPA has launched an approach to bring

about a shift in the way it assesses adverse effects and exposure; this was articulated in the strategic plan released by EPA (USEPA, 2009). This new strategy involves the incorporation of a wider suite of tools, (*e.g.*, “omics”, high throughput screenings, and predictive models) into the effects assessment process and the application of AOP-based approaches. The goals of this ambitious undertaking are:

1. To determine the relevance of EPA’s approach for developing a new testing strategy that is in concert with the vision laid down by the NAS (2007) for toxicity testing in the 21st century through the examination of existing classic toxicology results blended with newly generated toxicogenomic data;
2. To demonstrate the value of toxicogenomic data in achieving this goal through characterization of a cancer MOA/AOP;
3. To detect the perturbations (key events) within an MOA/AOP as a result of chemical exposure and to understand how they lead to apical effects;
4. To show how these generated data can be incorporated into knowledge-based profiling data models for predicting the hazards of new or untested pesticides;
5. To address the risk uncertainties associated with extrapolation of effect data from lower levels of biological organization to whole organisms and/or populations in a reliable, time- and cost-effective manner;
6. To show how animal usage can be minimized;
7. To provide a more mechanistically-based process for quantitative risk assessments;
8. To inform and encourage risk assessors on the utility of “omic” data in chemical-specific risk assessments.

To address these goals, the U. S. EPA research laboratory, NHEERL/ORD, developed a toxicogenomic dataset for several registered conazoles that lack MOA data for various toxicities, including tumorigenicity. Pesticides in this class are fungicides and are commonly used in the protection of fruit, vegetable and cereal crops. In addition to their widespread agricultural use, many conazoles are also available as pharmaceuticals for treatment of local and systemic fungal infections in humans. Antifungal activity is exerted through inhibition of a specific cytochrome P450, *i.e.*, CYP51 (lanosterol 14 α - demethylase), a critical step in the biosynthesis of ergosterol, a steroid required for formation of the fungal cell wall. In mammals, many of these conazoles induce a range of responses and toxicities that include induction or inhibition of hepatic CYP isoforms, hepatic hypertrophy, hepatotoxicity, and liver tumors. The present effort concentrates on propiconazole, which induces both liver adenomas and carcinomas in male CD-1 mice. It should be stressed that the regulatory status of propiconazole is not the focus of this undertaking; propiconazole was selected only as a model compound to test and illustrate an approach to incorporate toxicogenomic data into the risk assessment process. The data supporting the detailed case study for propiconazole are presented in Appendices A and B.

This Chapter is organized into two sections (Background and Approach). The approach section presents the strategy developed to examine a chemical with large datasets (*i.e.*, propiconazole) using both traditional and toxicogenomic data to investigate the MOA of mouse liver tumors. Additionally, ways in which these data can be incorporated into the risk assessment process (*e.g.*, definition of the MOA, defining AOPs, building of knowledge-based models for predicting the toxicity of new or untested pesticides) are discussed.

2.2 Approach

Propiconazole was selected based on discussions and on the wealth of toxicology studies submitted in response to FIFRA data requirements. The NHEERL studies of Sun *et al.* (2005), Allen *et al.* (2006), Ward *et al.* (2006), Goetz *et al.* (2006), Chen *et al.* (2008; 2009), Bruno *et al.* (2009), Nesnow *et al.* (2009; 2011), and Ortiz *et al.* (2010), Ross *et al.* (2009, 2010) represent the determination of traditional apical endpoints, non-traditional apical endpoints and the assessment of “omic” responses to support concerns regarding carcinogenicity.

A proposed phenobarbital type MOA, *i.e.*, microsomal enzyme induction, which is shown in Table 1, features the following apical endpoints: increase in body/liver weight (a result of hypertrophy); the livers contained increased levels of CYP proteins (*i.e.*, Cyp2b) and CYP enzymatic activity (PROD) which led to cell proliferation and tumors. Phenobarbital is known to act through a mitogenic MOA (Whysner *et al.*, 1996) where mitogenesis is the process that stimulates cell proliferation in the target organ without causing obvious cytotoxicity or cell death, and creates a favorable environment for tumor development partly because mitogens can act like tumor promoters.

Table 1. Possible Key Events Suggesting a Mitogenic Mode of Action (MOA) from the Toxicology Studies Findings with Propiconazole--Apical Endpoints

Key Events:

- Increased Liver /Body Weight
- Increased Liver Hypertrophy
- Increased CYP 2b Protein and Increased PROD Activity
- Increased Cell Proliferation
- Increased Liver Tumors

PROD = Pentoxoresorufin O-dealkylase; most frequently associated with the cytochrome P450 protein, Cyp2b

In the NHEERL studies, dose and time course evaluations were conducted using experimental conditions equivalent to those used in the 2-year chronic bioassay (Allen *et al.*, 2006). Briefly, groups of male CD-1 mice (17-21/dose) were administered dietary concentrations of 0, 100, 500 or 2500 ppm propiconazole for 4, 30 or 90 days.

Toxicological effects included: liver hypertrophy, increased AROD activities, increased hepatic cell proliferation, increased serum triglycerides, decreased serum high density lipids, and decreased serum cholesterol levels. Although propiconazole was determined to be non-genotoxic (non-DNA reactive as parent or metabolite) using short-term tests for genotoxicity, it was found to induce *in vivo* mutagenicity in the livers of Big Blue[®] transgenic mice after 4d of treatment at 2500 ppm (Ross *et al.*, 2009; Ross and Leavitt, 2010), a response possibly linked to oxidative stress-mediated endogenous DNA damage. Data that support this hypothesis have recently been presented (Nelson *et al.*, 2011).

Transcriptional analysis of liver tissue revealed: CAR and PXR nuclear receptor activation, CYP induction, oxidative stress, dysregulation (abnormal control) of cholesterol biosynthesis/metabolism, effects on retinoic acid metabolism, and alterations in cell signaling, cell growth, cell proliferation and apoptosis pathways (Ward *et al.*, 2006; Nesnow *et al.* 2009). Proteomic analyses of cytosolic liver fractions confirmed over half of the propiconazole-altered transcriptional pathways and identified additional pathways (Ortiz *et al.*, 2010). Metabolomic analysis of liver homogenates enhanced the knowledge-base on the process of metabolism and excretion of propiconazole, identified potential cancer biomarkers and extended the data on several key events within the MOA of propiconazole (Nesnow *et al.*, 2011).

Propiconazole-induced oxidative stress was confirmed by the identification of carbonylated proteins and by biochemical measurements (Bruno *et al.*, 2009). Studies confirmed that hepatic retinoic acid levels were reduced by propiconazole treatment and that this effect was related to specific CYP induction (Chen *et al.*, 2009). Dysregulation of cholesterol biosynthesis and metabolism was confirmed by metabolomic analysis (Nesnow *et al.*, 2011). Based on these findings, many of the toxicogenomic observations cited above were selected as key events and were synthesized into the proposed MOA that elucidated the toxicological effects of propiconazole (Figure 7).

The MOA analysis for propiconazole describes a series of key events that lead to mouse liver tumors. Many of the effects of these key events could generally be ascribed to either CYP induction or CYP inhibition. Key events related to CYP induction include: activation of nuclear receptors; hypertrophy, induction of CYP proteins, induction of oxidative stress, reduction in the hepatic levels of all-trans retinoic acid and induction of mutations (See Figure 7 [Key Events in blue boxes]). Events related to CYP inhibition are: dysregulation of cholesterol biosynthesis and metabolism and an increase in the levels of mevalonic acid (Figure 7 [Key Events in green boxes]). Propiconazole also decreases apoptosis; increases cell proliferation; and leads to liver tumors (Figure 7 [Key Events in orange boxes]). In this proposed MOA, propiconazole activates the CAR/PXR nuclear receptors, which in turn induce hypertrophy and increase levels and activities of Cyp2b and Cyp3a proteins, causing oxidative stress and mutations. Concomitant with this process is increases in Cyp2b and Cyp3a enzymatic activities, which trigger increased metabolism of all-trans retinoic acid (atRA), reduce atRA hepatic levels and increase cell proliferation. Propiconazole also inhibits CYP51, causing dysregulation of cholesterol biosynthesis and metabolism leading to increased cholesterol biosynthetic intermediates (*e.g.*, mevalonic acid), which results in increased cell proliferation. Propiconazole suppresses apoptosis,

which in concert with increased mutations and increased cell proliferation, can lead to tumor formation.

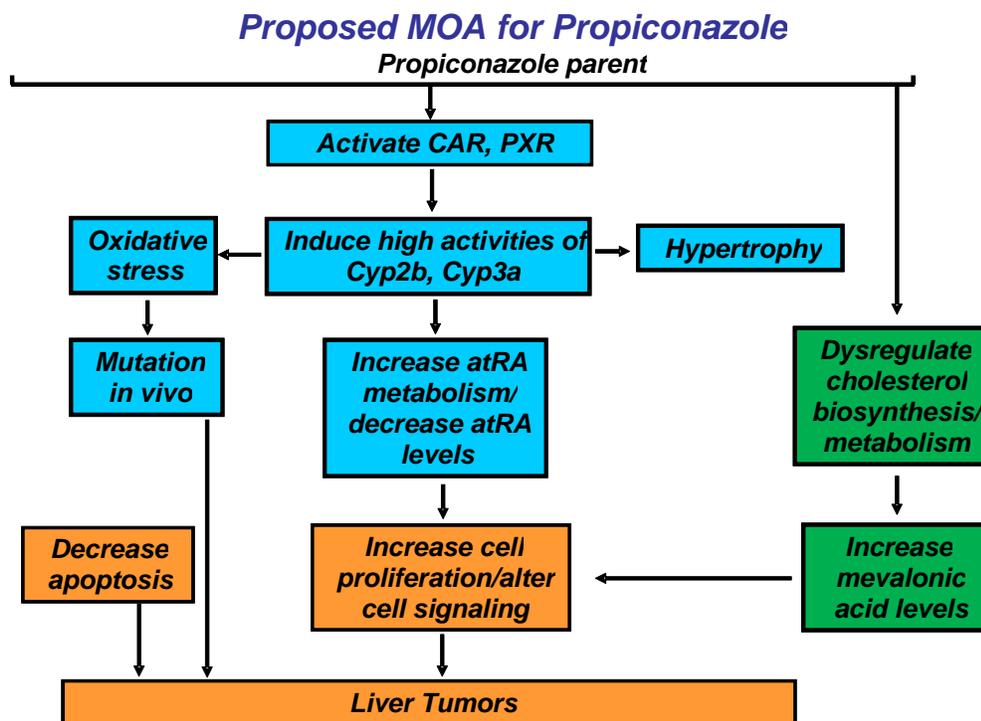


Figure 7 Proposed Mode of Action (MOA) of Propiconazole Using Toxicogenomic Data

Key Events enclosed in blue boxes are related to CYP induction; Key Events enclosed in green boxes are related to enzyme inhibition; Key Events enclosed in orange boxes are result of previous key events or are a direct result of propiconazole treatment.

2.2.1 Data elements supporting the MOA

As shown in Tables 2-5, propiconazole is a rich source of apical endpoints and toxicogenomic data. The classical toxicology studies serve as the point of reference to compare toxicogenomic responses to apical data and demonstrate the proof of concept of OPP's overall integrated approach to develop a MOA and an adverse outcome pathway (AOP) using these emerging toxicity testing strategies.

The apical endpoints along with the toxicogenomic data for propiconazole show a high degree of concordance with respect to adverse events, time and dose (Table 2). For example, CAR/PXR activation was identified by toxicogenomic analysis and was confirmed by the AROD data with marked increases in PROD activity, which occurred as early as 4 days at 500 and 2500 ppm (Allen *et al.*, 2006) (Table 3) and at 850 and 2500 ppm by day 14 in the sponsor's submitted mechanistic study (MRID 45215803, Table 4). *Cyp2b10* mRNA levels were increased 2.4X by 150 mg/kg/day (\approx 1000 ppm propiconazole) (Sun *et al.*, 2005) and *Cyp2b* protein levels were increased as well (Chen *et al.*, 2009). PXR/RXR activation was identified by toxicogenomic data. Levels of *Cyp3a11* mRNA were increased

at the same and higher doses after 4 or after 14 days of treatment (Sun *et al* 2005, Chen *et al.*, 2009). Cyp3a protein levels were increased (Chen *et al.*, 2009) and testosterone 16 β -hydroxylation (a measure of Cyp3a protein activity) was also increased (MRID 45215803). A 45% decrease in levels of hepatic all *trans*-retinoic acid (atRA) was evident in the study of Chen *et al.* (2009) after 4 days of treatment with 2500 ppm propiconazole and this was related to the induction of specific CYPs.

The apical data from the subchronic and chronic bioassays showed that reductions in the serum cholesterol level (-29 to -64% of control) were noted 4 days after mice received dietary preparations containing 850, 1450 or 2500 ppm propiconazole, respectively, for 4 days (Table 5). Allen *et al.* (2006) showed comparable activity at 2500 ppm after 30 and 90 days of treatment (Table 3).

Cell proliferation was markedly increased after 1 day's treatment with 850 or 2500 ppm; peaked at day 2 (37X at 850 ppm and 49X at 2500 ppm, respectively); started to decline at 3 days; and generally returned to background by day 14 (Table 4, MRID 45215802). The 4-day data by Allen *et al.* (2006) are also in good agreement with the findings from the sponsor's mechanistic study. The overall analysis indicated that the toxicogenomic studies conducted by NHEERL were instrumental in further delineating the initial MOA laid out for propiconazole, which only considered apical endpoints related to hepatocarcinogenesis. While "omic" studies are currently not a part of the typical toxicology studies reviewed by risk assessors, they provide important information to further characterize the MOA analysis as well as augment other areas of risk assessment. The application of "omic" data to refine the MOA and AOPs and other areas of the risk assessment is presented below:

Table 2. Temporal Association- Toxicology, Genomic, Proteomic and Metabolomics Studies with Propiconazole

Key event	Time, d (feed levels, ppm)										
	1	2	3	4	7	14	28/30	56	90	119	2yr
CAR/PXR Activation				G, IA (2500)			G/IA (2500)		G/IA (2500)	IA	
Increased Cyp2b Cyp3a				A, G 500, 2500)		A	A, G (500, 2500)		A, G (500, 2500)		
<i>Increased hypertrophy</i>				A (2500)		A	A (2500)		IA (2500)	A	A
Increased atRA metabolism- decreased hepatic atRA levels				A, G (2500)			IG (2500)		IG (2500)	IA	
Increased oxidative stress				A, G, P (2500)			IA, IG (2500)		IA, IG (2500)		
In vivo mutation				A, G (2500)			IG				
Inhibition of CYP51- dysregulation of cholesterol biosynthesis /metabolism and reduction in serum cholesterol levels				Increase hydroxylated cholesterol and bile acids. M (1250-2500) Decrease serum cholesterol levels. A (2500)			A (2500)	A	A (2500)	A	
Increase hepatic mevalonic acid levels				IG (2500)							
Increased cell proliferation	A	A	A	A (2500)			IG		IG		
Suppression of apoptosis				IG (2500)			IG		IG		
Tumors											A (2500)

Bold = causal key event; Italicized = associative event

Italicized = associative events

A= apical endpoint measured

G= genomic endpoint measured

P= proteomic endpoint measured

M=metabolomic endpoint

IA= inferred from apical endpoint

IG= inferred from genomic endpoint

Table 3. Summarized Key Events Associated with Tumor Induction in Male Mice Fed Diets Containing Propiconazole (Submitted Mechanistic Studies From the Open Literature) ^{a,b}

