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Minireviews

Adverse Outcome Pathways—Organizing Toxicological Information to Improve Decision Making

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ABSTRACT

The number of chemicals for which environmental regulatory decisions are required far exceeds the current capacity for toxicity testing. High-throughput screening commonly used for drug discovery has the potential to increase this capacity. The adverse outcome pathway (AOP) concept has emerged as a framework for connecting high-throughput toxicity testing (HTT) and other results to potential impacts on human and wildlife populations. As a result of international efforts, the AOP development process is now well-defined and efforts are underway to broaden the participation through outreach and training. One key principle is that AOPs represent the chemical-agnostic portions of pathways to increase the generalizability of their application from early key events to overt toxicity. The closely related mode of action framework extends the AOP as needed when evaluating the potential risk of a specific chemical. This in turn enables integrated approaches to testing and assessment (IATA), which incorporate results of assays at various levels of biologic organization such as in silico; HTT; chemical-specific aspects including absorption, distribution, metabolism, and excretion (ADME); and an AOP describing the biologic basis of toxicity. Thus, it is envisaged that provision of limited information regarding both the AOP for critical effects and the ADME for any chemical associated with any adverse outcome would allow for the development of IATA and permit more detailed AOP and ADME research, where higher precision is needed based on the decision context.

Introduction

At the turn of the 21st century, it was widely recognized that traditional methods for determining chemical toxicity were not adequate for the ever-increasing number of chemicals in commerce, which resulted in regulatory mandates from national and international governing bodies around the world. The U.S. Environmental Protection Agency (EPA) asked the National Research Council (NRC) of the U.S. National Academies of Science to develop a long-term vision to address the challenges with toxicity testing in the 21st century (TT21C). In particular, they sought to address the challenge of increasing the depth and breadth of toxicological information and understanding, while at the same time reducing cost, increasing efficiency, and reducing the use of animals. In 2007, a report was published that focused on the reduction, replacement, and refinement of animal-based toxicity testing and recommended the use of in vitro methods to examine chemical effects at molecular targets (NRC, 2007; Krewski et al., 2010). The basis for this recommendation was the concept of toxicity pathways, which are defined as "cellular response pathways that, when sufficiently perturbed in an intact animal, are expected to result

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ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; AO, adverse outcome; AOP, adverse outcome pathway; AOP-KB, adverse outcome pathway knowledge base; cpAOP, computationally predicted adverse outcome pathway; EPA, Environmental Protection Agency; HTT, high-throughput toxicity testing; IATA, integrated approaches to testing and assessment; KE, key event; KER, key event relationship; MIE, molecular initiating event; MOA, mode of action; NRC, National Research Council; OECD, Organisation for Economic Co-operation and Development; qAOP, quantitative adverse outcome pathway; QSAR, quantitative structure activity relationship; TT21C, toxicity testing in the 21st century.

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in adverse health effects" (NRC, 2007). Since some cellular response pathways are measurable via in vitro assays that can be scaled for high throughput, this new paradigm for toxicity testing has the potential to provide novel toxicologically relevant data, in which the cost and pace are tractable relative to the current chemical universe. In the short term, the NRC envisioned that high-throughput toxicity testing (HTT) would be paired with more traditional animal testing to clarify uncertainties, refine risk estimates, and fill gaps where assays are not available. Over time, as confidence in the HTT results improved, less and less animal testing would be needed.

Implementation of this vision has been led by the Tox21 consortium, which formed following the publication of the TT21C report (Collins et al., 2008; Tice et al., 2013). At the National Institutes of Health Chemical Genomics Center, the focus has been on adapting assays to run in a fully automated system capable of screening 10,000 chemicals per week (Attene-Ramos et al., 2013). The other members of the consortium have focused on identifying assays and evaluating the results from smaller-scale HTT efforts based on in vivo data. As an example, the EPA's ToxCast effort (http://epa.gov/ncct/toxcast/data.html) has screened approximately 2000 chemicals in approximately 800 assay measurements that covered a broad range of endpoints, and all results were made publicly available for evaluation and use (Kavlock et al., 2012). Many of these chemicals have a wealth of pre-existing toxicology data for use in evaluating the veracity of the HTT results [see the EPA online Aggregated Computational Toxicology Resource site (http://actor.epa.gov/)] (Judson et al., 2008). However, two challenges remain: 1) how do we interpret the results from in vitro perturbations in terms of the resulting consequences for a whole organism; and 2) how do we identify the full set of toxicity pathways that must be interrogated to ensure coverage of all potential adverse outcomes (AOs) in vivo? Both of these challenges can be overcome by better understanding the mechanistic basis for chemical toxicity in vivo. At least a portion of the information needed to address these questions is available within the vast body of toxicological research that has been conducted and published. However, to date, relatively little of that information has been systematically organized and summarized to better connect HTT and other results with the more apical AOs traditionally considered in risk assessment and regulation. Adverse outcome pathways (AOPs) (Table 1) are aimed at addressing that need.

The concept of AOPs emerged from the field of ecotoxicology as a means to enhance the utility of the quantitative structure activity relationship (QSAR), biomarkers, and other types of mechanistic data for both understanding and predicting potential adverse effects of chemical exposure in wildlife populations (Ankley et al., 2010). The basic premise is that toxicity is the result of generalizable motifs of biologic failure initiated by the interaction of a chemical with some biomolecule in the body. This molecular interaction elicits a perturbation in normal biology that ultimately impairs critical function of the organism, leading to toxicity, and eventually impacts the population of concern. Consequently, AOPs are described by identifying measurable key events (KEs) at varying levels of biologic organization beginning with molecular interactions of the chemical with the biologic system and proceeding through the organismal responses that impact population viability (Villeneuve et al., 2014a) (Fig. 1). This framework explicitly incorporates the TT21C concept of toxicity pathways by including KEs at the macromolecular and cellular levels of biologic organization. In an effort to better accommodate QSAR analysis, the AOP is anchored at one end by a molecular initiating event (MIE) (Table 1), which represents the direct interaction of a chemical with a biologic target. At the other end, the AOP is anchored by an AO (Table 1) at the organism level (e.g., disease or overt toxicity) or population level (i.e., inability to maintain a particular species in its native habitat). By providing a logical sequence of KEs (Table 1) connecting molecular and cellular events occurring early after chemical exposure to population-level outcomes, the AOP framework serves as a helpful foundation for both interpreting and applying HTT results.

