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Are Adverse Outcome Pathways Here to Stay?

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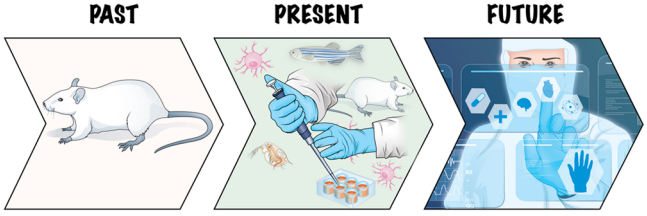

Social pressure to minimize the use of animal testing and the ever-increasing concern on animal welfare, together with the need for more human-relevant and more predictive toxicity tests, are some of the drivers for new approaches to chemical screening. These approaches must also be able to accelerate the screening and assessment of the thousands of chemicals that are currently in use and in development for potential hazards to human and ecological health. Ideally, approaches are needed that decrease (or eliminate) animal testing while increasing predictivity. Efforts in many countries have focused on a toxicological pathway-based vision for human health assessments relying on in vitro systems and predictive models,[1] vision equally applicable to ecological risk assessment.[2] A pathway-based analysis of chemical effects opens numerous opportunities to apply nontraditional approaches for understanding the risks of chemical exposure. Conservation of molecular initiating and key events leading to adverse outcomes of regulatory concern provide a defensible framework for extrapolating chemical effects across species, even if the specific adverse outcomes differ between them.[3]

PROGRESS TO DATE

An Adverse Outcome Pathway (AOP) is a linear pathway composed of a Molecular Initiating Event (MIE), Key Events (KE), and an Adverse Outcome (AO) causally linked together. It is a relatively new concept that has been rapidly gaining acceptance worldwide since its conception. The *AOP Revolution* started with the release of a National Research Council report in 2007 entitled "Toxicity Testing in the 21st Century"[4] that described a vision for the future of toxicity testing in order to support human health risk assessment. The report envisioned "*the steady evolution from apical end-point testing to a system based largely on toxicity-pathway batteries in a manner mindful of information needs and of the capacity of the test system to provide information*".[4] This vision was rapidly embraced by the ecotoxicology community, leading to the publication in 2010 by Ankley and collegues[5] of the concept that would be crucial for the future development of (eco)toxicity testing, and at that moment the AOP framework was born. As defined in Ankley et al.[5] an AOP is "*a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment*". While the concept was brilliant and very promising, little did they suspect that it was the beginning of the uphill path toward the AOP framework development. Luckily, the idea was rapidly embraced all over the world and international efforts to develop the framework began. Among these efforts, there were several workshops that explored the utility of AOPs for supporting predictive ecotoxicology[2] and the development of less animal-intensive alternatives to existing chronic ecotoxicity tests.[6] The AOP framework evolved and is maturing along a similar path as the mode of action framework (MOA), a complementary effort more focused on the human health arena that was developed by the World Health Organization (WHO) International Programme on Chemical Safety (IPCS).[7,8]

Some of the challenges in that proposed paradigm shift as identified in the summary of the Predictive Ecotoxicology workshop[2] were the establishment of credible links between responses measured at the cell or tissue level and adverse outcomes that are traditionally measured at the whole-animal or population level and the need to develop quantitative tools and models to extrapolate data from different levels of biological organization (i.e., cells or tissue to individual or population). Follow on manuscripts explored different solutions to overcome these challenges. For instance, Watanabe et al.[9] developed strategies to derive AOPs and design associated computational models from data present in the scientific literature. Perkins et al.[10] explored the use of computational approaches, including network inference, for the unsupervised discovery of key nodes (i.e., genes, proteins, metabolites, etc..) impacted by a perturbation in the system. Nichols et al.[11] emphasized the need to incorporate mathematical models in order to understand the mechanisms involved in the system recovery from an insult. Kramer et al.[12] showed how models can predict potential population impact once the data are transformed into the prediction of an adverse outcome of demographic significance (such as reproduction) at the organism level. Finally, Celander et al.[13] explored the use of applications such as sequence comparison for species extrapolation. Interestingly, while all these concepts are being intensely analyzed even now, three years later we are still facing some of the same challenges.