Key Event	Dose (ppm)	Dose (mg/kg/da)	Days				Reference
			4	14-15	30	90	
Activation of Nuclear Receptor: CAR/PXR^d							
EROD	500	50	1.7X ^c		1.8X		Allen et al., 2006
		75		1.2X		Sun et al., 2005	
		150		1.3X		Sun et al., 2005	
PROD	500	350	2.1X		3.1X	4X	Allen et al., 2006
		75		3.5X		Sun et al., 2005	
		150		5.4X		Sun et al., 2005	
Cyp2b10	500	350	13.3X		37.5X	35.9X	Allen et al., 2006
		75					
		150		2.4X 2.6X		Sun et al., 2005; Goetz et al., 2006	
Cyp2c55	2500	350	--	--	--	--	
		150		27.6X		Goetz et al., 2006	
		350	--	--	--	--	
Cyp3a11	2500	350	4	14-15	30	90	Sun et al., 2005
		150		5.2X			
		350	--	--	--	--	
↑ Liver Weight							
	500	50					
		75					
		150		1.5X 1.4X		Sun et al., 2005 Goetz et al., 2006	
	2500	350	1.4X 1.3X		1.9X	1.95X	Allen et al., 2006 Bruno et al., 2009
↑ Liver Hypertrophy							
	100	10	80%		60%	60%	Allen et al., 2006
	500	50	60%		80%	100%	Allen et al., 2006
		75					
		150					
	2500	350	100%		100%	100%	Allen et al., 2006
↓ Serum Cholesterol (Dysregulation of cholesterol biosynthesis and metabolism)							
	2500	350	--	--	-56 %	-55%	Allen et al., 2006
↓ Hepatic Retinoic Acid Levels							
	2500	350	45%↓				Chen et al., 2009

Key Event	Dose (ppm)	Dose (mg/kg/da)	Days				Reference
↑ Cell Proliferation							
	500	50					
	2500	350	9X		6X	1.8X	
						Allen et al., 2006	

^a Data were derived from Allen et al. (2006); Sun et al. (2005); Goetz et al.(2006); Chen et al. (2009) and Bruno et al. (2009),

^b N = 4-12 mice/group (Allen et al., 2006); 4 mice/group (Sun et al., 2005) 6 mice/group (Goetz et al., 2006); 3 mice/group (Chen et al., 2009); and 5 mice/group (Bruno et al., 2009).

^c Values expressed as X= fold increase over control: values expressed as % = %of control.

^d Abbreviations: CAR = Constitutive androstane receptor PXR = Pregnane X receptor EROD = Ethoxyresorufin O-dealkylase
 PROD = Pentoxyresorufin O-dealkylase

Table 4. Summarized Key Events Associated with Tumor Induction in Male Mice Fed Diets Containing Propiconazole (Submitted Mechanistic Studies) a b

Key Event	Dose (ppm)	Days								Reference
		1	2	3	4	7	14	28	60	
CAR^c Activation										
Total P450	850						3X ^d			45215803
	2500						3.9X			
EROD	850						2.2X			
	2500						3.9X			
PROD	850						30X			
	2500						55X			
Coumarin 7-hydroxylase	850						5X			
	2500						24X			
UDPGT	850						1.6X			
	2500						1.4X			
Cytosolic GST	850						1.6X			
	2500						1.8X			
Liver/Body Weight	850						1.3X			
	2500						2X			
Hypertrophy	850	1.1X	1.3X	1.3X	1.3X	1.3X	1.3X	1.4X	1.3X	45215802
	2500	1.2X	1.4X	1.5X	1.7X	1.9X	2.1X	2.3X	2X	
Hypertrophy	850	Mi	Mi	Mi	Mi	Mi	Mi	Mo-Ma	Mo-Ma	
	2500	Mi	Mi	Mi	Mi	Mi	Mi	Mo-Ma	Mo-Ma	
Cell Proliferation	850	19X	37X	6X	5X	<1X	<1X	<1X	<1X	
	2500	11X	50X	19X	10X	8X	1X	2X	1X	

^a Data were derived from MRID Nos. 45215802 and 45215803.

^b n generally = 5 mice/group.

^c Abbreviations:

CAR = Constitutive androsane receptor UDPGT = Uridine diphosphate glucuronosyl-transferase

EROD = Ethoxyresorufin O-dealkylase GT = Glutathione S-transferase

PROD = Pentoxyresorufin O-dealkylase

Mi = Minimal Mo-Ma = Moderate-Marked

^d Fold increase

Table 5. Summarized Key Events Associated with Tumor Induction in Male Mice Fed Diets Containing Propiconazole (Submitted Studies) ^{a b}

Key Event	Dose (ppm)	Weeks										
		4	8	9	13	14	17	52-53	78	100		
MRID		-502 ^a	-502 ^a	-401 ^a	-502 ^a	-501 ^a	-401 ^a	-501 ^a	-401 ^a	570 ^a	-401 ^a	-570 ^a
Liver/Body Weight	500 ^b	-	-	10%	18%	-	-	-	13%	12%	10%	6%
	850	-	-	33%	34%	-	-	-	29%	-	20%	-
	1450	-	-	-	56%	-	-	-	-	-	-	-
	2500	-	-	-	94%	-	-	-	-	41%	-	50%
Hypertrophy	500 ^b	100%	80% ^c	60% ^c	80%	-	-	20%	60%	18% ^d	56%	33% ^d
	850	90%	100%	100%	100%	-	-	70%	80%	-	58%	
	1450	100%	100%	-	100%	-	-	100%	-	-	-	
	2500	90%	100%	-	100%	-	-	100%	-	78% ^d	-	86% ^d
Cholesterol	500 ^b	NC	NC	-11%	NC	NC	-14%	-12%	-15%	-15%	-24%	-6%
	850	-29%	-9%	-25%-	-30%	-10%	-24%	-24%	-34%	-	-29%	-
	1450	-37%	-49%	-	-39%	-42%	-	-45%	-	-	-	-
	2500	-64%	-50%	-	-45%	-41%	-	-44%	-	-28%-	-	+44%
Single cell necrosis	500 ^b	40%	80%	10%	25%	-	-	0	0	0	0	0
	850	70%	40%	30%	65%	-	-	0	0		0	
	1450	90%	90%	-	90%	-	-	10%				
	2500	90%	90%	-	100%	-	-	60%		0		0
Necrosis	500 ^b	10%	10%	30%	25%	-	-	10%	0	0	0	0
	850	30%	0	-50%	15%	-	-	20%	0		0	
	1450	30%	50%	-	30%	-	-	40%				
	2500	70%	60%	-	30%	-	-	60%		0		0
Tumors^f	500 ^b										10%	37%
	850										24%	
	1450											
	2500											86%

^a Data were derived from MRID Nos. 42050502, 42050401, 42050501, 45215801, and 000129570.

^b Results for lower doses (≤ 100 ppm) were generally comparable to the vehicle control group. /10 = number of animals

^c MRID 45215801: reread of histological slides from MRID 42050502 (8-week) and 44381401 (9-week)

^d Listed as "hepatocyte enlargement, presumed to be hepatocyte hypertrophy.

^f Combined adenomas and carcinomas

2.2.2 Utility of “Omics” to Define the Propiconazole MOA

One of the obvious advantages of using “omics” data is to gain more insights into mechanisms of toxicity. The data generated in the propiconazole case study are illustrative of this point and clearly show how the “omics” data more completely clarify the operative MOA for propiconazole carcinogenesis (Figure 7) and enhance the knowledge base for propiconazole-induced hepatocarcinogenicity in the following ways:

2.2.2.1 Identify a series of potential molecular initiating and other key events that are plausibly linked to tumorigenesis (MOA/AOP).

The molecular initiating events that were identified from apical measurements were elevated expression of Cyp2b and Cyp3a (*i.e.*, CAR/PXR nuclear receptor activation) and inhibition of Cyp51 (*i.e.*, dysregulation of cholesterol biosynthesis and metabolism). Other key events include: liver cell hypertrophy, mutations *in vivo* (in transgenic mice), increased cell proliferation (hyperplasia), and liver tumors. The “omic” analyses were an equally rich source of potential key events when these results were associated with what is known about the carcinogenesis mechanisms. Intermediate key events identified by “omic” analyses include: oxidative stress, increase in atRA metabolism, dysregulation of cholesterol biosynthesis and metabolism, increased mevalonic acid formation, increased cell proliferation, and decreases in apoptosis (Ward *et al.*, 2006; Nesnow *et al.*, 2009) (Figure 7). The results of the microarray genomic analysis revealed the following:

- CAR and PXR canonical pathways (See page 4, Key Terms) altered by propiconazole were identified.
- The oxidative stress related canonical pathways altered by propiconazole exposure were: Nrf2-mediated oxidative stress response, and mitochondrial dysfunction;
- The Retinol Metabolism canonical pathway was identified as being altered;
- A series of genes in the cholesterol biosynthesis and metabolism pathways were also up regulated;
- Decreases in specific genes associated with apoptosis and the alterations of pathways associated with apoptosis: Apoptosis and Apoptosis Signaling; DNA-damaged-induced apoptosis; and Regulation of BCL2-associated agonist of cell death (BAD) phosphorylation were identified.

In addition to identifying new key events within the MOA, “omic” data provided greater depth to the understanding of certain apical key events (Ward *et al.*, 2006; Nesnow *et al.*, 2009). For example, pathways associated with DNA damage were altered supporting the *in vivo* mutation studies. These included Cdc25 and Chk1 Regulatory Pathways leading to DNA damage; Retinoblastoma Tumor Suppressor/Checkpoint Signal leading to DNA damage; and ATM/ATR regulation of G2/M checkpoint pathways, associated with cell

proliferation. Altered cell growth/signaling pathways were: PI3K/AKT Signaling; p38 Map Kinase Signaling; PTEN Signaling; TGF- β signaling; mTOR Signaling; and WNT Signaling. Other cell proliferation/cell cycle pathways altered by propiconazole were: Cell Cycle: G1/S Check Point; Influence of Ras and Rho on G1 to S Transition. Disruption of these pathways can eventually lead to tumors. The “omic” data also helped in identifying the distinct chemical-biological interactions of the molecular initiating events that are linked with tumor formation and are an integral part of the AOP analysis.

2.2.2.2 Link a continuum of precursor events that lead to apical responses.

One method of establishing a continuum of key events is to find differentially expressed genes (DEGs) common to each event. Examples are the key events of nuclear receptor activation and CYP induction in the livers of propiconazole-treated mice. CAR/PXR activation was significantly increased when mapped to the Ingenuity Pathway Analysis (IPA) Toxicity List [www.ingenuity.com] (Nesnow *et al.*, 2009). This list contained a number of cytochrome P450's (CYP) *Cyp1a2*, *Cyp2b*, and *Cyp2c* genes. The over expression of several of these genes was confirmed by Q-RT-PCR (Chen *et al.*, 2009). This shows that genes are common to both apical endpoints (CAR activation and Cyp induction), thus linking these events.

A second example describing a continuum of events is Cyp induction and retinoic acid metabolism. Mapping differentially expressed genes (DEGs) to the IPA Canonical Pathway, revealed that retinol and retinic acid metabolism were altered in livers from propiconazole-treated mice as evidenced by transcriptomic analyses. In addition, several Cyps known to be involved in the metabolism of retinoic acid were over expressed: *Cyp26a*, *Cyp3a*, and *Cyp2c*.

Retinoids are well-known anticancer agents that induce differentiation and growth suppressive signals in normal, premalignant, and malignant cells (Freemantle *et al.*, 2003). One of the major roles of retinoids is to control cell proliferation in epithelial cells. Follow-up experimental studies showed that Cyp3a protein levels and retinoic acid metabolism were also increased in the liver by propiconazole (Chen *et al.*, 2009). As retinoic acid metabolism was increased, it was hypothesized that retinoic acid levels in the liver would decrease. This hypothesis was confirmed by direct measurement of reduced retinol acid levels in liver tissues from propiconazole-treated mice (Chen *et al.*, 2009). These propiconazole data suggest that the decrease in atRA levels would provide a tumor promoting environment (Wang, 2003). Retinoic acid controls cell proliferation and lower levels of retinoic acid would be consistent with increases in cell proliferation, which were also observed in treated animals.

2.2.2.3 Identify cellular pathways and networks associated with these events for both linkage and coherence by enhancing information contained in apical key events.

An example of the linkage and coherence of the cellular pathways to key events is oxidative stress. Propiconazole induced a series of genes associated with oxidative stress through the Nrf2 pathway. This was observed in both pathway analysis and by the up-regulation of specific genes. Several aldo-keto reductases were also up regulated suggesting reactive oxygen species (ROS) formation, lipid peroxidation and reactive aldehyde formation. Many apical endpoints related to oxidative stress were increased by propiconazole treatment (*i.e.*, protein carbonyl formation, GSSG/GSH ratio; GSH levels and glutathione S transferase [GST] activities) (Bruno *et al.*, 2009; Nesnow *et al.*, 2011).

There is also linkage and coherence between the increased mutation frequency with a number of up regulated genes associated with DNA damage and repair as well as with the formation of ROS and its sequelae, lipid peroxidation and reactive aldehyde formation. Propiconazole treatment increased the mutation frequency in the livers of transgenic mice. Transcriptomic analysis identified both altered pathways associated with DNA damage and repair: Cdc25 and Chk1 Regulatory Pathway to DNA damage; Retinoblastoma Tumor Suppressor/Checkpoint Signal to DNA damage; and ATM/ATR regulation of G2/M checkpoint. A number of specific DNA repair genes were also up regulated: *Parp2*; *Atr*; *Apex1*; and *H2afx*. *Gadd45b* was significantly up regulated and is involved in the control of cell cycle, DNA repair and apoptosis (Thyss *et al.*, 2005).

2.2.2.4 Infer other key events not measured through apical approaches.

One example of this application is the decrease in apoptosis. A series of apoptosis-related genes with altered expression levels were detected by microarray analyses of livers from mice fed propiconazole in their diets. Anti-apoptotic genes (Li *et al.*, 2000; Pandey *et al.*, 2000) including *Gadd45b*, *Ikbkb*, *Ikbke*, *Ikbkg*, *Hsp70* *Hsp 90* and *Tmbim4* were up regulated (Ward *et al.*, 2006). A number of the pro-apoptotic genes, *Fas*, *Casp6* and *Bmf* were suppressed (Ward *et al.*, 2006). The data strongly suggest that propiconazole suppressed apoptosis in mouse livers. The high levels of *Gadd45b* mRNA induction at each time point indicates that high levels of protection against apoptosis occurred in the livers of propiconazole-treated mice. These results suggest a mechanism for reduced apoptosis in propiconazole-treated mouse liver, which focuses on *Gadd45b* as the indicator of an anti-apoptotic protein. Based on analyses of gene expression studies, the overall results suggest a decrease in apoptosis.

Another example of a key event identified by genomic analyses without a direct apical endpoint is the dysregulation of cholesterol biosynthesis and metabolism with increased mevalonic acid formation as a result of the overexpression of cholesterol biosynthetic genes. This is likely a compensatory effect related to cholesterol biosynthesis

inhibition by propiconazole, an effect attributed to the known ability of propiconazole to inhibit CYP51 (lanosterol 14 α -demethylase), a key step in the cholesterol, ergosterol biosynthesis pathway (Trosken *et al.*, 2006). This direct interaction is a distinct molecular initiating event for a second APO, in addition to the APO initiated by CAR/PXR activation.