In parallel with the development of the AOP concept in ecotoxicology, scientists and regulators concerned with the impact of chemicals on human health developed the mode of action (MOA)/human relevance framework (Table 1) as a basis to increase consistency and transparency when incorporating mechanistic data in human health risk assessment (Meek

TABLE 1
Glossary of terms

Term	Definition
AOP	"An AOP is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (e.g., a molecular interaction between a xeno-biotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment."[a]
MOA	A "biologically plausible series of key events leading to" an adverse effect.[b]
	A "sequence of Key Events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in" an adverse effect. "Mode of action is contrasted with 'mechanism of action,' which implies a more detailed understanding and description of events."[c]
KE	"A key event is an empirically observable step or its marker, which is a necessary element of the mode of action critical to the outcome (i.e., necessary, but not necessarily sufficient in its own right); key events are measurable and reproducible."[b]
	Measureable/observable biologic changes that are essential to the progression from the molecular interaction of a xenobiotic with the biologic system to an AO considered relevant to regulatory decision making. "KEs are, in essence, measurements of biological state or change in state with regard to a control or reference. Because KEs are measurements or observations of state, the confidence one has in a KE is dictated by the accuracy and precision with which that biological state can be measured."[d]
KER	The predictive and/or causal linkages between a pair of KEs. "KERs, in contrast, are a unit of inference or extrapolation. They are defined by the biological plausibility and evidence that provide a scientifically credible basis for inferring or predicting the state of a downstream KE based on the known state of an upstream KE and the confidence in that inference or prediction is defined by the weight of supporting evidence."[d]
MIE	The first KE within an AOP representing the biologic perturbation resulting from a molecular interaction between a xenobiotic and a specific biomolecule.
AO	Late stage KE in an AOP representing a biologic perturbation that would be considered adverse in a regulatory context. These typically occur at either the individual (e.g., cancer) or population (e.g., lack of reproductive carrying capacity) levels of organization.

[a]Ankley et al. (2010).
[b]Meek et al. (2014a).
[c]EPA (2005).
[d]Villeneuve et al. (2014a).

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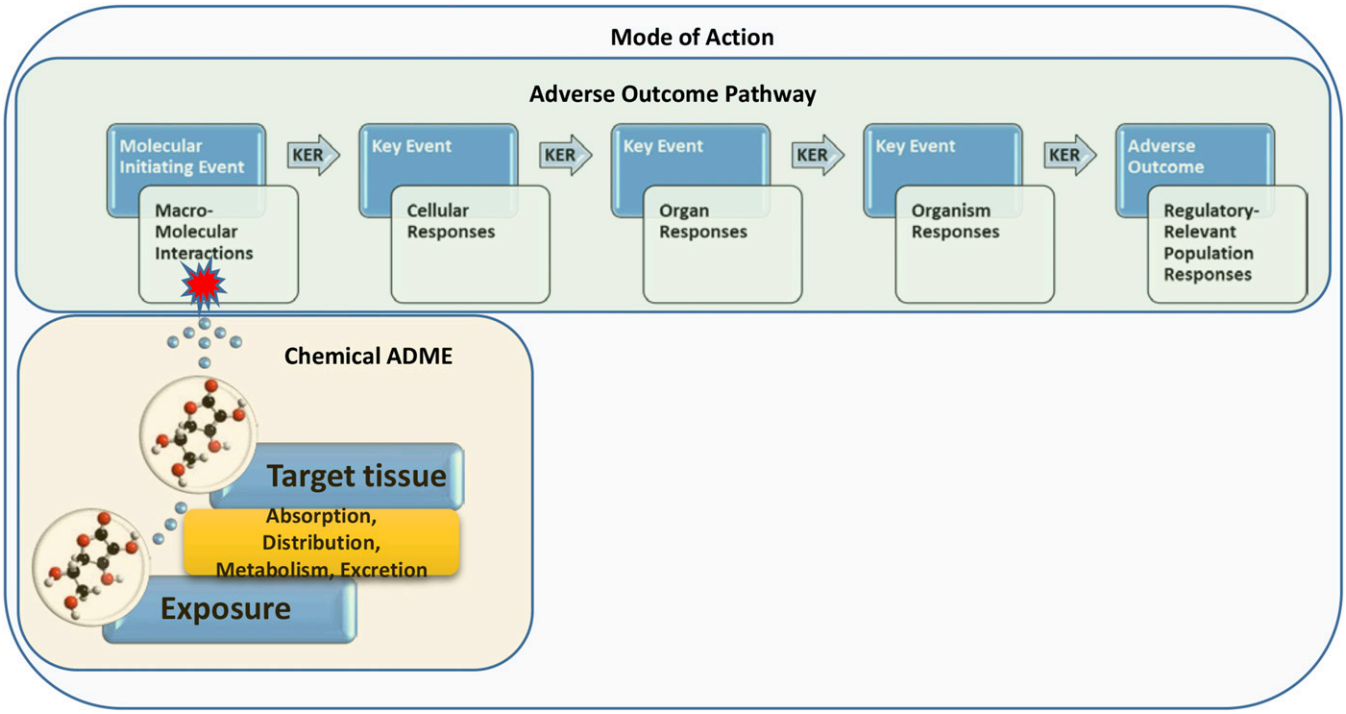

Fig. 1. AOP and MOA components. An AOP consists of KEs and KERs at different levels of biologic organization ranging from macromolecular interactions to population responses. The MIE represents the interaction of the chemical with the biologic system. The AO represents overt adversity at either the individual or population level, which in turn represents the endpoints used when determining safe levels of chemicals. KEs at molecular and cellular levels represent toxicity pathways that can potentially be evaluated using high-throughput screens. A MOA can be constructed from the AOP by including chemical-specific information such as ADME and a prediction of the relationship between the chemical concentration at the site of the MIE and the strength of perturbation of the MIE.

et al., 2014a). Initially, the focus was on assembling, evaluating, and determining the human relevance of toxicological data for cancer (Sonich-Mullin et al., 2001; Meek et al., 2003; EPA, 2005; Boobis et al., 2006). This framework was then extended to include noncancer outcomes (Seed et al., 2005; Boobis et al., 2008). The MOA framework has many similarities to the AOP framework. Most importantly, both frameworks rely heavily on the identification of KEs to allow the use of mechanistic information when knowledge of the mechanism of action is incomplete. KEs are biologic perturbations that are essential for toxicity, and they must be measurable because the measurements associated with these KEs are the basis for considering the weight of evidence or confidence in the MOA or AOP. In addition, there must be support for a causal relationship between an upstream KE and the subsequent KE. The evidence in some cases may not be definitive, but it should be documented in such a way as to allow informed use of the MOA for decision making. The purpose behind MOA analysis is to directly inform risk assessment for regulatory purposes, whereas the AOP is simply intended to describe the chemical agnostic toxicodynamic KEs underlying toxicity. This explains the incorporation of chemical-specific information, such as metabolism as a KE and toxicokinetics, in considering species concordance. With these subtle caveats, MOA analysis is conceptually identical to AOPs.