In order to help with the challenges that emerge from AOP development, the Organisation for Economic Co-operation and Development (OECD) initiated an AOP development workplan, published a guidance document on developing and assessing AOPs,[14] and also included the work of the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics OECD (EAGMST), which assists international collaborative efforts in the areas of Molecular Screening and Toxicogenomics, and now AOPs, with the aim of defining needs and possibilities for their application in a regulatory context. Participating scientists from member countries then began the discussions that eventually lead to an international

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workshop. The workshop focused on the AOP concept as a framework to characterize, organize, and define predictive relationships between measurable key events that reflect the progression from a molecular perturbation to an adverse outcome considered relevant to regulatory decision-making (https://aopkb.org/saop/workshops/somma.html). The objective of the workshop was to build upon previous efforts and provide expert opinion on the critical next steps required to advance the use and acceptance of the AOP framework to support integrated toxicology and regulatory decision-making. There are also some follow up efforts closely related, such as the "Adverse Outcome Pathways: from Research to Regulation", sponsored by the NIEHS (http://ntp.niehs.nih.gov/pubhealth/evalatm/3rs-meetings/past-meetings/aop-wksp-2014/index.html) or the "Application of Adverse Outcome Pathways in Environmental Risk Assessment", held in conjunction with the IEOS 2014 meeting (http://environmentalomics.org/ieos2014-workshops/).

CHALLENGES

One of the main topics addressed in the workshop was the need to identify research priorities for future development of AOPs. Groh et al.[15] explored how the AOP concept could be used to guide research aimed to improve our ability to predict adverse outcomes. They concluded that detailed mechanistic knowledge would facilitate alternative testing methods development and it would help prioritize higher tier toxicity testing. They also provided recommendations on a potential extension of the AOP framework to incorporate information on exposure and toxicokinetics, among others, that would be necessary for risk assessment and discussed interfaces that would allow the coupling of AOPs with modeling approaches. Furthermore, using fish growth as a case study, they demonstrated how the AOP concept can be used to critically assess the knowledge available for specific chronic toxicity cases in order to identify existing knowledge gaps and potential alternative tests.[16]

Many are interested in the development of AOPs, but not all know how to best proceed, or even get started, in the most productive manner emphasizing the need for a set of principles to help guide AOP development. After lengthy discussions, Villeneuve and colleagues[17] formulated a set of five principles: (1) AOPs are not chemical specific. Any stressor or chemical that triggers the MIE has the potential to activate the chain of KEs leading to an adverse outcome. Therefore, by definition, AOPs are not chemical specific. (2) AOPs are modular and composed of reusable components. Each AOP is composed of two fundamental units: KE and KE relationships (KER), which are not necessarily unique to a single AOP, but usually shared among AOPs. (3) An individual AOP is a pragmatic unit of AOP development and evaluation, as AOPs are not intended to be a complete representation of complex biological processes but to provide a simplified and structured framework to organize toxicological information. (4) Networks composed of multiple AOPs are likely to be the functional unit of prediction for most real-world scenarios, as in reality the prediction of adverse outcomes based on mechanistic or pathway-based data will often require consideration of multiple AOPs. Finally, (5) AOPs are not static, they are "living documents" and will evolve as new knowledge is presented.[17] These principles intend to address many of the current uncertainties in the AOP framework with the goal to increase consistency in AOP development. Villeneuve and colleagues[18] also developed a set of best practices to address many of the challenges in AOP development and to promote a consistent, but still flexible, approach.

Many people remain skeptical about the potential acceptance and application of the AOP framework in real-life risk assessment scenarios. In order to explore the issue of acceptance and usefulness of AOPs, Perkins et al.[19] reviewed the data requirements needed for an AOP to be usable in hazard or risk assessment. They demonstrated that "partial" AOPs could still be valuable and that confidence in these pathways could be increased through the use of unconventional information (e.g., computational identification of potential initiators). They concluded that for AOPs to be useful in a regulatory context as more than a categorization tool for screening or than a communication tool for relating lower level effects to outcomes of regulatory concern, they must be able to increase levels of confidence for risk decisions more than current approaches while reducing the use of animals.