Microarray analyses of the mRNA from the livers of mice treated with a number of conazoles show that the genes involved in the biosynthesis of cholesterol were consistently up regulated but to differing extents, presumably through a negative feedback compensatory mechanism producing more cholesterol (Ward *et al.*, 2006; Nesnow *et al.*, 2009). Mice treated with propiconazole (2500 ppm) for 4 days over expressed several key genes in the cholesterol anabolic pathway including squalene epoxidase (*Sqle*, 2.6-fold increase), lanosterol α -demethylase (*Cyp51*, 1.7-fold increase) and sterol-C4-methyl oxidase (*Sc4mol*, 1.6-fold increase), and 7-dehydrocholesterol reductase (*Dhcr7*, 1.5-fold increase) (See Table 12, Support Document for Case Study, Appendix B). Cholesterol is further metabolized to hydroxylated cholesterols and converted to bile acids. Several genes in these pathways were also up regulated at 4 days of treatment: *Cyp7a* (cholesterol 7 α -hydroxylase) 1.8-fold increase, aldo-keto reductase family 1, member D1 (*Akr1d1*, 2.1-fold increase), and aldehyde dehydrogenase family 1, subfamily A7 (*Aldh1a7*, 1.8-fold increase) (Ward *et al.*, 2006; Nesnow *et al.*, 2009). From proteomic analyses of livers of mice treated with propiconazole for 4 days, Aldo-keto reductase family 1, member D1 (*Akr1d1*) was increased 1.7-fold and aldehyde dehydrogenase family 1, subfamily A1 (*Aldh1a1*) was increased 1.3-fold, thus confirming the transcriptomic results (Ortiz *et al.*, 2010).

Concomitant with the microarray data, however, results from the submitted studies indicated that propiconazole treatment (2500 ppm) consistently reduced the serum cholesterol levels in the mice. The effect was first observed at 4 days and continued for at least 1½ years (Table 2). An explanation for these observations is the inhibition of cholesterol transport. Acyl-coenzyme A (CoA) cholesterol acyltransferases (ACATs) are membrane-bound proteins that utilize long-chain fatty acyl-CoA and cholesterol as substrates to form cholesteryl esters. ACATs play important roles in cellular cholesterol homeostasis in various tissues (Chang *et al.*, 2009), and the inhibition of ACAT activity has been associated with decreased plasma cholesterol levels (Leon *et al.*, 2005). Transcriptomic data from the livers of mice treated with propiconazole indicated significant decreases (-1.7 to -1.9-fold) in *Acat1* levels over a 90-day period (Nesnow *et al.*, 2009) suggesting a continued suppression in cholesteryl ester formation and transport of cholesterol out of the hepatocyte. Metabolomic data demonstrated that propiconazole treatment increased the levels of hydroxylated cholesterols and a number of bile acids (Nesnow *et al.*, 2011) suggesting an increased flux in cholesterol biosynthesis and metabolism. The increased flux of cholesterol biosynthesis increased the levels of mevalonic acid, an intermediate in the cholesterol biosynthesis pathway. Transcriptomic data after 4 days of treatment revealed significant increases in a series of genes within the cholesterol biosynthesis and metabolism pathways suggesting a dysregulation of cholesterol biosynthesis and metabolism. Cholesterol biosynthesis essentially begins with the action of HMG-CoA reductase on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to form mevalonic acid. Mevalonate production is a step in the cholesterol biosynthetic pathway

and leads to the formation of isopentenyl pyrophosphate, farnesyl pyrophosphate and lanosterol. Farnesylation of ras protein by farnesyl pyrophosphate is a posttranslational modification that allows attachment of ras to the plasma membrane. Eventually this triggers the ras/MAPK signaling pathway that drives malignant transformation through mutant ras GTP complexes (End, 1999). Ras regulates cyclin D1 expression and affects the G1 to S transition as well as the progression of cells through the G1 checkpoint into S phase, which leads to proliferation. Inhibition of mevalonate synthesis would block this process since it has been shown that inhibition of mevalonate prevents farnesylation of p21ras proteins and arrests cell growth of CHO cells (Goldstein and Brown, 1990). It is, therefore, hypothesized that propiconazole-induced cholesterol reduction brings about a negative feedback loop increasing hepatic mevalonic acid levels that can result in increased cell proliferation through increased ras farnesylation and activation of the MAPK signaling pathway. This hypothesis has been tested and verified using immortalized mouse hepatocytes (Murphy *et al.*, 2011).

2.2.2.5 Extend apical dose-response relationships to lower doses.

There are many examples where the apical responses can be extended to lower doses using transcriptomic data. A case in point is the *Gstm2* and *Gstt3* genes, which are associated with oxidative stress and exhibited a dose response of gene expression after 4 days of treatment with 500 or 2500 ppm propiconazole, glutathione S-transferase (GST) activity, which is encoded by these genes, was only evaluated at 2500 ppm propiconazole and found to be significantly increased. It is likely that GST activity would also be increased at 500 ppm since gene expression was also up regulated at this lower dose.

2.2.2.6 Consideration of alternate MOAs.

An example of the ability of toxicogenomic data to rule out potential alternative MOAs is illustrated in the case study for propiconazole. Propiconazole was compared to the prototypic reference compound phenobarbital to assess similarities and differences using transcriptomic profiling (Nesnow *et al.*, 2009). Targeted transcriptomic analyses were conducted on a global level using Principal Components Analysis (PCA) and on the gene level examining DEGs, and subsets of DEGs (cell cycle genes, genes associated with human hepatocellular tumors, and transcription factors). Analyses were also conducted on function, pathway and network levels by examining IPA Tox Lists and IPA Canonical Pathways maps, and GeneGo MetaCore dynamic networks and their central hubs www.genego.com. Genes expressed by propiconazole were also compared with genes associated with human hepatocellular cancer. The data indicated that although phenobarbital and propiconazole induced mouse liver tumors and exhibited similar apical responses, their transcriptional profiles were significantly different. This is shown as a Principal Components Analysis (PCA) plot (Figure 8). In this plot, each dot represents the total microarray data from one mouse after treatment with propiconazole or phenobarbital. Note, the propiconazole samples cluster in one area of space while the

phenobarbital samples cluster together in a separate area of space consistent with treatment group differences.

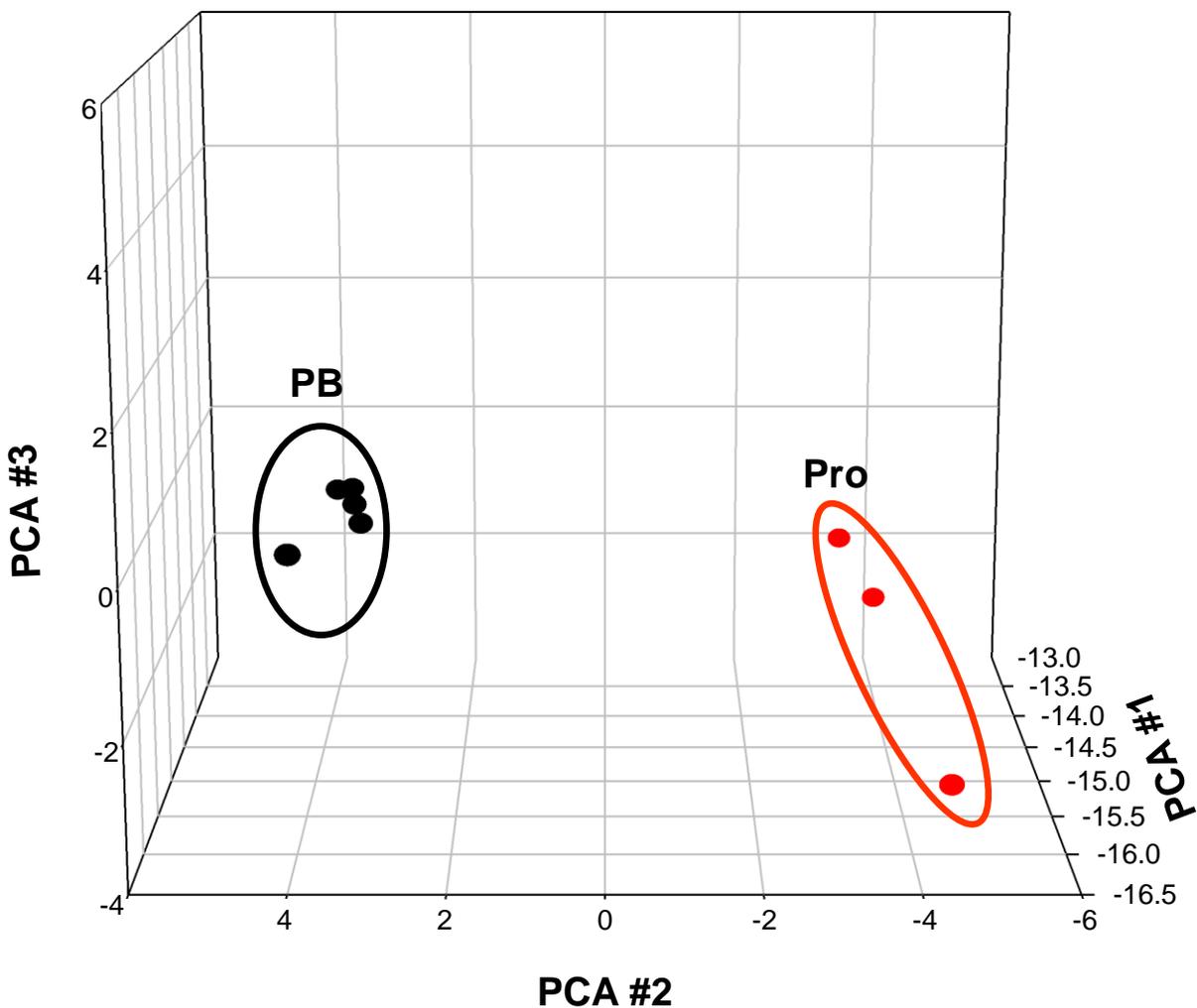


Figure 8. Principal Component Analysis (PCA) of DEGs from Phenobarbital (PB) and Propiconazole (Pro) Treatments at 30d. Data extracted from Nesnow et al. 2009.

2.2.3 Relationship of MOA to AOP/ Incorporation of the generated data into knowledge-based data models for prediction of toxicity for new or untested chemicals.

MOA and AOP are similar in concept. MOA is defined as the sequence of key events and cellular and biochemical events (measurable parameters), starting with the interaction of an agent with the target cell, through functional and anatomical changes, resulting in cancer or other adverse health effects (USEPA 2005; Boobis *et al.* 2008). An AOP represents existing knowledge concerning the linkage between a molecular initiating event and an adverse outcome at the individual or population levels (Ankley *et al.*, 2009). AOPs

are built in part on one of the key concepts of the MOA framework developed by EPA and IPCS. Once an MOA has been established, the key events can be used for read across to other chemicals, both qualitatively and quantitatively. If a new compound triggers the key events, it will potentially trigger the adverse effect identified in the MOA. The likelihood of an adverse outcome will then depend on a significant perturbation of key events (*i.e.*, dose response). This allows the use of existing knowledge and concepts while grounding the results of new methods in toxicologically relevant outcomes. This emphasizes the advantage of using "omics", *i.e.*, in structure-activity relationship (SAR) determinations both for chemicals with similar structural features and for chemicals with similar toxicity profiles. For example, propiconazole is structurally related to other triazole fungicides, as the rudimentary use of SAR presented in Table 16 of the Case Study indicates (See Support Document for Case Study, Appendix B). This SAR analysis relied solely on structure, tumor response in the mouse liver, and mutagenicity. Many parent conazoles have been shown to be tumorigenic in rodents, specifically liver tumors in the mouse, while a number of others are inactive. As a group, conazoles are known to inhibit ergosterol biosynthesis through inhibition of CYP51 (Vanden Bossche *et al.*, 1989). In mammals, many of these conazoles induce a wide range of effects that include induction or inhibition of CYP isoforms, hepatotoxicity, and liver tumors. With an increasing array of computational tools available, it seems reasonable that a more complex approach to calculate the physical and chemical properties of propiconazole would be enhanced with the incorporation of toxicogenomic data to inform SAR modeling. Similarly, with the expanded understanding of key toxicity pathways afforded by the "omic" findings, and the identification of the likely molecular initiating events, it is equally reasonable to assume that propiconazole would be an ideal candidate for inclusion as a training set in the computational, SAR and quantitative (Q)SAR databases to enhance their ability to predict potential interactions of similar structures with cellular molecules and linkage to the biological activities for AOPs. Using such an approach, we envisioned that compounds, such as phenobarbital, that exhibit toxicity behaviors similar to the conazoles but appear structurally different could be tested *in vitro* for their ability to interact with defined molecular initiating events. These data would then be available to develop SARs, group chemicals according to APOs and determine chemical properties that are not obvious from two-dimensional chemical structures. Using such a plan, chemicals could be prioritized for more apical and comprehensive testing. The ultimate goal being to shift away from the emphasis on apical endpoint testing towards a more in depth chemical characterization, afforded by the inclusion of computational tools early in the screening phase of pesticide registration. This would set the stage for the selection of more efficient "omic" assays designed to detect targeted biological perturbations.

2.2.4 Minimize animal usage

In addition to providing less of an in depth understanding than "omic" studies, traditional toxicology studies exact an enormous price in animal usage. For example, over 800 animals were used in the FIRFA-approved study designs for traditional toxicology testing (Table 5) and 240 mice were tested in the submitted mechanistic studies (Table 4). The overall number of sacrificed animals was upwards of 1000 rodents. By contrast, far fewer mice were involved in the microarray analysis of Ward *et al.* (2006). Our experience

in this project affirms the belief of many at EPA and the NAS that a new paradigm designed to be more efficient and instructive will also help minimize animal testing, and we foresee that the model of microarray analysis, which we have presented, has application as a proof of concept for 21st century toxicology testing.

2.2.5 *In vitro* testing

We further foresee that while targeted *in vivo* testing will continue to play a significant role in toxicology testing for the immediate future, movement away from whole animal testing is feasible and desirable over time. High throughput (HTP) *in vitro* screens at lower levels of biological organization could be used to estimate apical endpoints and/or target appropriate tier testing, thereby gradually reducing the need for more resource intensive tests in the future. Both rodent and human cell lines are amenable to generate important data. This is exemplified by the *in vitro* genomic data with primary mouse hepatocytes, immortalized mouse hepatocytes, and tumorigenic human and mouse cell lines, which have been used in propiconazole MOA studies (Chen *et al.*, 2008; Murphy *et al.*, 2011). Similarly, as discussed in the MOA analysis section of the case study, metabolism does not seem to be a necessary step to demonstrate the genomic activity of propiconazole (See Support Document for the Case Study, Appendix A). Thus, the need for an exogenous source of mammalian metabolic activation is not necessary. Based on these considerations, propiconazole is proposed as an ideal candidate for inclusion as a positive control in test systems constructed around mechanistic endpoints of its target organ responses. Such a HTP screening model could be designed to assess transcriptomic, proteomic and/or metabolomic alterations associated with one or more of the key events in conazole-mediated tumorigenesis. With such a prototype in mind, the need for animal testing could be circumvented. Thus, the overall goal of generating reliable data in a simple but efficient manner that is cost- and time-effective, while minimizing resources, could be realized.