Adverse Outcome Pathways—Evolution of the Concept

The AOP concept has continued to evolve and mature based on input from an ever-broadening group of experts, as well as lessons learned from early AOP development efforts and closer interactions with the MOA community. Shortly after the initial publication, a Society of Environmental Toxicology and Chemistry Pellston workshop was held to discuss a number of AOP-related topics, including the applicability of the TT21C paradigm for human health (NRC, 2007) to ecological risk assessment (Villeneuve and Garcia-Reyero, 2011). The participants in this workshop concluded that the AOP concept indeed represented a promising framework for connecting HTT (and information at other levels of biologic organization) to ecotoxicological endpoints. The AOP framework also allows the incorporation of biomarker information as a data bridge connecting the MIE with the AO (Kramer et al., 2011). Having a framework that integrates data across the different levels of biologic organization also improves our ability to develop and evaluate quantitative models. By highlighting the KEs underlying the AO, the AOP provides a basis for systematically approaching the question of species extrapolation. The KEs also allow more precision in defining the sources of inherent variability. For example, a genetic polymorphism that alters susceptibility for a certain AO can be modeled at the level of the KE rather than as a generic adjustment to the AO incidence. While the original TT21C report proposed to eliminate species extrapolation for human health risk assessment by testing compounds using human in vitro assays, ecological risk assessment will always require extrapolation from a handful of test species to all others.

In 2010, a workshop was held to consider the use of mechanistic information in forming chemical categories [Organisation for Economic Co-operation and Development (OECD), 2011]. This workshop in part stimulated thought

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about developing AOP libraries and consideration of a proof of concept for AOP development. Subsequent to this workshop, the concepts were used to develop the first proof of principle AOP, which was for skin sensitization. In 2012, the OECD launched an AOP Development Program to promote the development and use of AOPs (http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm). In 2013, the initial guidance for developing AOPs was released (OECD, 2013). This program now has close to 50 ongoing projects, with most focused on the development of AOPs covering various areas of toxicology. These projects have resulted in the greater practical understanding of the components needed to effectively describe an AOP. Another benefit coming from the OECD AOP program has been the close interaction between the AOP community and the MOA community. This interaction has resulted in both an increased understanding of the conceptual similarities and differences (relating to agnostic versus chemical focus) between the two concepts, as well as substantial improvements to the AOP framework with regard to evaluation of the evidence supporting the AOP. Finally, the OECD AOP development program has resulted in an AOP knowledge base (AOP-KB) that now serves as a single source for AOPs generated under this program (http://aopkb.org/). This effort assists with the sharing and dissemination of the AOPs, as well as improving the consistency in reporting among the different AOPs. As a result of these developments, it was determined that an AOP development handbook, herein referred to as the OECD handbook, was needed to expand the original OECD guidance and provide specific information about the best practices for defining AOPs (in particular, documenting the supporting weight of evidence as a basis for context-specific application, as described subsequently) and entering that information into the AOP-KB [users' handbook supplement to the guidance document for developing and assessing AOPs (https://aopkb.org/common/AOP_Handbook.pdf)].

The AOP-KB project consists of four independent modules that are connected via an underlying data hub. The AOP-Wiki module (http://aopwiki.org) was released in September 2014 and is designed to support formal AOP development. The emphasis in this module is capturing the evidence supporting the AOP in a prose format, while capturing KEs and their relationships in a structured form that can be readily used by the other components. The Effectopedia (http://effectopedia.org/) and AOPXplorer (http://aopxplorer.org/) modules are currently available as prerelease versions with a full release expected by February 2016. The Effectopedia module captures additional structured information such as the quantitative response-response relationships between KEs, and the assays and biomarkers available for measuring the KEs. The Effectopedia displays this information via a graphical interface to facilitate the decision-making process. The AOP evidence in the AOP-Wiki can be accessed from within Effectopedia or by following links to the Wiki itself. The AOPXplorer plays a dual role supporting both the development and use of AOPs. While the AOP-Wiki captures AOPs that have undergone a certain level of expert review, the AOPXplorer will collect AOP networks from automated processes such as the computationally predicted AOPs (cpAOPs) described subsequently. This information will be displayed in a graphical network allowing expert users to explore all the possible AOPs that may be relevant to their problem and then continue with either the decision-making process or continued development of critical AOPs. The fourth module, the intermediate effects database, will connect the AOP-KB to the OECD chemical screening information data sets database (http://webnet.oecd.org/HPV/UI/Search.aspx) via the International Uniform Chemical Information Database IUCLID software (http://iuclid.eu/) for use in a regulatory context and will connect AOPs to chemical-specific information. This module will be available with the release of IUCLID 6.

The AOP development effort has been greatly enhanced by a series of workshops designed to improve the collective understanding regarding the development, evaluation, and use of AOPs [National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and Physicians Committee for Responsible Medicine (http://ntp.niehs.nih.gov/pubhealth/evalatm/3rs-meetings/past-meetings/aop-wksp-2014/index.html)] (Becker et al., 2015; Garcia-Reyero, 2015). The broad feedback from these workshops has helped inform the development of the OECD handbook (https://aopkb.org/common/AOP_Handbook.pdf) and the ongoing AOP development under the OECD program.

Adverse Outcome Pathways—Where Are We Now?

Five fundamental principles (Table 2) have been defined to guide AOP development (Villeneuve et al., 2014a). The first two principles deal with the basic components of an AOP [KEs and key event relationships (KERs)], whereas the last three pertain more generally to the development and use of the construct. The MIE and other KEs included in AOPs are restricted to nonchemical-specific aspects (e.g., excluding metabolism and toxicokinetics) to increase their potential for application in a predictive context (i.e., to permit maximal use of mechanistic information at various levels of biologic organization for application in considering the effects of a wide range of stressors).

The first basic component of an AOP is the KE (Table 1). A KE must be measurable and causally linked to the AO, as described previously for the MOA (Villeneuve et al., 2014a). MIEs and AOs represent specialized KEs within the context of a single AOP (Fig. 1). A KE represents a description of a biologic process, and therefore is not specific to an AOP. Indeed, the reuse of common KEs in different AOPs streamlines the AOP development process in the long term and allows for a more comprehensive view of cumulative risk scenarios when using AOPs. Also, a KE can be a MIE or AO in certain AOPs, and an intermediate KE in others. For example,

TABLE 2
Five principles of AOP development[a]

Principle Number	Principle
1	AOPs are not chemical specific.
2	AOPs are modular (consisting of KEs and KERs).
3	An individual AOP is a pragmatic unit of development and evaluation.
4	For most real-world applications, AOP networks are the functional unit of prediction.
5	AOPs are living documents.

