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RESEARCH BRIEF

INNOVATIVE RESEARCH FOR A SUSTAINABLE FUTURE

ADVERSE OUTCOME PATHWAY (AOP) RESEARCH BRIEF

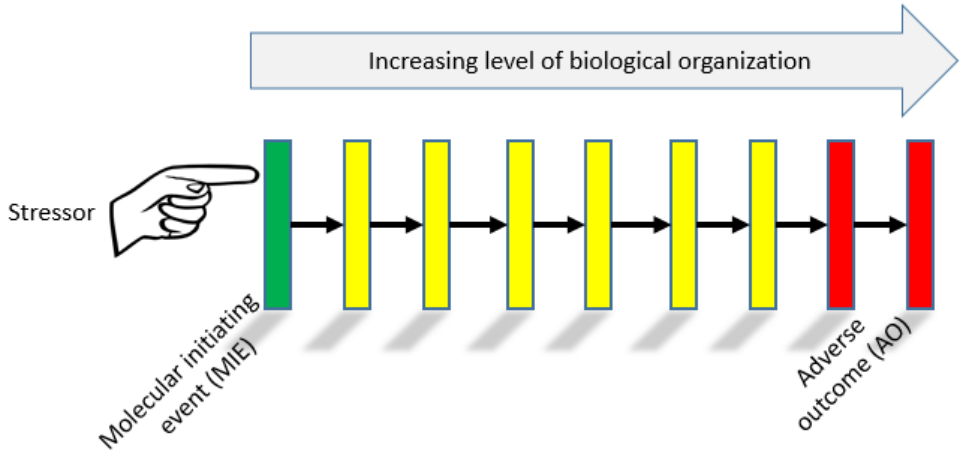

Figure 1. Conceptual representation of an AOP as a series of "biological dominos."

Context for Research Brief

The United States Environmental Protection Agency (US EPA) Chemical Safety for Sustainability National Research Program (https://www.epa.gov/chemical-research) has been advancing the discovery, development, and application of Adverse Outcome Pathways (AOPs) in collaboration with other EPA and federal partners as a framework to organize scientific information, support screening, prioritization, and risk-based evaluations, and inform regulatory decision-making. While EPA remains at the leading edge of this research, interest in this area is growing at a remarkable pace. The success of the approach and its enhanced application depends on broad and collaborative engagement across the international scientific community. To this end, EPA began a number of activities to advance and integrate common understanding, and to facilitate and encourage collaboration and shared learnings across the community.

This AOP Research Brief is intended primarily to communicate the context in which EPA is applying AOPs and promote engagement and contribution to advancing this integrative science. As the Research Brief is disseminated and also as the research progresses, the information and list of resources will be updated on: https://www.epa.gov/chemical-research/research-understanding-chemicals-interactions-biological-systems.

Overview of AOPs

Adverse outcome pathways (AOPs) are a conceptual framework intended to enhance the utility of pathway-based data for assessing hazards to human health and the environment. The framework promotes the systematic organization of toxicological information to support development of predictive, causal relationships between measures of the initiation or progression of a chemical-induced perturbation and adverse outcomes occurring at a level of biological organization relevant to regulatory decision-making. Accordingly, the AOP framework supports the use of different types of mechanistic data to complement, or in some instances potentially replace, traditional measures of apical toxicity outcomes.

Important Definitions and Concepts

An AOP can be conceptualized as a series of "biological dominos" (Figure 1). A stressor (e.g., **chemical initiator**) triggers some reversible or irreversible perturbation of normal biology via a molecular-level interaction (e.g., binding to a receptor, inhibition of an enzyme, or damage to DNA). This is termed a **molecular initiating event (MIE)** and represents the first "biological domino" in the sequence. If that perturbation is sufficiently severe, it can cause additional "biological dominos" to fall, where each domino represents a **key event (KE)** at increasing levels of biological organization or within other compartments in an organism (e.g., impacting cellular functions, which in turn impacts organ function, etc.). Each KE can be observed/measured (i.e., one can "see" the domino fall) to track progression towards the **adverse outcome (AO)**, which is a biological change considered relevant for risk assessment/regulatory decision making (e.g., impacts on human health/well-being or effects on survival, growth, or reproduction in wildlife). Additionally, each KE in the sequence is viewed as "essential" such that if the KE is not observed (domino does not fall), none of the downstream KEs in the pathway will occur (no additional dominos in the sequence will fall).