2.2.6 Conclusions

We believe that the approach is reliable, relevant, and offers an innovative means of informing and improving risk assessments. It has been presented at many international meetings and received enthusiastically. In summary, “omic” data have the capability to inform risk assessment in the following ways:

1. Identify potential molecular initiating and other key events that could be linked to adverse outcomes.
2. Link a continuum of precursor events that lead to apical responses.
3. Identify cellular pathways and networks associated with these events for both linkage and coherence by enhancing information contained in apical key events.
4. Infer events not measured through apical approaches.
5. Extend apical dose-response relationships to lower doses.

6. Link molecular initiating and other key events to adverse outcomes observed in animal models to humans thus enhancing human relevance.
7. Consider alternative MOAs.
8. Develop (Q)SAR databases to enhance their ability to predict potential interactions of similar structures with cellular molecules and linkage to the biological activities for AOPs .

This new technology can improve traditional approaches, which often fail to reveal mechanistic insights. Moreover, “omic” data can inform the relevance of a particular toxicity finding in animals with regard to what is biologically plausible in humans (Leighton, 2005; Reynolds, 2005). Toxicogenomic approaches have been used to address specific mechanistic questions in combination with standard toxicology assays and can provide detailed assessments of profiles that can distinguish molecular alterations in a target organ when compared to normal tissue. These technologies include the identification of predictive signatures for toxicity and carcinogenicity, support for low-dose extrapolation and characterization of susceptible human populations. There have been numerous reports detailing with the application of toxicogenomics methods in the assessment and prediction of chemical toxicity (Steiner *et al.*, 2004; Ruepp *et al.*, 2005; Ellinger-Ziegelbauer *et al.*, 2008; Ellinger-Ziegelbauer *et al.*, 2009) that serve to substantiate this approach.

While measurement of apical endpoints is considered important, they have inherent limitations particularly in terms of sensitivity. By contrast, “omics” can identify biological perturbations at the molecular level, the pathway level, and the toxicity pathway level (*e.g.*, groups of genes that interact giving a toxicity endpoint). These pathways can detect alterations in cell-signaling motifs, regulatory pathways and metabolic pathways. Additionally, “omics” can link seemingly disparate pieces of information into cellular-response dynamic networks that uncover new insights into a MOA. Finally, “omics” can detect early subtle changes farther upstream of a measurable physiological change in apical endpoints, and thus can identify early events associated with exposure. “Omics” is a developing field of science and is expected to impact the risk assessment of chemicals. It has the capability to provide both global high-level views of chemically induced alterations as well as more detailed views of altered pathways, networks genes, proteins and endogenous metabolites. It is envisioned that “omics” will impact risk assessment procedures in the hazard identification and dose- response phases within the MOA framework as well as within the framework for human relevance (Sonich-Mullin *et al.*, 2001; Boobis *et al.*, 2006). The advances in this technology that increase throughput screening at reduced costs and minimal usage of animals coupled with advances in bioinformatics will provide a significant benefit to the future of risk assessment.

OPP's strategic plan is to build on efforts such as those discussed in this document to augment the understanding of the MOA for toxicity pathways, to develop the knowledge of APOs, and to use this awareness as the basis for risk assessments. We further foresee a shift in the way toxicology testing is conducted, with emphasis on replacing the current resource intensive protocols with more efficient, informative and focused *in vitro* approaches.

3. Use of an Adverse Outcome Pathway (AOP) to Inform Risk Assessment - Triclosan as a Case Study

3.1 Background

This triclosan case study has been developed for the FIFRA Scientific Advisory Panel meeting on “Integrated Approach to Testing and Assessment Strategy: Use of New Computational and Molecular Tools”. This case study describes how EPA’s Office of Pesticide Programs (OPP) and the Office of Research and Development (ORD) have utilized *in vivo*, *in vitro*, and *in silico* research on triclosan to link key events and define an adverse outcome pathway (AOP) for triclosan’s effect on the thyroid hormone system. This approach may be more advantageous in comparison to use of conventional (i.e. apical) toxicity endpoints for defining potential adverse outcomes in humans. We intend to apply the AOP approach increasingly over time and, as described in this case study, we seek the SAP’s advice and recommendations with respect to the proposed approach. It is important to note that the purpose of this case study is to illustrate the approach we are taking to advance the risk assessment process by making it more efficient and effective and not to assess the risk of triclosan. Key terms used throughout this document are listed and defined as follows:

Key Terms Presented in This Document

- **Mode of action** is defined by the USEPA as the sequence of key events and cellular and biochemical events (measurable parameters), starting with the interaction of an agent with the target cell, through functional and anatomical changes, resulting in cancer or other adverse health effects (USEPA 2005).
- **Adverse outcome pathways (AOP)** represents existing knowledge concerning the linkage between the molecular initiating event and an adverse outcome at the individual or population level.
- **Key events** are empirically observable, necessary steps in the disease (cancer) process.
- **Apical endpoints** are tissue-based alterations observed *in vivo* and are often the first evidence that is observed after treatment-related chemical exposure and include tumors, loss of body weight, altered urinary proteins, and blood markers.
- **Toxicity pathways** are cellular response pathways that can lead to adverse health effects when sufficiently perturbed.
- **Molecular initiating event** is the initial point of chemical-biological intersection within the organism that starts the AOP. Additional key events further along the pathway that lead to, and are experimentally or toxicologically associated with the adverse outcome are referred to as “key events”.
- **Cytochrome P450:** Mixed function oxidases (MFO) also known as CYPs, are a host of enzymes, referred to as Phase I enzymes that metabolize chemicals, often as part of the body’s defenses to dispose of potentially harmful substances (pesticides and other toxins).
- **EROD/PROD:** Ethoxyresorufin/Pentoxeresorufin O-deethylase: These assays measure the enzymatic activities of individual and /or groups of CYPs.
- **CAR/PXR:** Constitutive androstane receptor/pregnane X receptor, are part of a nuclear receptor subfamily, functions as a sensor of xenobiotic substances and in response up-regulates the expression of target genes (CAR e.g, *Cyp2b10*; PXR e.g, *Cyp3a11*) to produce the gene product (e.g., *Cyp2b*, *Cyp3a*), which are responsible for the metabolism of harmful substances.
- **Pathways** are fixed and confined groups of genes (or proteins) associated with a specific biological function (e.g., retinal metabolism or CAR/PXR activation).

- **High-throughput screening:** *in vitro* biochemical or cellular assays that can be run efficiently on a large number of compounds to determine their activity on different biological targets such as ion channels, receptors, enzymes, proteins, and signaling pathways. Typically involves batch testing of chemicals for toxicological endpoints using automated liquid handling, detectors, and data acquisition methods.

The case-study presented here focuses on the use of IATA approaches as they relate specifically to use in human health risk assessment; however, their application is not limited to evaluating potential adverse effects in humans. The approaches have utility for informing ecological risk assessments across multiple taxa as well. For example, an understanding of the biology behind an AOP in mammals could be useful for understanding AOPs across a variety of other taxa. If the key molecular event(s) within an AOP are phylogenetically conserved across mammals and other taxa, then a mechanistic-based understanding of an AOP in mammals may be extrapolated to those other taxa. At a minimum, an understanding of an AOP in mammals could be a useful starting point for understanding and possibly identifying AOPs in other taxa (*e.g.*, birds, fish, invertebrates, and plants) particularly where there is sufficient information regarding specific deviations in highly conserved pathways. Successfully integrating emerging technologies such as *in vitro/in silico* high through-put assays based on omics, predictive tool such as QSARs and read-across, along with existing *in vivo* data across multiple taxa, will improve our mechanistic-based understanding of the underlying biology. Conversely, an understanding of the biology of these various taxa will also enable the use of these emerging technologies to establish linkages between various levels of biological organization that will allow increased use of data from lower levels of biological organization to estimate risk at the whole organism and population level with reduced reliance on *in vivo* testing.

3.1.1 Approach

Since 2008, the Agency, in collaboration with the Office of Research and Development's National Health and Environmental Effects Research Laboratory (NHEERL), conducted research investigations into the potential endocrine effects of triclosan, with an interest in identifying adverse outcome pathways that would facilitate characterization of precursor events for human health risk assessment. These research efforts have involved investigation of triclosan's effect on the estrogen, androgen, and thyroid hormone systems using *in vitro and in vivo* testing methodologies consistent with the Agency's goal under the Endocrine Disruptor Screening Program (EDSP) to screen pesticides, commercial chemicals, and environmental contaminants for their potential to disrupt the endocrine system. Research results on triclosan to date have identified precursor events involving the effect of triclosan on the thyroid hormone system that form the basis for the adverse outcome pathway for this case study. This proposed pathway is outlined below (Figure 9).

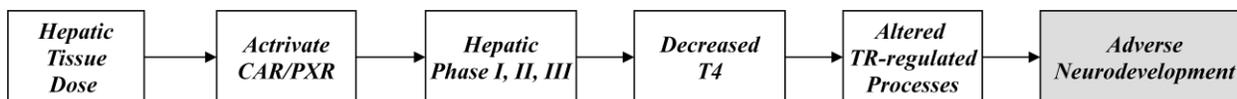


Figure 9. Proposed adverse outcome pathway (AOP) for triclosan effects on the thyroid hormone system.

From these data, it is proposed that the molecular initiating event in the adverse outcome pathway for triclosan's effect on the circulating thyroxine (T4) is activation of the pregnane-X-receptor (PXR) and/or the constitutive androstane receptor (CAR) in the liver. From activation of these receptors, up-regulation of hepatic phase I and phase II enzymes and hepatic transporters occurs. The up-regulation of these enzymes and transporters results in increased catabolism of thyroxine (T4) with a resulting decrease in circulating T4, tissue T4, and T3. The significance of this AOP for humans is that adverse neurodevelopmental outcomes in children is a well known adverse outcome from thyroid hormone insufficiency during brain development (Haddow, 1999; van Wassenaer, 2002). Thus, identification of this AOP for triclosan represents a significant step to refinement of the existing human health assessment, where the AOP is relevant to potential adverse effects in humans in comparison to apical endpoints. The use of the experimental evidence to define an AOP and to use it in assessment of human health is consistent with the Agency's goal of moving toward 21st century toxicity principles that emphasize a mechanistic understanding of how chemicals perturb normal biological pathways and subsequent reliance on assays that are diagnostic of key events within an adverse outcome pathway. This approach begins with formulation of evidence-based hypotheses about the plausible toxicological potential of a chemical or group of chemicals based on their physical-chemical and biological properties, and using knowledge of key events on a causal path to target animal testing focuses on the most likely hazards of concern. The Agency is seeking the Science Advisory Panel's advice on the scientific soundness of using AOP and knowledge of the qualitative and quantitative relationship of the causal key events identified in the triclosan case study.

3.2 Endocrine Effects of Triclosan

The endocrine effects of triclosan have been studied in a variety of systems, including *in vitro* systems (Gee et al. 2007; Ahn et al. 2008; Chen et al. 2007; Christen et al. 2010; James et al. 2008) and in sub-mammalian species (Foran et al, 2000; Ishibashi et al. 2004; Dodson 2005; Veldhoen et al. 2006; Fort et al 2010). The focus of this case study is in identification of an adverse outcome pathway involving disruption of thyroid hormone levels and downstream consequences, most significantly adverse neurodevelopmental outcomes in humans.

3.2.1 Effects on Thyroid Hormone -- *in vivo* Studies

Triclosan's structural similarity with thyroxine, the evidence for interaction of triclosan with hormone systems in non-mammalian species, and published evidence of widespread environmental occurrence all led to the need for further investigation of triclosan's potential effect on mammalian hormone systems. Alterations in thyroid homeostasis is of particular concern as disruption of circulating concentrations of thyroid hormones have been associated with adverse outcomes in humans, including altered neurodevelopment (Zoeller and Crofton, 2000; Mitchell and Klein, 2004; Morreale de Escobar et al., 2004; Koibuchi and Iwasaki, 2006).

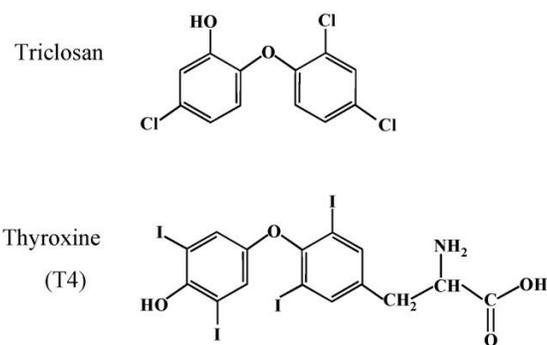


Figure 10. Structure of Triclosan and Thyroxine

Crofton et al., (2007) examined the effects of short-term (4-day) oral exposure to triclosan on thyroxine (T4) levels in adult rats at doses of 0, 10, 30, 100, 300, and 1000 mg/kg. At doses of 100 mg/kg and higher, a dose-related decrease in serum total thyroxine concentrations was observed. Decreases of 28, 34, and 58% were observed at doses of 100, 300, and 1000 mg/kg/day (Figure 11).

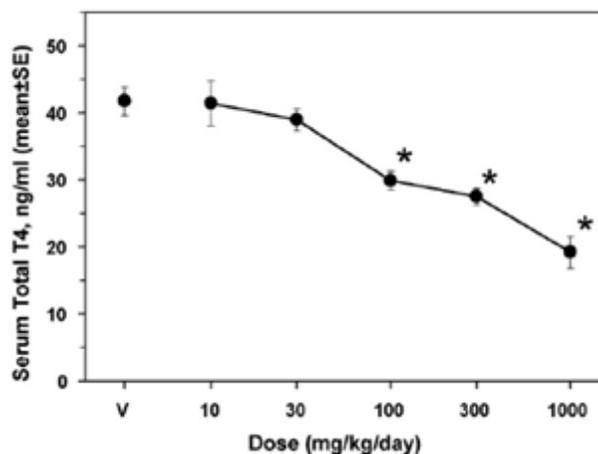


Figure 11. Short-term oral exposure to triclosan decreased serum total T4 concentration.

Data are expressed as group mean values (\pm S.E.), V, corn oil vehicle-only control. Absolute T4 group mean value for the control group (0 mg/kg/day triclosan) is 41.8 ± 2.2 ng total T4/ml serum. The Symbol (*) shows significant difference from the respective control ($p < 0.05$); $n = 16$ for all groups except 10 and 1000 mg/kg/day where $n = 8$ /group.

At that time, there were several possible mechanisms suggested by which this effect could be produced, including increases in sulfation and glucuronidation activity through activation of PXR-linked genes. Activation of hepatic xenobiotic receptors (eg., PXR, CAR) results in induction of Phase I enzymes (e.g., cytochrome P-450s), and Phase II enzymes (e.g., UGTs, SULTs), as well as Phase III enzyme induction (e.g., cellular transporters) (Omiensinsky, 2011). Upregulation of these enzymes and transporters can result in decreased circulating hormone levels via increased turnover rates (Capen, 1997; McClain, 1995; Hood and Klassen, 2000).