[a]Villeneuve et al. (2014a).

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binding to the estrogen receptor would be the MIE for an AOP that describes direct binding to the estrogen receptor, but it would be an intermediate KE for an AOP describing a perturbation that impacts circulating levels of endogenous estrogen, such as an inhibitor of the aromatase enzyme, since the binding of estrogen to the receptor is not directly perturbed by the chemical (Fig. 2A) (Ankley et al., 2010). When describing a KE, emphasis is placed on describing the underlying biologic changes, as well as the methods available for measuring changes in the biologic state (Villeneuve et al., 2014b).

The second basic component of an AOP is the KER (Table 1). A KER represents the connection between an upstream KE and the subsequent KE in the pathway (Fig. 1). The KERs within an AOP capture the evidence supporting the causal relationship between any pair of KEs, and therefore are critical to the overall evaluation of the evidence supporting

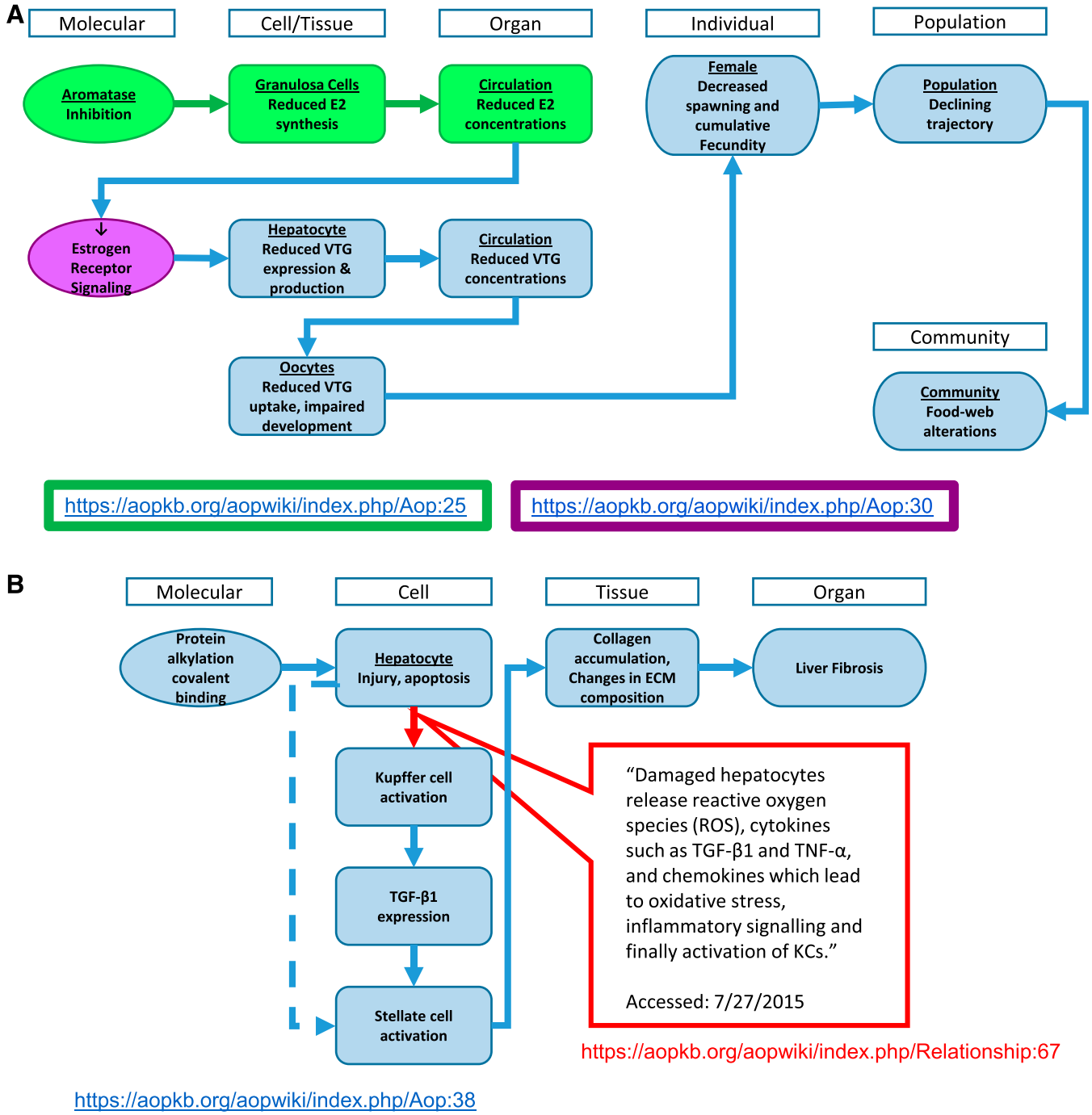

Fig. 2. AOP examples. (A) AOP network containing two AOPs linked to a decrease in signaling through the estrogen receptor. Estrogen receptor antagonists bind directly to the estrogen receptor, resulting in a reduction of estrogen receptor signaling (purple node) as the MIE. The AOP initiated by the inhibition of the aromatase enzyme and reduction of estrogen synthesis includes three additional KEs (green nodes) upstream of the decrease in estrogen receptor signaling. In this case, decreased signaling by estrogen receptors would be secondary to decrease circulating estrogen levels and the MIE would be the inhibition of the aromatase enzyme. (B) AOP example highlighting the capture of several biologic processes within a single KER.

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the AOP (Villeneuve et al., 2014b). Where appropriate information is available, the KER also contains the quantitative description of the relationship between KEs (i.e., the change in the downstream KE that would be expected given a measured/predicted change in the upstream KE) and factors known to modulate that relationship. While the emphasis in describing a KER is on the weight of evidence and quantitative understanding of the relationship, it is important to note that KERs can embed a more detailed mechanistic description of any biologic processes that connect the upstream KE to the downstream KE, especially when these processes are not readily measurable. In other cases, measurable biomarkers are not specific for the AOP, and therefore would not be informative for monitoring a separate KE in the AOP. An example for the latter would be the AOP for protein alkylation leading to liver fibrosis that folds formation of reactive oxygen species and release of cytokines and chemokines in response to hepatic cell death into the KER that leads to the activation of Kupffer cells (Fig. 2B) (Landesmann et al., 2012; Willett et al., 2014). KERs should be described to connect a specific pair of KEs independent of the AOP such that they can be reused where possible.