Key Event Relationships (KERs; arrows in Figure 1) describe the likelihood and conditions under which a particular biological change, represented as a KE, will trigger the next KE in the sequence (i.e., cause the next domino to fall). As such, KERs are defined on the basis of:

1) **biological plausibility** – understanding of biology that suggests a change in the upstream event will trigger an alteration in the next event in the sequence, based on known structural or functional relationships;

2) **empirical support** – evidence showing that when the upstream KE is triggered, the downstream KE also tends to occur in a manner consistent with assumptions of causality (evaluated using modified Bradford-Hill considerations); and

3) **quantitative understanding** – knowledge concerning the conditions under which a change in the upstream KE will cause change in the downstream KE, including mathematical description of response-response relationships and the influence of known modulating factors (e.g., adaptive/feedback responses, effects of diet or environmental factors).

KERs provide the basis for inferring/predicting the likelihood of downstream "biological dominos" in the sequence falling based on observation of one or more upstream dominos. Transparent presentation of the weight-of-evidence (WoE) supporting each KER defines the confidence/certainty along the pathway and helps determine the types of risk assessment/regulatory decisions the AOP can be used to support.

Key AOP Attributes

1. **AOPs are not chemical specific.** Rather, they depict a generalized sequence of biological effects that can be expected for any chemical that perturbs a particular biological target (defined by the MIE), assuming the concentration and duration of that perturbation is sufficient to trigger the next KE and the timing is appropriate. Any given chemical (or even non-chemical stressor) may trigger one or more MIEs and thereby the associated AOPs. This is important because it allows mechanistic information from well-studied chemicals to be leveraged when making toxicity decisions for new chemicals.

2. **AOPs are modular.** Any AOP can be represented as a sequence of KEs and KERs linking those KEs together. Key events provide verifiability (i.e., represent assays and endpoints that can be measured or modeled), while KERs provide a scientifically-based rationale for inference/extrapolation from one KE to another.

3. **AOPs are a pragmatic functional unit of development and evaluation.** AOPs are a deliberate simplification of complex biological systems intended to support regulatory decision-making and help identify uncertainties that can be a focus of additional testing. They provide a tractable unit for both knowledge assembly and WoE assessment.

4. **AOP networks are the functional unit of prediction.** Multiple AOPs sharing common KEs (nodes) and/or KERs (links/edges) can be assembled into networks (Figure 2). AOP networks capture complexity of real biological systems and become more complete as more AOPs are defined. These networks offer the opportunity to consider the effects of chemical mixtures that cause common AOs via different MIEs, or single chemicals that might produce multiple AOs via a single MIE. This attribute enables consideration of pathway interactions in a systems biology context.

5. **AOPs are living documents.** AOPs are primarily a way of organizing information. As new evidence and understanding supporting KERs and/or new methods for measuring KEs emerge, AOPs can be continually expanded or refined. Consequently, there is no objective "complete" AOP. Rather, their application is guided by "fit for purpose" relative to the level of confidence required for a given regulatory application. Some applications, such as screening or prioritization, may only require modestly supported AOPs and will rely heavily on direct observation of KEs for subsequent decision-making. Other uses, such as predictive application to quantitative risk assessment, demand well understood and strongly supported relationships. The AOP description provides systematic and transparent assembly of the supporting information to inform regulatory decisions and highlights data gaps to guide future research in cases where the existing data are insufficient to support those decisions.

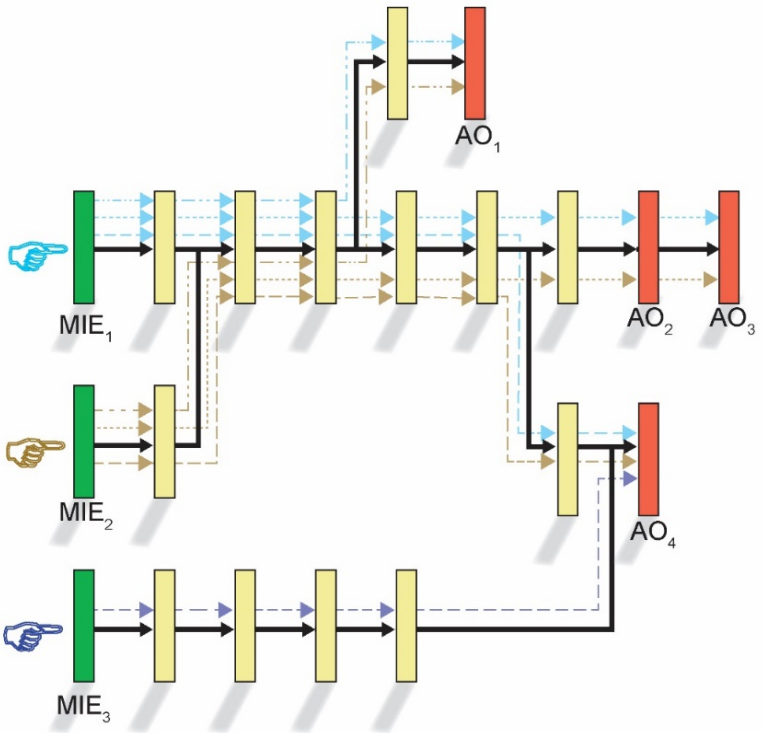

Figure 2. Conceptual representation of an AOP network of seven AOPs. AOP_1 linking MIE_1 to AO_1. AOP_2 linking MIE_1 to AO_3. AOP_3 linking MIE_2 to AO_1. AOP_4 linking MIE_2 to AO_3. AOP_5 linking MIE_1 to AO_4. AOP_6 linking MIE_2 to AO_4. AOP_7 linking MIE_3 to AO_4.