3.2.1.1 *In vivo* studies in weanling/young adult animals

Zorilla et al., (2008) extended the findings of Crofton (2007) by investigating the effects of oral triclosan exposure in weanling male rats using the Tier I Endocrine Disruption Screening Program (EDSP) male pubertal protocol (http://www.epa.gov/endo/pubs/pubertalmale_fs.pdf). Rats were administered triclosan orally from post-natal day (PND) 23 to PND 42. Two experiment blocks were used: Block one received doses of 0, 3, 30, and 300 mg/kg/day, block two received doses of 0, 100, and 200 mg/kg/day. Assessments included preputial separation beginning on post-natal day 33, measurement of serum testosterone, androstenedione, leutenizing hormone (LH), thyroxine (T4), triiodothyroxine (T3), thyroid stimulating hormone (TSH), and prolactin following treatment, histology of the testes, epididymides, and thyroid gland, and hepatic EROD, PROD, and UGT activities. No visible signs of toxicity or effects on body weight were observed in this study. Liver weight was significantly increased at 100 and 300 mg/kg as well as anterior pituitary weight at 3 and 300 mg/kg. Preputial separation was not affected at any dose in this study. Serum testosterone was significantly decreased in a non-dose-dependent manner at 200 mg/kg, but not at 300 mg/kg, as compared with the controls (Figure 12), and serum LH and prolactin were not affected at any dose level. No significant histological lesions were observed in testes or epididymides following triclosan exposure. Mean serum T4 concentrations were observed to be decreased in a dose-dependent manner from 30-300 mg/kg (Figure 13), while serum T3 was affected only at 200 mg/kg. No effect on serum TSH was observed in this study.

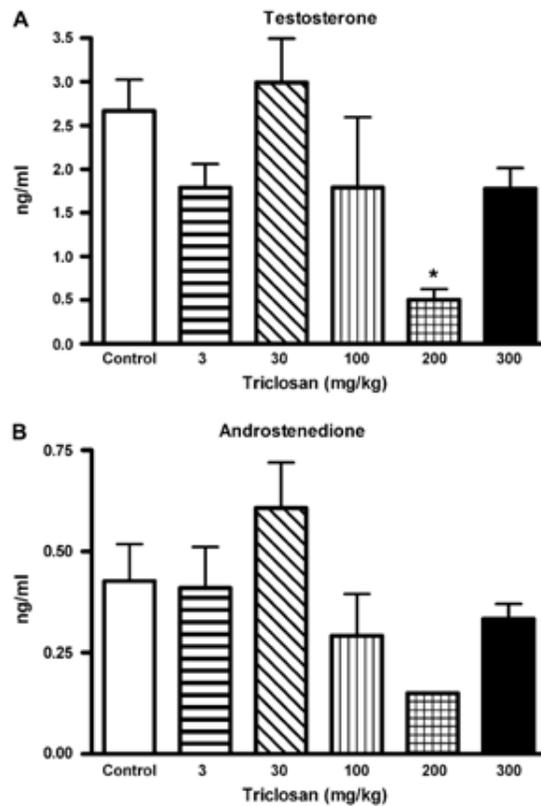


Figure 12. The effect of a 31-day exposure to triclosan (mg/kg/day) on mean (A) total serum testosterone and (B) serum androstenedione concentrations in the male Wistar rat.
*p < 0.05 as compared with total mean.

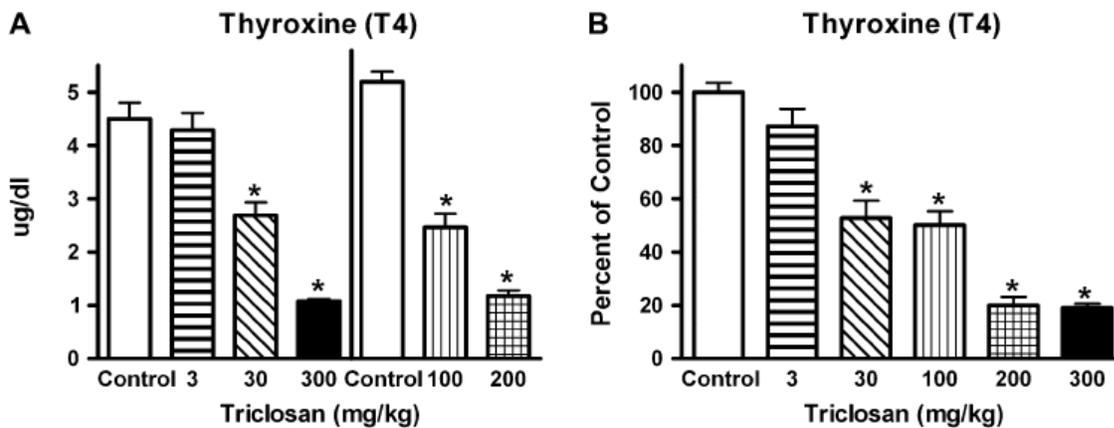


Figure 13. The effect of a 31-day exposure to triclosan (mg/kg/day) on (A) mean total serum T4 and (B) mean total T4 shown as percent of control.

In a more detailed mechanistic study, Paul et al., (2010) characterized the mode-of-action of the hypothyroxinemia induced by triclosan. This work hypothesized that triclosan activated hepatic CAR or PXR receptors, leading to upregulation of catabolic enzymes (i.e., UGTs or SULTs), that caused increased biliary excretion of conjugated thyroxine, with subsequent declines in serum concentrations. In this study, female Long-Evans rats 27-29 days of age were

exposed to oral doses of triclosan for four days at dose levels of 0, 10, 30, 100, 300, and 1000 mg/kg/day. Measurements post-treatment included serum total T4 and total T3, hepatic microsomal EROD and PROD activity, UGT activity for T4, and mRNA gene expression of specific genes involved in hepatic catabolism and transport. Results of this study showed increased liver weight (12%) at 1000 mg/kg, and decreased total serum T4 and T3 levels in a dose-responsive manner at 100, 300, and 1000 mg/kg, but no change in TSH levels relative to controls (Figure 14).

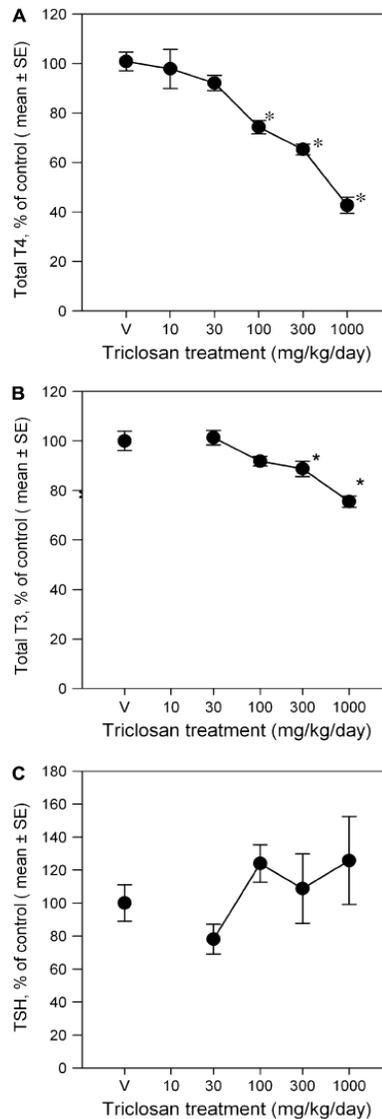


Figure 14. Triclosan treatment results in dose-dependent decreases in total serum T4 (A) and T3 (B), with no change in TSH (C), represented as group mean values (\pm SE) percent of vehicle control for each study cohort V $\frac{1}{4}$ vehicle control, corn oil; for T4: n $\frac{1}{4}$ 24 for V, 30, 100, and 300 mg/kg/day dose groups, n $\frac{1}{4}$ 16 for 1000 dose group, and n $\frac{1}{4}$ 8 for 10 dose group; for T3 and TSH: n $\frac{1}{4}$ 8 for V, 30, 100, 300, and 1000 mg/kg/day dose groups; *significantly different from vehicle controls, $p > 0.05$.

As noted in Paul (2010), “expression levels of hepatic microsomal CYP mRNA were differentially altered by triclosan exposure.” Cyp1a1 mRNA expression was not altered by triclosan treatment, but Cyp 2b1/2 and Cyp 3a1/23 were increased in a dose-dependent manner. Triclosan increased hepatic pentoxyresorufin-o-deethylase (PROD) activity, but not ethoxy-o-deethylase (EROD) activity (Figure 15). T4-glucuronidation activity increased in a dose-dependent manner (Figure 16), and isoform-specific increases in UGT and SULT mRNA expression were also observed in triclosan-treated rats (Figure 17). These findings are consistent with the hypothesized MOA, and the pattern of effects on hepatic Phase 1 and 2 mRNA and enzyme activities and supports the conclusion that the molecular initiating event in the mode of action for triclosan-induced hypothyroxinemia is activation of hepatic CAR and/or PXR receptors.

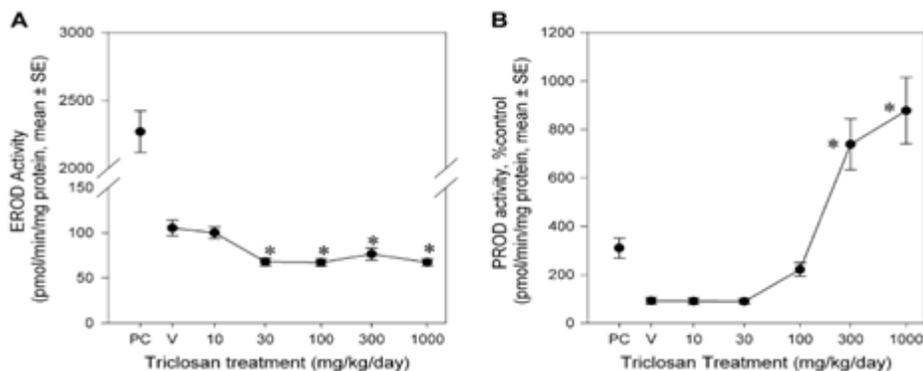


Figure 15. Dose-effect curves for the effects of a 4-day exposure to triclosan on liver microsomal EROD activity (A) and PROD activity (B). Data are presented as group mean (\pm SE) percent of vehicle control for each respective study block. Activity $\frac{1}{4}$ picomoles resorufin/mg protein/min; V $\frac{1}{4}$ vehicle control, corn oil; PC $\frac{1}{4}$ positive control, single 10 lg/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin dose and single 300 mg/kg Aroclor dose; n $\frac{1}{4}$ 24 for V, 30, 100, and 300 mg/kg/day dose groups, n $\frac{1}{4}$ 16 for 1000 dose group, and n $\frac{1}{4}$ 8 for 10 dose group; *significantly different from vehicle control, p > 0.05.

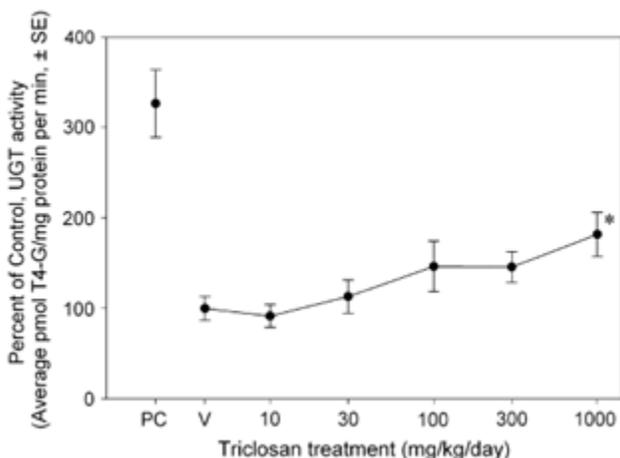


Figure 16. Dose-response curves for the effects of a 4-day exposure to triclosan on liver mRNA expression of CYP isoforms. Data are plotted as the mean (\pm SE) fold change from control for the genes assayed: Cyp1a1 (A), Cyp2b2 (B), and Cyp3a1/23 (C). V $\frac{1}{4}$ vehicle control, corn oil; n $\frac{1}{4}$ 8 per dose group; *significantly different from vehicle control, p > 0.05.

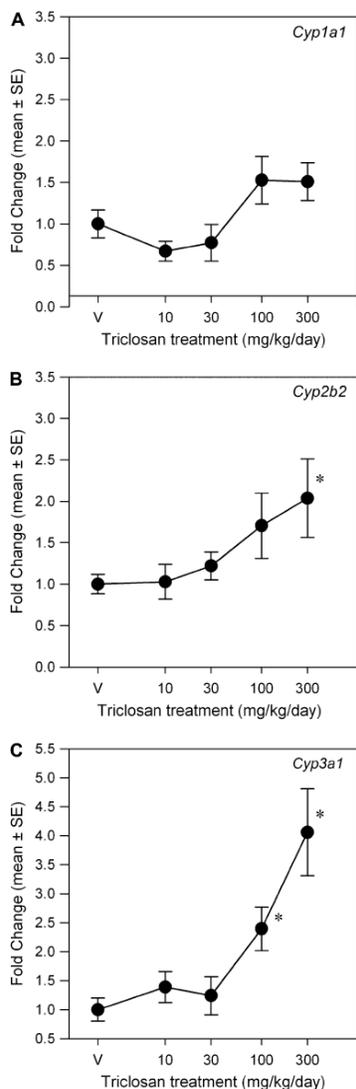


Figure 17 Dose-response curves for the effects of a 4-day exposure to triclosan on liver mRNA expression of CYP isoforms. Data are plotted as the mean (\pm SE) fold change from control for the genes assayed: *Cyp1a1* (A), *Cyp2b2* (B), and *Cyp3a1/23* (C). V $\frac{1}{4}$ vehicle control, corn oil; n $\frac{1}{4}$ 8 per dose group; *significantly different from vehicle control, $p > 0.05$.

3.2.1.2 *In vivo* studies in pubertal, maternal, and neonatal animals

Additional investigations were conducted in reports published by Paul et al., (2010) and Stoker et al., (2010) that extended the research on the effects of triclosan on thyroid hormone levels by examining the effect in pubertal and maternal animals, as well as offspring. It is known that regulation of thyroid hormones during development is critical to development and maturation of the nervous system in mammalian species, including humans, and that there are adverse impacts of subclinical thyroxine concentrations during

human development (cited in Paul et al., 2010). Therefore, investigation into the effect of triclosan on the developing mammalian organism is of significance.

Stoker et al. (2010) conducted measurement of thyroid hormones as part of a Tier I EDSP female pubertal study with triclosan. In this study, weanling female rats received triclosan by gavage at doses of 0, 9.375, 37.5, 75, or 150 mg/kg from PND 22 through PND 42. Total and free serum T4 and serum TSH as well as serum estradiol were measured at the end of the 21-day exposure period.

In the female pubertal assay, mean serum estradiol concentrations were significantly decreased in females from 37.5-150 mg/kg triclosan following the 21-day exposure. Mean serum T4 concentrations were also decreased in a dose-dependent manner from 37.5 to 150 mg/kg following the 21-day exposure, while free serum T4 was decreased at 75 and 150 mg/kg. There was no significant effect on serum TSH concentrations on PND 42 (Figure 18). In the uterotrophic assay, there was a dose-dependent decrease in both mean serum total T4 and free T4 at doses of 18.75 mg/kg and higher (Figure 19).