The final three principles for AOP development (Table 2) are designed to facilitate development while not hindering the use of AOPs for decision making (Villeneuve et al., 2014a). AOPs are generally described as a single sequential pathway beginning with a particular MIE and ending with an AO. This construct is to keep the description of the AOP simple as it is being developed and to facilitate the evaluation of the evidence. Each AOP can then be considered a building block within a larger AOP network that more comprehensively describes the biologic processes involved in real-world scenarios (Knapen et al., 2015). It is anticipated that these more realistic AOP networks will better represent the complexity required for many decision-making contexts. When KEs and KERs are shared among multiple AOPs, these networks naturally assemble as the AOPs are developed. For this reason, it is anticipated that the richness of AOP network descriptions will continue to improve over time as more AOPs are described and included in the AOP-KB. In addition, AOPs will be defined in terms of the information available at any given point in time. As new information becomes available, the AOP will continue to evolve and the evidence supporting the AOP should improve. Since the evidence supporting the AOPs is explicitly described at each stage, there should be no barrier to the use of the AOP network at any stage of development as long as the uncertainties are clearly articulated.

The framework proposed to systematically evaluate the evidence underlying a given AOP for regulatory purposes is based on the evolved Bradford-Hill considerations, which are used for comparative analysis of weight of evidence in the MOA context (Meek et al., 2014a,b). However, these have been modified, when appropriate, to address the context of chemical agnostic AOPs (https://aopkb.org/common/AOP_Handbook.pdf) (Becker et al., 2015). The three primary considerations are biologic plausibility, essentiality of KEs, and empirical support. When evaluating an AOP, the biologic plausibility and empirical support are both evaluated for each KER separately. This process not only supports sharing of components among AOPs, as described previously, but also helps explicitly identify critical data gaps within the AOP as a basis to facilitate targeted research and regulatory uptake. The biologic plausibility assessment relies on an understanding of the fundamental biologic processes involved and whether they are consistent with the causal relationship being proposed. The empirical support typically comes from experiments using one or more reference chemicals, where the dose-response and temporal concordance for the KE pair can be assessed. Unlike the other two, the essentiality of KEs is considered in the context of an entire AOP. Once the weight of evidence (composed of biologic plausibility and empirical support) has been evaluated for each KER and the essentiality has been evaluated for each KE, the evidence in support of the overall AOP can be summarized, often in the form of a summary table as recommended by the OECD handbook (https://aopkb.org/common/AOP_Handbook.pdf). The assessment of the AOP on "Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations" (https://aopkb.org/aopwiki/index.php/Aop:15#Overall_Assessment_of_the_AOP) provides a good example of this type of summary (Yauk et al., 2015).

The final consideration when defining and evaluating AOPs is the applicability (or relevance) of that AOP across various species, life stages, or sexes (Villeneuve et al., 2014b; Groh et al., 2015). In defining the AOP, this is typically restricted to those species for which empirical evidence exists. A notable exception to this common practice would be AOPs that have been designed to address human health risks where little, if any, direct evidence is available in humans. In those cases, the human relevance/species concordance should be determined using the MOA human relevance framework (Meek et al., 2014a,b). While this same approach could be applied when assessing ecological risk as well, in practice the data available to support the inference are typically not as extensive for species other than humans. The evaluation of the taxonomic relevance of any particular AOP involves determining the level of conservation of the various key events and their underlying molecular components and functionality across the levels of biologic organization beginning with the molecular target and proceeding through cellular processes, organ similarity, and overall phylogenetic diversity. The sequence similarity of homologous proteins across a range of species can be used to estimate the conservation of the molecular target (LaLone et al., 2013). Another example is the Web-ICE tool (http://www.epa.gov/ceampubl/fchain/webice/), which provides information at the organism level for acute toxicity. Life stage and sex applicability is based on direct empirical support and biologic plausibility, and is generally anticipated to be the same across species unless otherwise noted. While these methods provide information on a qualitative level, they do not address the possibility of estimating quantitative differences in response as evaluated in the MOA species concordance framework.

AOP development is an iterative process. While early efforts have focused on AOPs that are more easily defined and will provide good examples, the long-term goal would be to have the level of detail for a given AOP be driven by the information needs associated with its envisaged use (Fig. 3, left triangle). Expert-driven AOP development can be generally broken into three broad phases of development (Villeneuve et al., 2014a). Putative AOPs represent a basic description of the AOP without extensive citations or evaluation of the evidence. These are not intended to be hypothetical constructs but instead should be based on documentable, but not necessarily documented or evaluated, evidence. By not exhaustively

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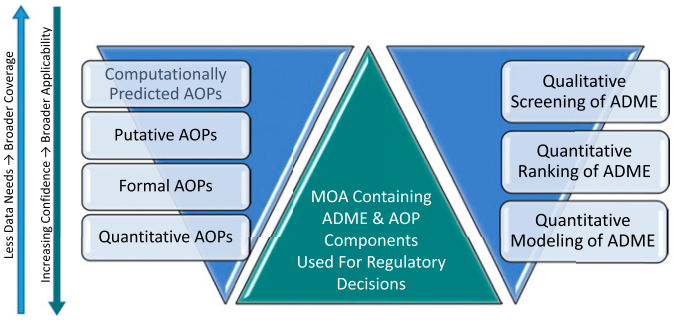

Fig. 3. Tiered structures for defining AOPs and ADME allow for MOA at varying levels of confidence. The left side shows the phases of AOP development including three expert-derived types of AOPs and one cpAOP type. The left triangle represents the relative number of AOPs expected for any given type based on the time and effort required to reach that phase of development. This type of phased structure allows the acceptable uncertainty based on the intended use of the AOP to determine the effort expended to define and evaluate the AOP. The right side shows a tiered approach to defining the ADME for a chemical, which provides the same benefits as the phased AOP development. The triangle in this case represents the relative number of chemicals for which an ADME prediction at a given tier can be made. The tiered approaches for both chemical-specific ADME and chemical-agnostic AOPs allow for the development of MOAs for a wide array of chemicals with lower confidence. As more confidence is required (represented by the width of the green triangle in the middle), the number of chemical/AO pairs that can be characterized will decrease.

reviewing and documenting the literature supporting these AOPs, it should be possible to assemble them relatively quickly, thereby providing some mechanistic information, and hopefully encouraging further development, to cover a broader range of biology in cases where the information is not captured in more rigorously documented AOPs. More formal documentation of the supporting evidence for AOPs allows the user to determine where there are confidence gaps that preclude its use in particular applications. In cases where the decision rests on knowing the magnitude of change at early KEs required for an AO, a quantitative AOP (qAOP) may be developed that is based on computational modeling—although given their significant data requirements, qAOPs will likely represent a small fraction of the formal AOPs in the foreseeable future. However, existing qAOPs should contribute by inference to quantitation of effects associated with chemicals or groups acting by similar MOAs.