Purpose and Applications of AOPs

The AOP framework is intended to enhance communication between scientists involved in generating biological/toxicological data and the potential end users of this information, such as modelers or risk assessors. Information comprising an AOP is captured in a consistent and organized manner, using a harmonized terminology, making it easier for decision-makers to access and understand comparatively complex datasets/relationships. Further, connecting measurable biological responses across biological levels of organization to the AO allows confidence in the use of alternative types of data for regulatory decision-making. The AOP framework plays a central translational role in the application of this science to the challenge of assessing and managing human health and ecological risks associated with the tens of thousands of chemicals entering or present in the environment.

There are a number of areas in which a pathway-based understanding of chemical effects can substantially enhance chemical safety assessment:

- **Enhanced use of mechanistic data**: Because AOPs are chemical agnostic, data from many chemical and non-chemical perturbations can be used to define the AOP. This wealth of information provides added confidence when using mechanistic data from computational models, in vitro systems, and/or short-term in vivo assays to predict possible adverse effects of chemicals when traditional apical data are lacking. This is the case for many chemicals under regulatory programs lacking statutory authority to require extensive testing.

- **Explicit evaluation of uncertainty**: AOP descriptions provide qualitative WoE calls for each pair of key events in the pathway. This allows the decision maker to compare the strengths or gaps in confidence in the AOP with the chemical-specific data available for a given risk assessment and decide on the suitability of that AOP for supporting different types of decisions.

- **Hypothesis-driven testing**: Knowledge of apical endpoints likely to be affected by perturbation of a given MIE, can help focus in vivo testing that may need to be done for an untested chemical in terms of identifying appropriately sensitive species, life-stage(s), and toxicity endpoint(s). This attribute is useful for regulatory applications, such as pesticide registration, that would benefit from the identification of sensitive tests most likely to affect estimates of risk. This mechanism-based testing strategy could also provide the information needed to support alternative tests for other chemicals that operate via this mechanism, thereby greatly reducing future testing costs.

- **Cross-species extrapolation:** A significant uncertainty in both human health and ecological risk assessment involves the extrapolation of toxicity data from tested to untested species. The use of AOP knowledge to directly evaluate conservation of pathways and quantitative differences in toxicological response across species can help address this challenge.

- **Evaluation of complex mixtures**: An additional challenge in chemical risk assessment involves forecasting or understanding the toxicity of new or existing chemical mixtures. Insights provided by AOP networks can help address uncertainties associated with prediction of mixture effects and facilitate a more hypothesis-driven approach to mixture assessment.

What AOPs are Not

- **AOPs are not risk assessments** – AOPs inform the characterization of hazard or effect in a risk assessment, starting at the perturbation of a biological system. They do not explicitly address exposure.

- **AOPs are not synonymous with high-throughput testing or pathway-based assays** – AOPs are assemblies of knowledge designed to aid the interpretation of high-throughput testing or pathway-based data in the context of relevant apical hazards. However, they do not represent actual biological assays.

- **AOPs are not Mode of Action analyses** – The mode of action framework, as applied in human health risk assessment, represents a systematic description and analysis of the means through which a specific chemical elicits an adverse effect in an organism. AOPs, which are intended to be generalizable to any chemical acting on a particular MIE, can be applied in mode of action analysis, but the terms are not synonymous.

- **AOPs are not computational models** – AOPs are intended to help facilitate quantitative understanding of how alteration in one KE impacts downstream KEs. While this information may be represented in the form of one or more computational models, AOPs are not, in and of themselves, computational models. Computational models that align with AOPs, simulate the KERs along the AOP, and quantitatively predict the state of KEs under various conditions or scenarios are described as quantitative AOPs (qAOPs).

- **AOPs are not a "silver bullet" –** AOPs may not comprehensively predict all toxicological outcomes. They do not solve all the challenges of in vitro to in vivo extrapolation. AOPs do not describe every detail of adverse and adaptive biology underlying an organism's response to a stressor. They cannot account for every aspect of individual variability nor every environmental or life-history variable that may affect a toxicological outcome in real-world settings. They are, in short, simply a means to help organize what we know about how biological perturbations can lead to apical adverse outcomes, and use that information to aid regulatory decision-making.