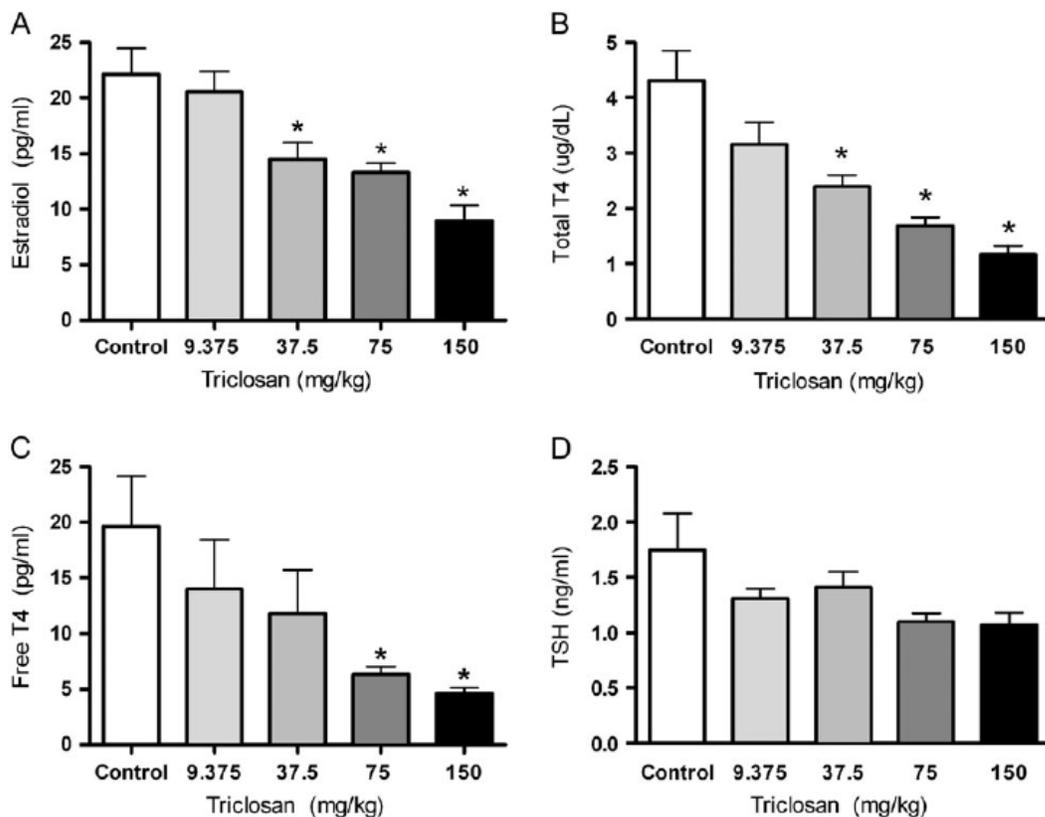


Figure 18. Mean serum (A) estradiol (B) total T4 (C) free T4 and (D) TSH on PND 42 following a 21-day exposure to triclosan in the female pubertal assay.

Mean \pm SEM. *p < 0.05 for significance compared with control mean.

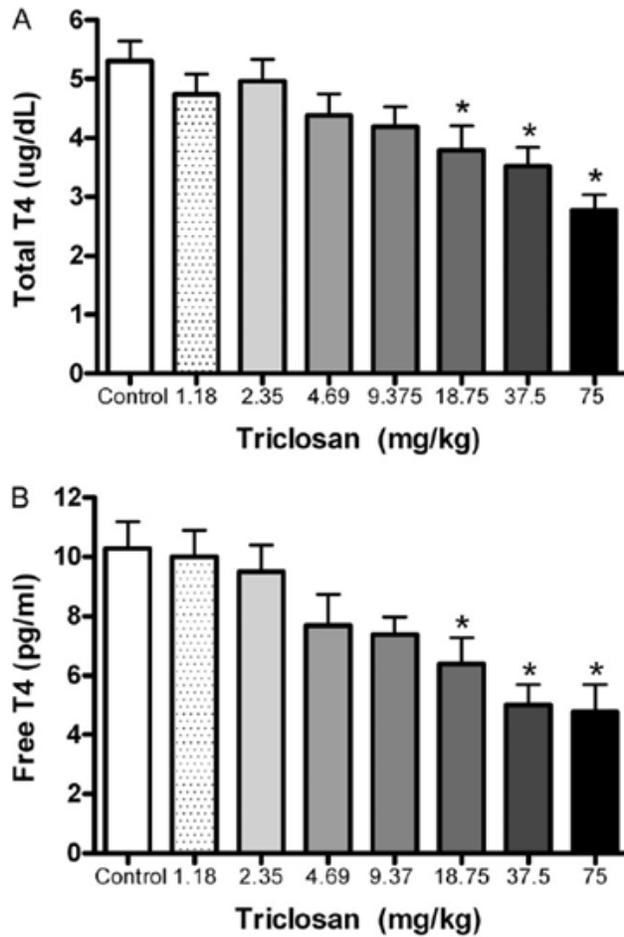


Figure 19. Mean serum total (A) and free (B) T4 concentrations following a 3-day oral exposure in the uterophic assay.

Mean \pm SEM. * $p < 0.05$ for significance compared with control mean.

In both the uterotrophic study and the pubertal study, there was a dose response suppression of total and free T4. In the uterotrophic study, a dose of 18.75 mg/kg triclosan decreased total and free thyroxine by 20% six hours following the third daily exposure in the weanling female rat. This effect was dose-dependent, with the dose of 75 mg/kg suppressing T4 by more than 50%. A no observed adverse effect level (NOAEL) value of 9.38 mg/kg was identified in this study for the thyroid effect of triclosan.

3.2.1.3 *In vivo* studies in maternal and perinatal animals

Paul et al., (2010) investigated the hypothesis that perinatal triclosan exposure would alter circulating thyroid hormone levels in pups during early postnatal development and in dams at the conclusion of lactation. In this study, dams received oral doses of 0, 30, 100, and 300 mg/kg/day from gestation day 6 through post-natal day 21. Pups were sacrificed on PND 4, 14, and 21 for examination of serum T4. Dams were sacrificed on PND

22. EROD, PROD and uridine diphosphate glucuronyltransferase (UDPGT) activities were also measured in liver microsomes in both maternal animals and offspring.

Perinatal exposure to triclosan in this study had no effect on gestation length, litter size, viability, or sex ratio. Body weight decrease of 7-10% were observed in dams at the 300 mg/kg dose level. There were no effects on pup body weights at any dose level. At 300 mg/kg, serum thyroxine was decreased in maternal animals on GD20 and PND 22 by approximately 30%. Decreases in thyroxine in maternal animals were not observed at lower doses. Serum T4 in pups was decreased 27% on PND4 at 300 mg/kg triclosan, but not on PND14 or PND21 at any dose level. Maternal exposure to triclosan resulted in decreased serum T4 in neonates, but only on PND 4 at the 300 mg/kg dose, where a 27% decrease in serum T4 was observed relative to controls. No effects of triclosan were observed in pups on PND 14 or PND 21 at any dose level tested in this study (Figure 20). At the 300 mg/kg dose, triclosan concentrations in fetal and neonatal serum as well as liver were observed to decrease with animal age from PND4 to PND21, suggesting that the lack of effect on T4 at PND14 and PND21 are due to lower exposures at these ages.

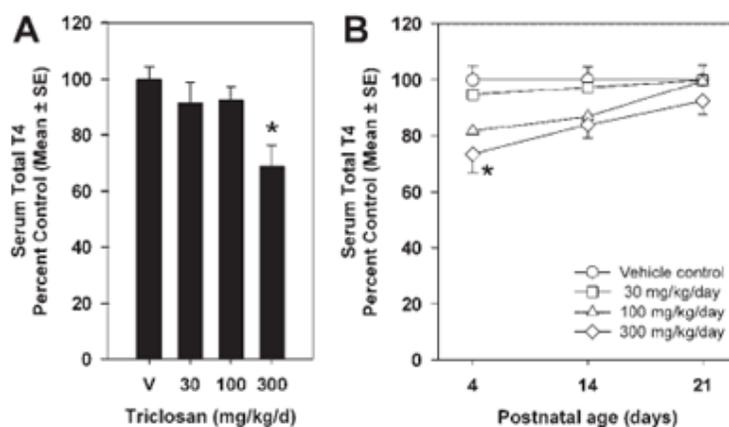


Figure 20. Percent of control serum total thyroxine (T4) for dams (A) and pups (B), by age and oral maternal dose (mean ± SE).

For dams and postnatal day (PND) 21, n = 10, 10, 9, 8 for the 0, 30, 100, and 300 mg/kg/d, respectively; for PND14, n = 10, 10, 8, 8 for the 0, 30, 100 and 300 mg/kg/day, respectively; for PND4, n = 9, 9, 8, 8 for the 0, 30, 100 and 300 mg/kg/day, respectively; * = significantly different from vehicle control by ANOVA, $p > 0.05$. Raw T4 values in ng/ml (mean ± SE) for the 0 mg/kg/d and 300 mg/kg/d groups, respectively, were 49.6 ± 2.2 and 34.1 ± 3.7 for PND22 dams, 9.63 ± 0.49 and 7.08 ± 0.64 for PND4 pups, and 48.4 ± 2.2 and 40.6 ± 2.3 for PND14 pups, and 38.9 ± 2.0 and 36.0 ± 1.8 for PND21 pups. V= vehicle control.

PROD activity was observed to be increased in pups on PND4 to 220% of control and in dams on PND22 (309% of control) at 300 mg/kg triclosan. There were no effects on PROD observed in neonates on PND14 or PND21. Hepatic UDPGT activity was observed to be increased by 1.5-fold in dams on PND22 at 300 mg/kg triclosan. Up-regulated expression of Cyp2b and Cyp3a isoforms in dams was observed in dams only. Measurement of serum triclosan levels in both maternal animals and neonates showed decreased concentrations in offspring on PND14 and PND21 compared to concentrations found in offspring on PND4 and fetuses on GD20. The results of this study showed a significant effect of triclosan on serum T4 levels in neonates on PND 4, but not at later post-natal sampling periods. This could be the result of toxicodynamic and/or toxicokinetic factors

that require additional examination, such as the toxicokinetic distribution of triclosan into maternal milk that may limit its exposure with increasing post-natal age, the degree of placental transfer to the fetus, and effects of triclosan on biotransformation enzymes that have been shown to be upregulated in adult animals.

3.2.1.4 *In vitro/in silico* studies

Triclosan has also been observed to affect a number of biochemical processes involved in thyroid hormone homeostasis in mammalian systems. As summarized in Crofton et al. (2007), triclosan has been shown to induce hepatic EROD and PROD activity, and cytochromes P450 2B1/2 (Hanioka et al., 1996; Jinno et al., 1997). Inhibition of iodothyronine sulfotransferase activity has also been demonstrated in both rat and human hepatic microsomes (Schuur et al., 1998; Wang et al., 2004). Activation of human pregnane-X receptor (PXR) by triclosan in a transient transfection study with human PXR in a human hepatoma cell line has also been shown (Jacobs et al., 2005). PXR activation leads to increases in Phase I and II hepatic biotransformation enzymes, including glucuronidases that catabolize thyroid hormones (THs) (You, 2004). These enzymes are important in maintaining euthyroid hormone concentrations (Capen, 1994; Hill et al., 1998; DeVito et al., 1999).

Recent work from Paul (2011) investigated the molecular basis of the up-regulation of the biomarkers Cyp2b2, Cyp3a1, Ugt1a1, Sult1c1, and T4 glucuronidation. As the expression of these Phase I and Phase II enzymes is regulated by nuclear receptors including CAR and PXR, cell-based nuclear reporter assays were used to measure the ability of triclosan to activate human and rat PXR, the biologically active variants of human CAR, and rat CAR. Human PXR was shown to be moderately activated by triclosan at 10 μ M, while the human CAR2 variant was strongly activated by triclosan and human CAR3 was moderately activated. At the human CAR1 receptor, triclosan acted as an inverse agonist. There was no activation of rat PXR by triclosan, but triclosan instead acted as a modest inverse agonist. These data suggest that triclosan may be capable of interacting with rat CAR and can activate human CAR and PXR to up-regulate hepatic catabolism of triclosan in both species, consistent with *in vivo* observations in the rat.

Additional evidence for this action of triclosan comes from data collected through EPA's ToxCast™ program (Judson et al., 2010; <http://www.epa.gov/ncct/toxcast/>), where triclosan was shown to be active in a Nova Screen™ Biosciences PXR binding assay. In addition, there was increased transcription of target genes for PXR and CAR nuclear receptors in the quantitative nucleic acid protection assay (qNPA) run by CellzDirect on primary human hepatocytes. Although cytotoxicity of triclosan was found to be a confounding factor at some concentrations tested, the results suggest that triclosan may have PXR and possibly CAR agonist activity, which are generally consistent with the results observed from *in vivo* and *in vitro* testing of triclosan.

3.3 Benchmark Dose Analysis for Effect on T4

Knowledge of the AOP can provide useful information in the dose–response assessment for a substance. If the quantitative relationships are understood for precursor events or key events within a causal path leading to a disease or adverse outcome, the key event(s) can serve as the basis of for the dose response analysis. In this case, the reduction of serum thyroid hormones was subjected to benchmark dose (BMD) modeling. This approach is preferred over use of the NOAEL/ loest adverse effect level (LOAEL) approach in this particular case, as it is suspected that a certain level of perturbation is necessary for disruption of homeostatic mechanisms of thyroid regulation leading to an adverse outcome.

Benchmark doses were identified that corresponded to low- and no-effect levels in the rat for potential use in dose response analysis from the data of Zorrilla et al. (2009) and Stoker et al. (2010) that investigated the effect of triclosan on thyroid hormone concentrations in male and female weanling and pubertal rats . The data of from these two studies were used for BMD analysis because the studies were considered representative of the most sensitive lifestage with respect to perturbation of thyroid hormone levels and provided data at the lower region of the dose response curve. These studies consisted of two blocks of male rats and one block of female rats. The first block included 10 males per treatment at doses of 0, 100 or 200 mg/kg triclosan, and the second block included 15 males per treatment at doses of 0, 3, 30 and 300 mg/kg. One block of females was administered 0, 9.375, 37.5, 75 or 150 mg/kg triclosan (n=10) for 20 days.

Serum T4 data from all three blocks of triclosan exposure were evaluated using EPA's Bench Mark Dose Software (BMDS) to determine the BMD and lower limit estimate (BMDL). Because the doses were run in three blocks, the T4 was rescaled for control values in each block. The unconstrained Hill coefficient is a useful model to make an approximation to be used in the observed range. Setting the bench mark response (BMR) at 10% using an unconstrained Hill estimate to fit the data, the BMD was 10.37 mg/kg and the BMDL was 3.47 and at 20% BMR using the same model, the BMD was 19.05 mg/kg and the BMDL was 8.07 mg/kg (Table 6).

Response (BMR)	BMD	BMDL	BMDU
0.10	10.37	3.47	35.18
0.20	19.05	8.07	46.85

Table 6. Results of benchmark dose analysis using data from Zorrilla et al. (2009) and Stoker et al. (2010).

Both the BMR10 and BMR20 are represented.

The 20% BMR was included in the BMD modeling results based on the clinical literature indicating perceived toxicological significance of a change in the measured endpoint (decreased T4 concentration) and general clinical knowledge about adverse outcomes associated with decreases in circulating thyroid hormones. The 10% BMR is selected as a default approach and to provide a lower end of biological changes. US EPA

guidance on setting the BMR suggests using “a minimal level of change in the endpoint that is generally considered to be biologically significant (for example, a change in average adult body weight of 10%, or the doubling of average level for some liver enzyme”.