In cases where existing information is not sufficient to support the current application of the AOP, the confidence and/or quantitative understanding of the AOP can be improved via a combination of experimental and computational approaches (Groh et al., 2015). It is anticipated that recording of the evidence and essentiality at the KER and KE levels, respectively, will facilitate identification of important data gaps and foster experiments to explicitly address them. For early KEs at the molecular and cellular levels, a combination of in vitro and in vivo experiments is probably most appropriate to establish the relationship between in vitro and in vivo response, thereby potentially allowing future extrapolation from in vitro to in vivo. If data gaps exist in relation to intermediate KEs at the level of organs or organ systems, in vivo studies will likely be required. In these cases, experiments that can identify noninvasive bioindicators that are causally linked with the KEs are ideal. Once established, the bioindicators can be used in future studies to evaluate the applicability of the AOP in different species, at different life stages, and in the presence of modifying factors such as genetic predisposition or pre-existing disease. If the goal is to support estimation of quantitative dose-response relationships, then data on response-response relationships and the time course between KEs are needed. Collectively, this information can provide direct data to support the linkage between MIE perturbation and apical endpoint measurements associated with an AO when that level of confidence or quantitative understanding is required.

Developing a MOA from an AOP and Absorption, Distribution, Metabolism, and Excretion (ADME) Information

As with AOPs, MOA analysis has evolved as the number of case studies has increased (Meek et al., 2014a,b). However, the underlying purpose of the two frameworks is slightly different, which leads to some interesting distinctions between the two. The driver for MOA has always been to facilitate the application of mechanistic data in risk assessment of specific chemicals or chemical groups. This motivation has resulted in MOA case studies that are primarily defined by the chemical(s) used to elicit the organismal response. Since MOA analysis has focused on the application of mechanistic data in the assessment of specific chemicals, it addresses both toxicokinetics (in species concordance analysis) and metabolism (as a KE) (Boobis et al., 2006, 2008; Meek, 2008; Meek et al., 2014a,b). Indeed, several highly cited MOA case studies include metabolic activation of the chemical (Meek et al., 2003; Seed et al., 2005) as a KE in the MOA. In contrast, consideration of ADME is not incorporated directly into an AOP description, which is intended to be applicable to any chemical that triggers a given MIE. However, it is recognized that ADME must be considered whenever AOPs are applied to address chemical-specific questions, including the use of any single or combination of early KEs at different levels of biologic organization to infer potential toxicological outcomes associated with a specific exposure scenario.

A second key distinction between MOA and AOP is the use of the terms species concordance versus taxonomic applicability. The difference in this case is very nuanced but interesting. In determining human relevance for a MOA, the species concordance is documented and evaluated based largely on direct experimental evidence from both environmental and medical research designed to address the relevance of certain animal and in vitro models for specific human diseases (Meek et al., 2014a). Concordance is an appropriate word in this case since the results are for an explicit test species compared with humans. On the other hand, AOPs often have been intended for ecological risk assessments, where there are generally few (if any) data available for the majority of species of concern. In these cases, the term taxonomic applicability is used in a more generic sense to highlight all species (or taxa) for which evidence exists (based on direct empirical evidence or biologic plausibility) that the AOP would be relevant. The user then determines the likelihood that the AOP might apply to a species for which no direct evidence exists. While the taxonomic applicability for humans is likely based on a human relevance analysis of the species concordance, most other species will only be represented if direct experimental evidence exists for the AOP in that species.

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Given the purpose for which these two different frameworks were developed, it makes the most sense to retain both as separate but related entities and clearly define the subtle distinctions between the two. Therefore, we propose that AOPs are one of the basic components from which a MOA can be developed (Fig. 1). The AOP assembles the evidence supporting the description of the biologic response and any knowledge concerning the quantitative relationships between neighboring KEs. The AOP may be developed and documented based on studies using reference chemicals to define the AOP, but the chemical-specific aspects such as toxicokinetics and metabolism are addressed in the MOA analysis. With an AOP so defined, the MOA could start with an AOP and a chemical of interest and proceed to assemble ADME and chemical-specific toxicity prediction for informed decision making (Meek et al., 2014a).

If the chemical-specific toxicity predictions involve HTT, read-across, or QSAR linked specifically to the MIE of an AOP, it is essential to understand the chemical's ADME parameters, which will dictate the probability of a chemical and/or its active metabolites reaching the molecular target in vivo. Once ADME is characterized, the in vitro concentration expected to perturb a MIE could be used to extrapolate to an in vivo target tissue concentration, which is used in turn to estimate an external exposure concentration of possible concern (Yoon et al., 2015). The dose would ideally be calculated at the cellular level, but this level of detail will seldom be possible in the short term. In reality, the information regarding ADME for a specific chemical may be lacking. In view of this challenge, a tiered approach (Thomas et al., 2013) can be used to connect chemical-specific ADME to the biology-based AOP based on the availability of ADME data (Fig. 3, right triangle).

In the lowest tier where no ADME data are available, computational chemistry and cheminformatics tools could provide the opportunity to identify molecular fingerprints that characterize the probability that the chemical will reach the location required for perturbing the MIE, allowing for qualitative refinement of HTT or other results. In this tier, qualitative evaluation of ADME potential could aid in the identification of false positives, which are in vitro active chemicals that cannot reach the molecular target in vivo due to certain ADME characteristics (Phillips et al., 2015). For example, some chemicals (e.g., anthralin) can be sequestered in skin tissues; therefore, they are unlikely to enter the systemic circulation—and thus molecular targets in internal organs—when exposed via a dermal route. In addition, chemical structures or molecular descriptors could potentially be used to detect possible false negatives that are either inactive parents of active metabolites or in vivo active chemicals not detected in vitro (Phillips et al., 2015).

In higher tiers where some ADME data are attainable, measured or estimated ADME rates could potentially be used to rank chemicals in conjunction with in vitro potency measurements. Again, a MIE is triggered by a sufficiently high dose of a chemical at the target site to elicit a biologically relevant interaction between the chemical and the molecular target for the required length of time. Such a quantitative approach allows chemicals that have higher binding affinity, are rapidly absorbed and distributed to the target tissues, and are slowly metabolized and excreted to be prioritized over those that have lower binding affinity, are slowly absorbed and distributed to the target tissues, and are rapidly metabolized and excreted (Leonard et al., manuscript in preparation). In the highest tier where more certainty is required, exposure and ADME data could be used as a basis to develop quantitative exposure-to-dosimetry models that would contribute to prioritizing chemicals (Terry et al., 2015; Wetmore, 2015). Such models also have the capability of examining factors other than dose, such as timing of exposure, which is particularly important when developmental effects are of concern.