There is undoubtedly a need for timely and robust decision-making. Therefore, toxicity testing needs to become more efficient and cost-effective, which could result on the need to direct resources and focus efforts. Hypothesis driven Integrated Approaches to Testing and Assessment (IATA) have been proposed as practical solutions to that strategic testing. The AOP framework could then offer the biological context necessary to facilitate the development of IATA for regulatory decision-making, as it was discussed by Tollefsen and colleagues.[20]

MOVING FORWARD

On September 25th 2014, a major milestone in the AOP history was achieved with the public release of the AOP Knowledge Base (AOP-KB, https://aopkb.org/). The AOP-KB is a joint collaboration between the OECD, the US Environmental Protection Agency (EPA), the European Commission Joint Research Center (JRC), and the U.S. Army Engineer Research and Development Center (ERDC). The AOP-KB consists of several modules including the AOP Wiki (https://aopkb.org/aopwiki/index.php/Main_Page), a central repository for all AOPs and an open-source interface to facilitate collaborative AOP development and sharing of AOPs. The AOP Wiki was developed as part of the OECD AOP development effort lead by the OECD EAGMST. Other components of the AOP-KB are Effectopedia (http://www.effectopedia.org/), AOP-Xplorer (http://aopxplorer.org/), and the Intermediate Effects Database (developed by JRC). Concurrently, a User's Handbook Supplement to the OECD guidance document for developing and assessing AOPs was released (https://aopkb.org/common/AOP_Handbook.pdf). The User's handbook better clarifies the information needed for AOP development and includes practical instructions for entering the information into the AOP-KB. The importance of the moment is captured in the words of Prof. Maurice Whelan, Head of the Systems Toxicology unit at JRC, cochair of EAGMST, and one of the main promoters of the collaborative effort: *"I see the AOP-KB as a 'big knowledge' project, the first one of its kind, to simply, yet elegantly, combine what an international scientific community actually knows about how toxicological process work. What will emerge from this unique crowd-sourcing exercise will not only pave the way for completely new approaches to the way we assess chemical hazard, but in my view the AOP-KB will break the mold in terms of how we use collective scientific knowledge to better society."*