(http://www.epa.gov/ncea/bmds/bmds_training/toc.htm). There are some data that associate the degree of disruption of thyroid function to adverse neurodevelopmental outcomes where the perturbations are mild to moderate. Haddow et al (1999) reported a 25% decrease in maternal free T4 during the second trimester in women and associated this with neurodevelopmental and cognitive deficits in children. Henrichs et al. (2010) associated maternal hypothyroxinemia with higher risk of verbal and nonverbal cognitive delay in early childhood. It should be noted that a 10% change is within normal experiment-to-experiment variation in control values, whereas the BMR of 20% provides a more reliable BMR. The range of 10-20% is interpreted as a balance between the biological and statistical significance of the reported changes in T4 and associated adverse outcomes. This range is consistent with previous work in both animals and humans for changes in thyroid hormones and precursor events such as iodine uptake inhibition (Strawson et al., 2004; Zorrilla et al., 2008; Zhou et al., 2001; Paul et al., 2010). For this reason, the BMD at both 10% and 20% were calculated to provide a lower and upper range of BMRs.

3.4 Adverse Outcome Pathway Development

The effect of triclosan on the thyroid hormone system has been studied in *in vivo* experiments in both adult and weanling experimental animals as well as fetal/neonatal animals. Additional evidence for the key events and molecular initiating event of this effect comes from *in vitro* and *in silico* studies examining the effect of triclosan on up-regulation of specific hepatic receptors (PXR and CAR) leading to downstream induction of enzymes involved in biotransformation of thyroid hormones and reduction in circulating levels *in vivo*. It is known that the developing nervous system is dependent upon adequate amounts of thyroid hormones and that significant neurological damage can occur when the deficiency is present during brain development (Obregon, 2007). It has also been demonstrated that deficiencies of thyroid hormone at birth can result in deficiencies in mental and neurologic outcome at 2 and 5 years of age (van Wassenaer, 2002). Indeed, 25% decreases in maternal T4 levels during the second trimester are associated with a 7 point decreases in IQ at 7 to 9 years of age (Haddow et al., 1998). Thus, the thyroid effects observed from studies on triclosan in experimental animal systems are relevant for interpreting potential adverse neurodevelopmental effects in humans. Data presented in this case study have shown that triclosan causes hypothyroxinemia, while *in vitro* studies have provided supporting data on the molecular initiating event and downstream molecular events resulting in decreased circulating T4 levels, events that are plausible in humans. This case study that presents the AOP proposed for triclosan and supported by the experimental evidence allows for refinement of the current human health assessment and is significant for its relevance to human health.

Additional published data prompt the discussion of relative species sensitivity to perturbations of thyroid hormone levels. In humans and other primates, thyroxine-binding globulin (TBG) is the principal protein that binds T4 (Dohler et al., 1979). It has a very high affinity for T4 (only about 0.03% of the T4 in serum is in the free unbound form (Hill et al.

1989)). By comparison, most T4 in adult rat serum is bound to albumin and transthyretin (TTR). There is a developmental switch in binding proteins in rats where TBG is the major binding protein until approximately postnatal day 35 when the TTR becomes the dominant binding protein. The binding affinity of T4 for TBG is more than 100-fold greater than that of albumin or transthyretin (Hill et al., 1989) and this difference contributes to the higher rate of T4 clearance in adult rats. The increased clearance also contributes to the need for a higher rate of production of T4 per unit of body weight in rats to maintain normal concentrations of T4 (Dohler et al., 1979). These differences are hypothesized to be responsible to the increased susceptibility of rats to thyroid follicular tumors compared to humans (Capen, 1997; McClain, 1995). In addition, it has also led to speculation that that rats may be a more sensitive species to perturbations of circulating thyroid hormone compared to humans.

There is also evidence for species differences in the metabolism and toxicokinetics of triclosan. Data in both rats and humans using single and repeated doses of triclosan (Scientific Committee on Consumer Products, Opinion on Triclosan, 2009; van Dijk, 1994, 1995, 1996; cited in SCCP, 2009; Lucker et al., 1990; cited in SCCP, 2009) that have measured kinetics of triclosan show that rats attain higher values for area under the curve (AUC) [63.9 $\mu\text{g}\cdot\text{hr}/\text{mL}$] compared to humans [0.2-7.2 $\mu\text{g}\cdot\text{hr}/\text{mL}$] and higher plasma levels (4,450 ng/mL in rats vs 23-574 ng/mL in humans) at comparable oral doses. These data suggest a higher overall exposure to triclosan in rats compared to humans. The disposition of triclosan in rats also differs from humans, with the majority of an oral dose of triclosan (81-84%) eliminated in feces, whereas urinary elimination is the major route in humans (57-87%). Humans also demonstrate a pronounced first-pass effect from triclosan exposure, resulting in less systemic exposure to free triclosan than in rats (SCCP, 2009). Enterohepatic circulation of triclosan has also been observed from experimental studies in rats (consistent with feces as the major excretory route in rats vs. urine in humans), which could result in longer residence time for triclosan in blood and higher tissue exposures to triclosan in rats compared to humans (SCCP, 2009).

In the only data available on the impact of triclosan exposure on thyroid hormones in humans, Allmyr et al. (2009) reported no significant effect on serum T4 after repeated (14 day) use of triclosan-containing toothpaste in adults. These data, while from a short-term dosing study, suggest that normal use of toothpaste containing triclosan is not likely to cause adverse effects on thyroid function.

In addition to understanding of the molecular and key events for the proposed AOP for thyroid hormone perturbation between experimental animals and humans, the understanding of the physiological differences in thyroid hormone regulation and pharmacokinetic differences in rats versus humans is important to informing the factors applied for interspecies and intraspecies extrapolation.

References

Section 1. Integrated Approaches to Testing and Assessment Strategy: Use of New Computational and Molecular Tools - References

- Ankley, G. T., *et al.* (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry*, 29, 730-741.
- Blackburn, K., Stickney, J. A., Carlson-Lynch, H. L., McGinnis, P. M., Chappell, L., & Felter, S. P. (2005). Application of the threshold of toxicological concern approach to ingredients in personal and household care products. *Regulatory Toxicology and Pharmacology*, 43, 249-259.
- Boobis, A. R., *et al.* (2006). IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Critical Reviews in Toxicology*, 36, 781-792.
- Boobis, A. R., *et al.* (2008). IPCS Framework for Analyzing the Relevance of a Noncancer Mode of Action for Humans. *Critical Reviews in Toxicology*, 38, 87-96.
- Bradbury, S. P., Feijtel, T., & van Leewan, C. (2004). Meeting scientific needs of ecological risk assessment in a regulatory context. *Environmental Science and Technology*, 38(23), 63a-470a.
- Carmichael, N. G., *et al.* (2006). Agricultural Chemical Safety Assessment: A Multisector Approach to the Modernization of Human Safety Requirements, *Crit Rev Tox.* 36:1-7
- Dellarco, V. L., Henry, T., Sayre, P., Seed, J., & Bradbury, S. P. (2010). Meeting the common needs of a more effective and efficient testing and assessment paradigm for chemical risk management. *J Toxicol Environ Health B Crit Rev*, 13(2-4), 347-360.
- Dix D. J., Houck K. A., Martin M. T., Richard A. M., Setzer R. W., & Kavlock R. J. (2007). *Toxicol Sci.* Jan;95(1):5-12. Epub 2006 Sep 8. The ToxCast program for prioritizing toxicity testing of environmental chemicals.
- [Federal Insecticide, Fungicide and Rodenticide Act \(FIFRA\)](#) (7 U.S.C. 136), [Federal Food, Drug and Cosmetic Act \(FFDCA\)](#) (21 U.S.C. 346a), [Food Quality Protection Act of 1996 \(FQPA\)](#) (7 U.S.C. 136d)
- Felter, S., *et al.* (2009). Refining the threshold of toxicological concern (TTC) for risk prioritization of trace chemicals in food *Food and Chemical Toxicology*, 47, 2236-2246.
- Hill, Austin Bradford (1965). "The environment and disease: association or causation?". *Proceedings of the Royal Society of Medicine*. 58: 295–300.
- Judson, R. S., *et al.* (2010). In Vitro Screening of Environmental Chemicals for Targeted Testing Prioritization: The ToxCast Project. *Environmental Health Perspectives*, 118(4), 485-492.
- Judson, R. S., *et al.* (2008). ACToR--Aggregated Computational Toxicology Resource. *Toxicol Appl Pharmacol*, 233(1), 7-13.
- Kroes, R., Kleiner, J., & Renwick, A. (2005). The threshold of toxicological concern concept in risk assessment. *Toxicol Sci*, 86(2), 226-230.
- Kroes, R., *et al.* (2004). Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem Toxicol*, 42(1), 65-83.
- Kroes, R., *et al.* (2007). Application of the threshold of toxicological concern (TTC) to the safety of cosmetic ingredients. *Food and Chemical Toxicology*, 45, 2533-2562.
- Mekenyan O., Dimitrov S., Schmieder P., & Veith G. (2003). In silico modelling of hazard endpoints: current problems and perspectives. *SAR and QSAR in Environmental Research* 14(5-6), 361-371.

- Munro, I. C. (1996). A procedure for the safety evaluation of flavouring substances. Toxicological evaluation of certain food additives and contaminants. Prepared for the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, Number 35, Annex 5.
- Munro, I. C., Ford, R. A., Kennepohl, E., & Sprenger, J. G., (1996). Correlation of structural class with no-observed effect levels: a proposal for establishing a threshold of concern. *Food Chem. Toxicol.* 34, 829–867
- Munro, I. C., Renwick, A. G., & Danielwska-Nikiel, B., (2008). The threshold of toxicological concern (TTC) in risk assessment. *Toxicol. Lett.* 180, 151–156
- National Research Council (2007). *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Washington, DC, USA. http://www.nap.edu/catalog.php?record_id=11970#toc
- National Research Council (2009). *Science and Decisions: Advancing Risk Assessment*. Washington DC: Committee on Improving Risk Analysis Approaches Used by the US EPA.
- Nesnow, S., Padgett, W. T. Ren, H., & Hester, S.D. (2009). Discrimination of tumorigenic triazole conazoles from phenobarbital by transcriptional analyses of mouse liver gene expression. *Toxicol Sci* 110, 68-83
- OECD (2008). Workshop on Integrated Approaches to Testing and Assessment. Paris, France: Organisation for Economics Co-operation and Development. <http://www.oecd.org/dataoecd/45/52/40705314.pdf>
- OECD (2009). *Report of the expert consultation to evaluate an estrogen receptor binding affinity model for hazard identification*. Paris, France: Organisation for Economics Co-operation and Development,.
- Richard, A. M., & Williams, C. R. (2002). Distributed structure-searchable toxicity (DSSTox) public database network: a proposal. *Mutat Res*, 499(1), 27-52.
- Richard, A. M., Yang, C., & Judson, R. S. (2008). Toxicity Data Informatics: Supporting a New Paradigm for Toxicity Prediction. *Toxicology Mechanisms and Methods*, 18(2-3), 103-118.
- Schmieder, P. K., *et al.* (2004). Use of trout liver slices to enhance mechanistic interpretation of estrogen receptor binding for cost-effective prioritization of chemicals within large inventories. *Environ Sci Technol*, 38(23), 6333-6342.
- Schultz, T. W. (2010). Adverse outcome pathways: A way of linking chemical structure to in vivo toxicological hazards. In M. T. D. Cronin & J. C. Madden (Eds.), *Issues in Toxicology No.7 In Silico Toxicology: Principles and Applications* (pp. 351-376): Royal Society of Chemistry.
- Seed, J., *et al.* (2005). Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Crit Rev Toxicol*, 35(8-9), 664-672.
- Sonich-Mullin, C., *et al.* (2001). IPCS Conceptual Framework for Evaluating a Mode of Action for Chemical Carcinogenesis. *Regulatory Toxicology and Pharmacology*, 34 (2), 149-152.
- U.S. Environmental Protection Agency (USEPA) (2005). *Guidelines for Carcinogen Risk Assessment*. EPA/630/P-03/001B, March 2005, Risk Assessment Forum U.S. Environmental Protection Agency Washington, DC Washington DC. (http://www.epa.gov/ttn/atw/cancer_guidelines_final_3-25-05.pdf)
- U.S. Environmental Protection Agency (USEPA) (2009a). *The Use of Structure Activity Relationships of Estrogen Binding Affinity to Support Prioritization of Pesticide Inert Ingredients and Antimicrobial Pesticides for Screening and Testing*. Washington DC, USA: United States Environmental Protection Agency (EPA).
- U.S. Environmental Protection Agency (USEPA) (2009b). *The U.S. Environmental Protection Agency's Strategic Plan for Evaluating the Toxicity of Chemicals*. from http://www.epa.gov/spc/toxicitytesting/docs/toxtest_strategy_032309.pdf.
- U.S. Environmental Protection Agency (USEPA). (2008). Draft Science Issue Paper: Chlorpyrifos Hazard and Dose Response Characterization. Prepared for the September 16 - 19, 2008FIFRA Scientific Advisory Panel. Document available at http://www.epa.gov/scipoly/sap/meetings/2008/091608_mtg.htm
- U.S. Environmental Protection Agency (USEPA). (2010a). Re- Evaluation of Human Health Effects of Atrazine: Review of Experimental Animal and In Vitro Studies and Drinking Water Monitoring Frequency. Prepared

for the April 26-29, 2010 FIFRA Scientific Advisory Panel. Document available at <http://www.epa.gov/scipoly/sap/meetings/2010/042610meeting.html>

U.S. Environmental Protection Agency (USEPA). (2010b). Re-Evaluation of Human Health Effects of Atrazine: Review of Non-Cancer Epidemiology, Experimental Animal and *In vitro* Studies and Drinking Water Monitoring Frequency. Prepared for the September 14-17, 2010 FIFRA Scientific Advisory Panel. Document available at <http://www.epa.gov/scipoly/sap/meetings/2010/091410meeting.html>

van Leeuwen, K., Schultz, T. W., Henry, T., Diderich, B., & Veith, G. D. (2009). Using chemical categories to fill data gaps in hazard assessment. *SAR QSAR Environ Res*, 20(3-4), 207-220.

Section 2. Use of "Omic" Technology to Inform the Risk Assessment A Case Study: Propiconazole - References

Allen, J. W., *et al.* (2006). Toxicity profiles in mice treated with hepatotumorigenic and non-hepatotumorigenic triazole conazole fungicides: Propiconazole, triadimefon, and myclobutanil. *Toxicol Pathol* **34**, 853-862.

Ankley, G.T., *et al.* (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ Tox and Chem* 29(3): 730-741.

Boobis, A. R., *et al.* (2006). IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Critical reviews in toxicology* **36**, 781-792.

Boobis, A. R., *et al.* (2008). IPCS Framework for Analyzing the Relevance of a Noncancer Mode of Action for Humans. *Critical Reviews in Toxicology*, 38, 87-96.

Bruno, M., Moore, T., Nesnow, S., & Ge, Y. (2009). Protein Carbonyl Formation in Response to Propiconazole-Induced Oxidative Stress. *J Proteome Res* **8**, 2070-2078.

Chang, T. Y., Li, B. L., Chang, C. C., & Urano, Y. (2009). Acyl-coenzyme A:cholesterol acyltransferases. *Am J Physiol Endocrinol Metab* **297**, E1-9.