Applications for the AOP/MOA Framework

The primary application of the AOP and MOA frameworks to date has been to support environmental regulatory decision making. The MOA framework has been adopted by regulatory agencies around the world for considering mechanistic information (Meek et al., 2008). The OECD AOP development program is closely linked to both the OECD QSAR Toolbox (OECD, 2009) and efforts to harmonize the use of HTT and other potentially more predictive measures across member countries (OECD, 2013). Specific applications include chemical grouping for read-across, design of efficient testing strategies, prioritization for testing, and quantitative risk assessment (Meek et al., 2014a; Perkins et al., 2015), with an emphasis on the replacement, refinement, and reduction of animal-based testing (Burden et al., 2015). The use of the AOP is tightly coupled with the evidence evaluation and associated confidence discussed previously. In addition, most cases of regulatory decision making will require more than one AOP within an interconnected network to fully account for the biologic processes that may influence the final outcome.

The level of confidence needed for the AOPs within the AOP network under consideration is dependent on the regulatory application (Patlewicz et al., 2015b; Perkins et al., 2015). Patlewicz et al. (2015b) proposed a seven step process for establishing scientific confidence for an AOP. After the AOP has been developed, assays for the KEs within the AOP should be identified or developed followed by an analytical validation of those assays. An advanced example of this process is the evaluation of assays monitoring the KEs from the skin sensitization AOP (Reisinger et al., 2015). Once assays have been analytically validated, development and qualification of models that incorporate the assay data can begin. Qualified models can then be used for specific purposes to document the sufficiency of the model for that purpose. This provides the information needed for an open, transparent review and eventual regulatory acceptance of the model.

In cases where limited data exist for the chemical or chemicals in question, even a low-confidence AOP can provide important mechanistic information that can aid in interpretation of HTT and other information. If, instead, HTT results are being proposed to be an influential component of considerations for quantitative risk assessment in lieu of further testing, high confidence is needed in the AOP to link upstream KEs to the AO of regulatory concern. In addition, qAOP development requires more extensive information on the response-response relationships and time course for each pair of KEs to improve the overall quantitative understanding of the AOP. However, when considering the replacement of an existing animal test, the confidence in the new test may be equivalent to—but need not be any higher than—that of existing animal tests. For example, assays like the uterotrophic

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assay employed in the Endocrine Disruptor Screening Program (EPA, 2009) essentially serve as a screen for a MOA, which is implicitly linked to an anticipated AO. HTT assays that evaluate the same MOA should not be held to a higher standard in terms of linkage to the AO. However, when considering the MOA, the in vivo assay might have additional advantages from an ADME perspective, as would be the case with the previous uterotrophic example. This can be addressed via in vitro/in silico models in which we have sufficient confidence to make ADME predictions, e.g., physiologically based pharmacokinetic models that are being used in the pharmaceutical industry.

AOPs are valuable in cases where decisions must be made with available data because the framework provides a structured way to evaluate and communicate the existing information, and they are particularly useful when additional testing options are available. AOPs can contribute significantly to the development of integrated approaches to testing and assessment (IATA) (Tollefsen et al., 2014; OECD, 2015), which iteratively evaluate existing information and identify data needs to make effective regulatory decisions (EPA, 2011; OECD, 2015). While the decision context will determine the details of an IATA, the general flow will be to consider existing data for the chemical of interest, followed by in silico approaches coupled with existing data from structurally related chemicals (e.g., read-across), followed by the generation of new data from high- and medium-throughput assays, with extensive in vivo testing reserved as a last resort. As confidence grows in a given AOP, it should allow more decisions to be made at earlier stages in the workflow. A workshop was held in November 2014 to consider the AOP/MOA concept as a framework for the development and use of IATA (OECD, 2015). The participants in this workshop agreed that the AOP framework informed the toxicity-related elements of the IATA while not addressing other components such as exposure, ADME, and chemical-specific information. Consistent with the definitions used in this review, the MOA was assumed to include ADME as well as the AOP.

In cases where existing information on the chemical in question is insufficient for a decision, in silico approaches such as QSAR (Patlewicz et al., 2014; van der Veen et al., 2014) merged with data on structurally related chemicals via read-across (OECD, 2014; Patlewicz et al., 2015a) can be used prior to the generation of new data. The use of AOPs in a read-across context was considered at a workshop held as part of the SEURAT-1 project (Berggren et al., 2015). The goal of the workshop was to increase confidence in read-across using results from alternative methods. Specifically, the proposed read-across justification would be based on mechanistic understanding of the toxicokinetics and toxicodynamics and strengthened by supporting in vitro data. It was concluded that the key elements are the decision context, mechanistic information, structural similarity, and data availability for chemicals in the group. The European Chemical Council Long Range Initiative reached a similar conclusion based on explicit evaluation of a series of case studies (Patlewicz et al., 2015a). They highlighted the importance of evaluating the supporting assays and resulting data in addition to the confidence required for the mechanistic description of the AOP itself. As the first and only OECD-endorsed AOP to date, the AOP for skin sensitization has been incorporated into the OECD Toolbox (http://www.oecd.org/env/ehs/risk-assessment/Tutorial_17_TB%203.3_AOP%20for%20Skin%20Sens.pdf). The skin sensitization AOP has also been used by several groups to effectively illustrate the IATA concept and integrated testing strategies (Jaworska et al., 2013; Patlewicz et al., 2014).

Beyond simple hazard assessment, the AOP framework provides a basis for response-response characterization, which in combination with chemical-specific data can contribute to dose-response assessment for specific chemicals (MacKay et al., 2013). This process can be broken down into three components: 1) toxicokinetics for the chemical of interest; 2) chemical-specific dose-response information, which could come from high-throughput screening assays; and 3) chemical-independent toxicodynamics information provided by the AOP. While these can be modeled separately or jointly depending on the purpose, separate models would be expected to facilitate reusability. Modeling the toxicokinetics for the chemical in question can provide an estimate of chemical concentration at the site of action based on an external exposure concentration. A dose-response model of the MIE perturbation at specific levels of the chemical provides the chemical-specific data required for predicting the activity of the chemical. A quantitative model describing the response-response relationships among the KEs in the AOP allows AO predictions based on the MIE measurements with caveats regarding the potential for modulating factors that modify the response-response relationships in certain sensitive groups. The skin sensitization AOP has been incorporated into a combined model including toxicokinetics and dose-response informed by high-throughput screening assays as a proof of concept for this application (Maxwell et al., 2014).