AOP Development and Availability

One of the primary objectives of the AOP framework is knowledge assembly -- specifically, making information attained through scientific research by subject-matter experts and distributed in the body of scientific literature accessible to regulators during the decision-making process. Efficient knowledge transfer is facilitated through international harmonization and formalization of the AOP framework and its implementation via the AOP Knowledge Base (AOP-KB). In 2012 the Organization for Economic Cooperation and Development (OECD) established its AOP development program under the oversight of its Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). This program developed guidance detailing internationally-accepted approaches for describing and documenting AOPs and evaluating their technical robustness. In coordination with member organizations, the program also recently implemented the AOP-KB (https://aopkb.org/), which provides a global resource for accessing and searching AOP information in a systematic format consistent with OECD guidance and the principles described above. This knowledge base serves as a centralized platform for both accessing AOP descriptions and crowd-sourced development of additional AOP content. At present, most of that content is available through the AOP-wiki (https://aopwiki.org). Other components of the knowledge base that will facilitate AOP network visualization and analysis, study of quantitative key event relationships, and implementation of predictive models are under development. The AOP guidance, knowledge base, and associated tools aim to provide a broad spectrum of stakeholders with systematically organized knowledge that can aid the use of new data streams in regulatory toxicology and chemical safety assessment.

Example Publications and Resources:

Additional resources can be found at https://www.epa.gov/chemical-research/research-understanding-chemicals-interactions-biological-systems.

Original AOP Manuscript

1. Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem*. 29(3):730-741. doi: 10.1002/etc.34.

AOP Overview

2. Edwards SW, Tan YM, Villeneuve DL, Meek ME, McQueen CA. (2016). Adverse Outcome Pathways-Organizing Toxicological Information to Improve Decision Making. *J Pharmacol Exp Ther*. 356(1):170-81. doi: 10.1124/jpet.115.228239.

AOP Definition and Development Principles

3. Villeneuve DL, Crump D, Garcia-Reyero N, Hecker M, Hutchinson TH, LaLone CA, Landesmann B, Lettieri T, Munn S, Nepelska M, Ottinger MA, Vergauwen L, Whelan M. (2014). Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicol Sci*. 142(2):312-320. doi: 10.1093/toxsci/kfu199.
4. Villeneuve DL, Crump D, Garcia-Reyero N, Hecker M, Hutchinson TH, LaLone CA, Landesmann B, Lettieri T, Munn S, Nepelska M, Ottinger MA, Vergauwen L, Whelan M. (2014). Adverse outcome pathway development II: best practices. *Toxicol Sci*. 142(2):321-330. doi: 10.1093/toxsci/kfu200.

AOP Networks

5. Knapen D, Vergauwen L, Villeneuve DL, Ankley GT. (2015) The potential of AOP networks for reproductive and developmental toxicity assay development. *Reprod Toxicol*. 56:52-5. doi: 10.1016/j.reprotox.2015.04.003.

AOP Evaluation

6. Becker RA, Ankley GT, Edwards SW, Kennedy SW, Linkov I, Meek B, Sachana M, Segner H, Van Der Burg B, Villeneuve DL, Watanabe H, Barton-Maclaren TS. (2015). Increasing Scientific Confidence in Adverse Outcome Pathways: Application of Tailored Bradford-Hill Considerations for Evaluating Weight of Evidence. *Regul Toxicol Pharmacol*. 72(3):514-537. doi: 10.1016/j.yrtph.2015.04.004.
7. Yauk CL, Lambert IB, Meek ME, Douglas GR, Marchetti F. (2015). Development of the adverse outcome pathway "alkylation of DNA in male premeiotic germ cells leading to heritable mutations" using the OECD's users' handbook supplement. *Environ Mol Mutagen*. 56(9):724-750. doi: 10.1002/em.21954.

AOP Training Resources

8. Villeneuve DL, Edwards SW, Martinovic-Weigelt D. (2015). Developing and Applying Adverse Outcome Pathways What You Need to Know - Part 1. Society of Environmental Toxicology and Chemistry Conference. http://setac.sclivelearningcenter.com/index.aspx?PID=9484&SID=215605
9. Villeneuve DL, Edwards SW, Martinovic-Weigelt D. (2015). Developing and Applying Adverse Outcome Pathways What You Need to Know - Part 2. Society of Environmental Toxicology and Chemistry Conference. http://setac.sclivelearningcenter.com/index.aspx?PID=9484&SID=215606

OECD Effort and Guidance for AOP Development

10. OECD AOP Development Programme: http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm
11. OECD. (2014). Users' handbook supplement to the guidance document for developing and assessing AOPs**:** https://aopkb.org/common/AOP_Handbook.pdf.
12. OECD. (2013). Guidance document on on deveoping and assessing adverse outcome pathways. Paris, Organisation for Economic Cooperation and Development, Environment Directorate. ENV/JM/MONO(2013)6:45.