Chen, P. J., Moore, T., & Nesnow, S. (2008). Cytotoxic effects of propiconazole and its metabolites in mouse and human hepatoma cells and primary mouse hepatocytes. *Toxicol In Vitro* **22**, 1476-1483.

Chen, P. J., *et al.* (2009). Three conazoles increase hepatic microsomal retinoic acid metabolism and decrease mouse hepatic retinoic acid levels in vivo. *Toxicol Appl Pharmacol* **234**, 143-155.

Cohen, S. M., Meek, M. E., Klaunig, J. E., Patton, D.E., & Fenner-Crisp, P. A. (2003). The human relevance of information on carcinogenic modes of action: Overview. *Critical reviews in toxicology* 33 (6); 581-589.

Ellinger-Ziegelbauer, H., Aubrecht, J., Kleinjans, J. C., & Ahr, H. J. (2009). Application of toxicogenomics to study mechanisms of genotoxicity and carcinogenicity. *Toxicol Lett* **186**, 36-44.

Ellinger-Ziegelbauer, H., Gmuender, H., Bandenburg, A., & Ahr, H. J. (2008). Prediction of a carcinogenic potential of rat hepatocarcinogens using toxicogenomics analysis of short-term in vivo studies. *Mutation research* **637**, 23-39.

End, D. W. (1999). Farnesyl protein transferase inhibitors and other therapies targeting the Ras signal transduction pathway. *Investigational new drugs* **17**, 241-258.

Fielden, M. R., Brennan, R., & Gollub, J. (2007). A gene expression biomarker provides early prediction and mechanistic assessment of hepatic tumor induction by nongenotoxic chemicals. *Toxicol Sci* **99**, 90-100.

Freemantle, S. J., Spinella, M. J., & Dmitrovsky, E. (2003). Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* **22**, 7305-7315.

Goetz, A. K., *et al.* (2006). Gene expression profiling in the liver of CD-1 mice to characterize the hepatotoxicity of triazole fungicides. *Toxicol Appl Pharmacol* **215**, 274-284.

Goldstein, J. L., & Brown, M. S. (1990). Regulation of the mevalonate pathway. *Nature* **343**, 425-430.

- Leighton, J. K. (2005). Application of emerging technologies in toxicology and safety assessment: regulatory perspectives. *International journal of toxicology* **24**, 153-155.
- Leon, C., Hill, J. S., & Wasan, K. M. (2005). Potential role of acyl-coenzyme A:cholesterol transferase (ACAT) Inhibitors as hypolipidemic and antiatherosclerosis drugs. *Pharmaceutical research* **22**, 1578-1588.
- Li, C. Y., Lee, J. S., Ko, Y. G., Kim, J. I., & Seo, J. S. (2000). Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J Biol Chem* **275**, 25665-25671.
- Murphy, L., Moore, T., & Nesnow, S. (2011). Propiconazole Enhances Cell Proliferation by Dysregulation of Ras Farnesylation and the MAPK pathway In Proceedings of the 2011 Annual Meeting of the Society of Toxicology, pp. Abstract No. 2109. Society of Toxicology, Washington, DC.
- NAS (2007). Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment National Academy Press, Washington, DC.
- Nelson, G., Leavitt, S., & Ross, J. (2011). Quantitative changes in endogenous DNA damage correlate with conazole mutagenicity and tumorigenicity. In Proceedings of Chemistry in Cancer Research, a Joint Meeting of the AACR and ACS, pp. Abstract A4, p50. American Association for Cancer Research, San Diego, CA.
- Nesnow, S., Padgett, W. T., & Moore, T. (2011). Propiconazole induces alterations in the hepatic metabolome of mice: relevance to propiconazole-induced hepatocarcinogenesis. *Toxicol Sci* **120**, 279-309.
- Nesnow, S., Ward, W., Moore, T., Ren, H., & Hester, S. D. (2009). Discrimination of tumorigenic triazole conazoles from phenobarbital by transcriptional analyses of mouse liver gene expression. *Toxicol Sci* **110**, 68-83.
- NRC (1994). Science and Judgment in Risk Assessment. *The National Academies Press*, Washington, D.C., 1-652.
- Ortiz, P. A., Bruno, M. E., Moore, T., Nesnow, S., Winnik, W., & Ge, Y. (2010). Proteomic analysis of propiconazole responses in mouse liver: comparison of genomic and proteomic profiles. *J Proteome Res* **9**, 1268-1278.
- Pandey, P., *et al.* (2000). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *The EMBO journal* **19**, 4310-4322.
- Reynolds, V. L. (2005). Applications of emerging technologies in toxicology and safety assessment. *International journal of toxicology* **24**, 135-137.
- Ross, J. A., & Leavitt, S. A. (2010). Analysis of the mutations induced by conazole fungicides in vivo. *Mutagenesis* **25**, 231-234.
- Ross, J. A., Moore, T., & Leavitt, S. A. (2009). In vivo mutagenicity of conazole fungicides correlates with tumorigenicity. *Mutagenesis* **24**, 149-152.
- Ruepp, S., *et al.* (2005). Assessment of hepatotoxic liabilities by transcript profiling. *Toxicol Appl Pharmacol* **207**, 161-170.
- Sonich-Mullin, C., *et al.* (2001). IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul Toxicol Pharmacol* **34**, 146-152.
- Steiner, G., Suter *et al.* (2004). Discriminating different classes of toxicants by transcript profiling. *Environmental health perspectives* **112**, 1236-1248.
- Sun, G., *et al.* (2005). Propiconazole-induced cytochrome P450 gene expression and enzymatic activities in rat and mouse liver. *Toxicol Lett* **155**, 277-287.
- Thyss, R., Virolle, V., Imbert, V., Peyron, J. F., Aberdam, D., & Virolle, T. (2005). NF-kappaB/Egr-1/Gadd45 are sequentially activated upon UVB irradiation to mediate epidermal cell death. *The EMBO journal* **24**, 128-137.
- Trosken, E. R., *et al.* (2006). Comparison of lanosterol-14 alpha-demethylase (CYP51) of human and *Candida albicans* for inhibition by different antifungal azoles. *Toxicology* **228**, 24-32.
- U.S. Environmental Protection Agency (USEPA) (2005). Guidelines for carcinogen risk assessment. EPA/630/P-03/001F, 1-166, http://oaspub.epa.gov/eims/eimscomm.getfile?p_download_id=439797.

- U.S. Environmental Protection Agency (USEPA). (2007). Interim Guidance for Microarray-Based Assays: Data Submission, Quality, Analysis, Management, and Training Considerations, Washington, DC.
- U.S. Environmental Protection Agency (USEPA). (2009). The U.S. Environmental Protection Agency's Strategic Plan for Evaluating the Toxicity of Chemicals, Washington, DC. CAN NOT DELETE THE SPACE
- Vanden Bossche, H., Marichal, P., Gorrens, J., Coene, M. C., Willemsens, G., Bellens, D., Roels, I., Moereels, H., and Janssen, P. A. (1989). Biochemical approaches to selective antifungal activity. Focus on azole antifungals. *Mycoses* **32 Suppl 1**, 35-52.
- Wang, X. D. (2003). Retinoids and alcohol-related carcinogenesis. *J Nutr* **133**, 287S-290S.
- Ward, W. O., *et al.* (2006). Transcriptional profiles in liver from mice treated with hepatotumorigenic and nonhepatotumorigenic triazole conazole fungicides: Propiconazole, triadimefon, and myclobutanil. *Toxicol Pathol* **34**, 863-878.
- Whysner, J., Ross, P. M., & Williams, G. M. (1996). Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion. *Pharmacol. Ther.* **71**: 153-191.

Submitted MRIDs

- 45215801 Hardisty, J. (1997) 13-Week Dietary Toxicity Study with CGA-64250 in Male Mice: Final Report: (Supplement for MRID No.42050502): Lab Project Number: 140-081: F-00107: 799-97. Unpublished study prepared by Experimental Pathology Laboratories, Inc. 51 p. MRID Nos.
- 42050502 Potrepka, R. & Turnier, J. (1991a) 13-Week Dietary Toxicity Study with CGA-64250 in Male Mice: Lab Project Number: F-00107. Unpublished study prepared by Ciba-Geigy Corp. 226 p.
- 42050501 Potrepka, R. & Turnier, J. (1991b) Subchronic Dietary Toxicity Study with CGA-64250 in Mice: Lab Project Number: F-00098. Unpublished study prepared by Ciba-Geigy Corp. 302 p.
- 44381401 Gerspach, R. (1997) CGA-64250 Technical: 18-Month Oncogenicity Study in Mice: Lab Project Number: 943126. Unpublished study prepared by Novartis Crop Protection Ag. 453 p.
- 0129570 Hunter, B., *et al.* (1982) CGA 64 250: Long-term Feeding Study in Mice: CBG/196/81827. Final rept. (Unpublished study received Jul 21, 1983 under 100-641; prepared by Huntingdon Research Centre, Eng., submitted by Ciba-Geigy Corp., Greensboro, NC; CDL:250784-A; 250785; 250786)
- 0151503 Ciba-Geigy Corp. (1985) Response to the EPA Review of the Long-Term Feeding Study in Mice with CGA-64250 Technical: [Includes Chemistry Data of Test Material, Details of Diet Preparation, Summary of Incidence of Clinical Signs, and Addendum to Report CBG 196/81827]. Unpublished compilation. 214 p.
- 93194037 Gillis, J. & Tisdell, M. (1990) Ciba-Geigy Corp. Phase 3 Summary of MRID 00129570 and Related MRIDs 00084153, 00151503, 00130844. Long-Term Feeding Study in Mice: Propiconazole: Study # 196/81827. Prepared by Huntingdon Research Centre. 14 p.
- 45215802 Weber, E. (1999) Assessment of Hepatic Cell Proliferation in Male Mice (Propiconazole): Final Report: Lab Project Number: CB 97/23: 539-98. Unpublished study prepared by Novartis Crop Protection AG. 59 p.
- 45215803 Beilstein, P. (1998). CGA-64250 Technical (propiconazole): Final Report Effects on biochemical parameters in the liver following administration to male mice. Toxicology/Cell Biology, Novartis Crop Protection AG, CH-4002 Basel, Switzerland. Study No.: CB 97/22. April 7, 1998.

Section 3. Use of an Adverse Outcome Pathway (AOP) to Inform Risk Assessment - Triclosan as a Case Study - References

- Ahn, K. C., *et al.* (2008): In Vitro Biologic activities of the antimicrobials Triclocarban, its analogs, and Triclosan in Bioassay Screens: Receptor-Based Bioassay Screens. *Env. Hlth. Persp.* 116(9): 1203-1210.
- Allmyr, M., Panagiotidis, G., Sparve, E., Diczfalusy, U., & Sandborgh-Englund, G. (2009): Human exposure to triclosan via toothpaste does not change CYP3A4 activity or plasma concentration of thyroid hormones. *Basic Clin. Pharm. Tox.*
- Australian Government, Department of Health and Ageing (NICNAS) (2009): Priority Existing Chemical Report No. 30: Triclosan.
- Capen, C., (1997). Mechanistic Data and Risk Assessment of Selected Toxic End Points of the Thyroid Gland. *Toxicol. Pathology.* Nov. 1(25): 39-48
- Chen, Jiangang *et al.* (2007): Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. *Toxicol. Appl. Pharmacol.* 221: 278-284.
- Christen, V., Crettaz, P., Oberli-Schrammli, A., & Fent, K. (2010): Some flame retardants and the antimicrobials triclosan and triclocarban enhance the androgenic activity in vitro. *Chemosphere* 81: 1245-1252.
- Crofton, K., *et al.* (2007): Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine. *Env. Tox Pharm.* (24): 194-197.
- Fort, D. J. Rogers, R. L., Gorsuch, J. W., Navarro, L. T., Peter, R., & Plautz, J. R. (2010): Triclosan and Anuran Metamorphosis: No Effect on Thyroid-mediated Metamorphosis in *Xenopus laevis*. *Tox. Sci.* 113(2): 392-400.
- Gee, R. H., Taylor, C. N., & Darbre, P. D. (2008): Oestrogenic and androgenic activity of triclosan in breast cancer cells. *J. Appl. Toxicol.* 28: 78-91.
- Haddow, J. E., *et al.* (1999): Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *N. Eng. J. Med.* 341(8): 549-555.
- Henrichs, J. *et al.* (2010): Maternal Thyroid Function during Early Pregnancy and cognitive functioning in Early Childhood: The Generation R Study. *J. Clin. Endocrinol. Metab.* 95(9): 1-8.
- Hood, A. & Klaassen, C. (1999). Differential Effects of Microsomal Enzyme Inducers on *in Vitro* Thyroxine (T4) and Triiodothyronine (T3) Glucuronidation. *Toxicol. Sci.* (55):78-84.
- Ishibashi, H, *et al.* (2004). Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin. *Aquatic Toxicology* 67:167–179
- Matsumura, N, *et al.* (2005). Effects of nonylphenol and triclosan on production of plasma vitellogenin and testosterone in male South African clawed frogs (*Xenopus laevis*). *Biol. Pharm. Bull.* 28: 1748–1751.
- McClain, R. M. (1995). Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment, *Mutation Research*, 333: 131- 41.
- Miller M. D., Crofton K. M., Rice D. C., & Zoeller R. T. (2009). [Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes.](#) *Environ Health Perspect.* Jul;117(7):1033-41. Epub 2009 Feb 12. Review.
- Palenske, N. M., & Dzialowski, E. M (2005). Effects of the Environmental Contaminant Triclosan on the Physiology of Developing *Xenopus Laevis* Tadpoles. *Integrative and Comparative Biology.* 45(6): 1175. Published.
- Paul K. B., Hedge J. M., Devito M. J., & Crofton K. M. (2010a). Developmental triclosan exposure decreases maternal and neonatal thyroxine in rats. *Environ. Toxicol. Chem.* 29(12):2840-4.

- Paul K. B., Hedge J. M., Devito M. J., & Crofton K. M. (2010b): Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in young Long-Evans rats. *Tox. Sci.* 113(2): 367-379.
- Scientific Committee on Consumer Products (SCCP) (2009): Opinion on Triclosan.
- Stoker, T. E., Gibson, E. K., & Zorrilla, L. M. (2010). Triclosan exposure modulates estrogen-dependent responses in the female Wistar rat. *Toxicol. Sci.* 177(1):45-53.
- Tamura, H., *et al.* (2006). Use of Structural basis for androgen receptor agonists and Antagonists: Interaction of SPEED 98-listed chemicals and Related compounds with the androgen receptor based on an In vitro reporter gene assay and 3D-QSAR. *Bioorganic & Medicinal Chemistry.* 14:7160-7174.
- Van Wassenaer, A. G. *et al.* (2002): Free thyroxine levels during the first weeks of life and Neurodevelopmental outcome until the age of 5 years in very preterm infants. *Pediatrics* 109(3): 534- 539.
- Veldhoen, N., *et al.* (2006). The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquatic Toxicology* 80: 217–227.
- Woodruff T. J., *et al.* (2008). Meeting report: moving upstream-evaluating adverse upstream end points for improved risk assessment and decision-making. *Environ Health Perspect.* Nov;116(11):1568-75. Epub 2008 Jul 10. Review.
- Zorrilla, L. M., *et al.* (2009) The effects of triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol. Sci.* 107 (1): 56-64.