Implications for chemical-specific dose-response relationships have been formalized in MOA species concordance analysis (Julien et al., 2009). This includes the chemical-specific ADME components that map external exposure to a target tissue dose for a specific chemical, the interaction with the biologic system in the target tissue, and the progression toward an effect of concern. The approach explicitly incorporates the homeostatic mechanisms that influence the propagation of the perturbation to the downstream KEs, which has been developed more recently by the International Life Sciences Institute RISK21 project (Simon et al., 2014) to incorporate modulating factors as well as associative events that allow for the use of correlative biomarkers in cases where direct measurement of the causal events are not feasible. The objective is the integration of data collected across many KEs, which can then be compared with predicted exposure concentrations. This information can be visualized by a heat map with predicted exposure along one axis and predicted toxicity along the other to provide a broader context in which to interpret a specific comparison such as a benchmark dose value compared against a chronic dietary exposure range (Simon et al., 2014).

The EPA has recently taken a similar approach when incorporating HTT data from the ToxCast and Tox 21 programs into the Endocrine Disruptor Screening Program (http://www2.epa.gov/sap/meeting-materials-december-2-4-2014-scientific-advisory-panel). The basis for this approach is a model for estrogen receptor activation that integrates data from 18 separate in vitro assays based on the early KEs common to perturbations in the estrogen receptor signaling AOP (Judson et al., 2015). This model was evaluated by comparison against independently confirmed estrogen receptor

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bioactivity as well as against the results from current Endocrine Disruptor Screening Program Tier 1 assays (Browne et al., 2015). The predictivity for the new model was superior to previous results with the Tier 1 assays when compared against the estrogen receptor reference chemicals identified by the Interagency Coordinating Committee on the Validation of Alternative Test Methods. The results were comparable to existing assays when considering a larger literature-based list of reference chemicals as well. A scientific advisory panel reviewing these results recommended that the models incorporate uncertainty and sensitivity analyses. The scientific advisory panel also reviewed a proposal to connect the results from the dose-response predictions from the in vitro model with exposure estimates from a complementary rapid exposure prediction model (http://www2.epa.gov/sap/meeting-materials-december-2-4-2014-scientific-advisory-panel) (Isaacs et al., 2014; Wambaugh et al., 2014). While the panel recognized the promise of this approach, they suggested that further refinement was needed to gain better understanding of how monitoring data can inform the predictions.

The IATA workshop (OECD, 2015) established three types of AOP from the application perspective: qualitative, semi-quantitative, and quantitative. These would fall into the spectrum from the formal AOP to the qAOP, as shown in Fig. 3. An additional type called a correlative AOP was proposed and could be considered analogous to the putative AOPs from Fig. 3. The difficulty with this terminology is that the weight of evidence supporting the KERs, and by extension the causal support for the AOP as a whole, includes both empirical support and biologic plausibility. While biologic plausibility is generally weighted heavier than empirical support, one could imagine a case where the empirical evidence is quite strong, and while there is no evidence to question the biologic plausibility it has not been firmly established. In contrast, many biologically plausible pathways exist that are unlikely to ever result in an AO because of homeostatic mechanisms that may or may not be currently defined. The putative AOP terminology used here allows for the combined contribution of biologic plausibility and empirical support to be considered, which provides consistency with the formal evaluation of weight of evidence that represents the next stage of AOP development. It must also be emphasized that AOPs will exist on a continuous gradient from poorly defined to extensively documented. While labels such as putative, formal, and quantitative allow us to define characteristics of AOPs at different points along the continuum, each AOP must be evaluated individually based on the evidence provided and the purpose for which it would be used.

Conclusions and Future Directions

The AOP and MOA frameworks collectively provide a helpful construct that facilitates systematic organization of mechanistic data to support decisions relating to toxicology and therapeutics. Both frameworks have established a process for systematic incorporation of mechanistic information. Integrating HTT and in silico predictions with traditional toxicological measurements in a knowledge base designed to provide a consistent means of evaluating and communicating the information should increase the efficiency of translating research data for use in regulatory applications. The small number of AOPs currently limits this impact, although the streamlined approach to integrating the disparate data streams still provides a tangible benefit. The AOP-KB is also designed to automatically generate the global AOP network through the identification of shared KEs as new AOPs are developed. This will ensure that the value of the resource increases over time, and that the AOP-KB can potentially provide insights on emergent behaviors ranging from interactions between environmental chemicals and/or pharmaceuticals to impacts of genetics and/or pre-existing disease on chemical or pharmaceutical risk/benefit. In addition, exploration of the resulting AOP networks will provide a structured way of identifying new biologic processes relevant to disease endpoints of interest.

The phased structure of AOP development will encourage the use of AOP networks by focusing more comprehensive AOP development on those AOPs where the decision-making context for their use requires a high degree of certainty. This process can be further improved by using data mining approaches such as those commonly used in the drug discovery arena (Schadt et al., 2009) to assemble cpAOPs, which can be evaluated by experts as needed to support the decision-making process (Fig. 3). These approaches are solely limited by data availability and challenges associated with data integration, although the resulting cpAOPs will require expert evaluation prior to use under almost all circumstances. Once established, these methods should provide AOPs to cover any biologic space in which sufficient high-content or public annotation data exist. cpAOPs and putative AOPs can be evaluated and improved as they are needed, thereby focusing the more labor-intensive work where it can have the most impact.

With a phased structure of AOP development and a similar tiered approach to link ADME information to AOPs, a MOA can be assembled rapidly regardless of the level of information available at the time (Fig. 3). Having knowledge bases to categorize both the chemical-agnostic AOPs and chemical-specific ADME will make it easier in the long term to determine what additional data would have the largest impact on reducing uncertainty in decision making, thereby promoting efficient use of resources on targeted testing. If chemical-specific ADME is the major source of uncertainty, there may be no need to specifically address the data gaps that exist in the AOP. If additional research on the AOP is deemed necessary, the granular description of the evidence supporting the AOP should make the data gaps apparent.

While the use of AOPs and MOA analysis has primarily been focused on environmental risk assessment, the concepts could more consistently capture the mechanistic information that is already applied extensively in early safety assessment during pharmaceutical development. As the AOP networks become more extensive, they become a platform to incorporate genetics and other factors that might impact the safety and efficacy of pharmaceuticals as we move toward precision medicine (Collins and Varmus, 2015). In fact, since the AOP concept considers only biologic perturbations that result in AOs such as disease, it applies equally well to target discovery as it does to safety assessment. The systems biology–based network approaches to understanding disease and identifying pharmaceutical interventions (Schadt and Björkegren, 2012; Friend and Schadt, 2014) stand to benefit from the AOP framework as well. The AOP can serve as a convenient construct for summarizing the complex networks into more

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tractable components that are more amenable to follow-up experimentation. Efforts are already underway in the environmental science arena to assemble AOP networks from genomics (Bell, manuscript in preparation) and public annotations (Oki, manuscript in preparation).

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