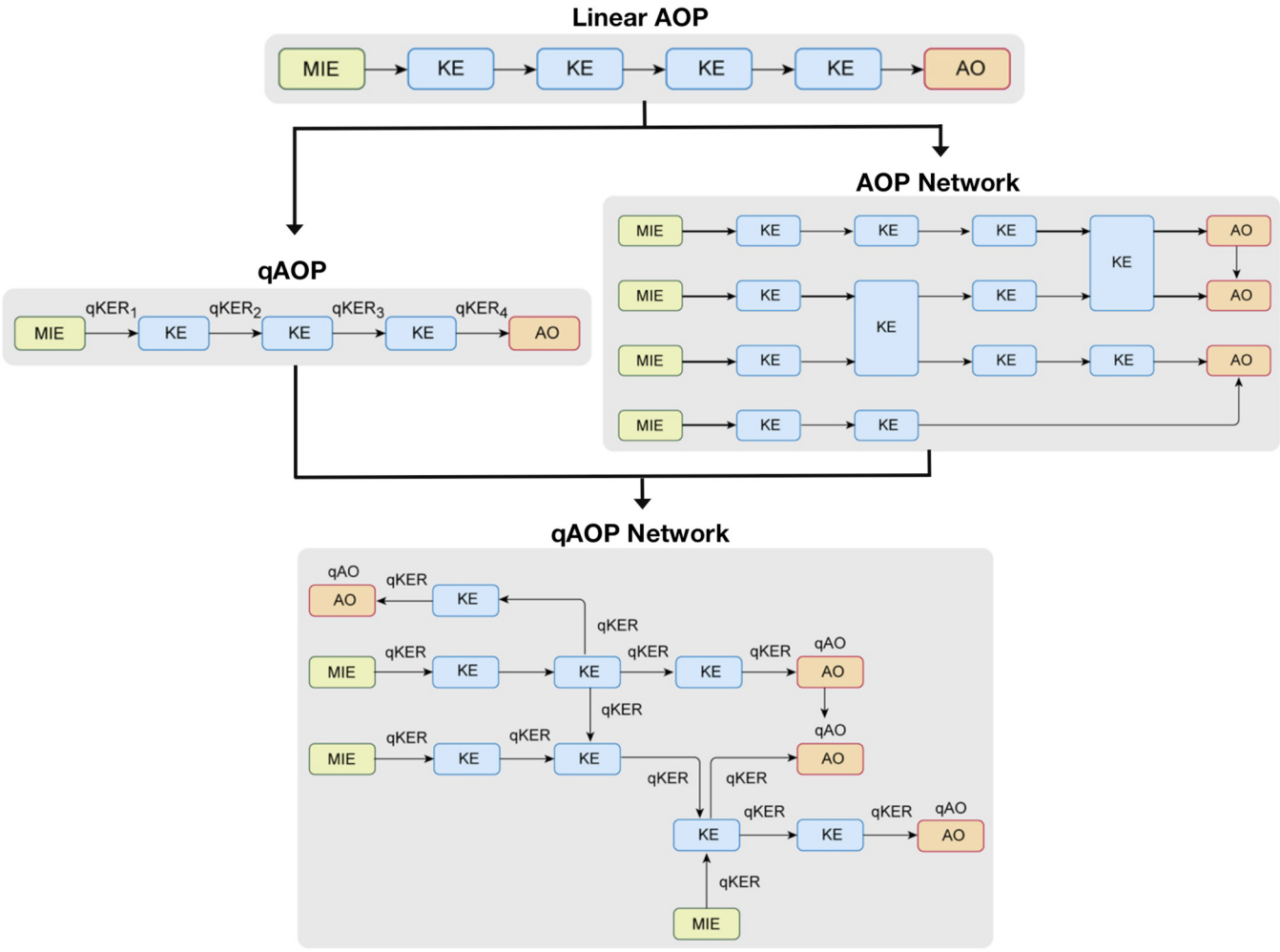

Figure 1. AOPs as pragmatic units and linear structures are rapidly evolving toward AOP networks and quantitative AOPs (qAOPs). In qAOPs, the linkages between two KEs, that is, KER, will be given a weight/value, forming the quantitative KER (qKER).[25] The merging of qAOPs and AOP networks will eventually lead to qAOP networks.

Other interesting concepts are emerging to support the AOP framework. For instance, one of the most critical (and criticized) aspects of AOP development is the ability to define scientifically sound linkages between the MIE, the KEs, and the adverse outcomes. This means that the relevant responses at the macromolecular, cellular/tissue, organ, organism, and population should be identified.[8,14,21−23] Another important aspect of AOP development is the evaluation of the scientific evidence that supports these linkages. The implementation of the (evolved) Bradford Hill considerations has been recommended to assess the weight-of-evidence supporting the AOP or mode of action. Simultaneously, evaluating the supporting evidence for these linkages, helps identify gaps and uncertainties in an AOP, defines the confidence with which the AOP can be used in predictive (eco)toxicology, and identifies areas where research is needed to support or reject these relationships.[24] These ideas naturally lead to the concept of a quantitative AOP (qAOP), the "ideal" form of AOP to be used in risk assessment. Perkins et al.[25] explored the use of weighting, probabilistic, and mechanistic approaches to quantitatively characterize the response-response relationships among the key events within an AOP.

Another interesting concept gaining momentum is the network of AOPs. As discussed by Villeneuve and colleagues,[17] while linear AOPs can be considered discrete units for AOP development, in reality, prediction of adverse outcomes will often require consideration of multiple AOPs with shared KEs. These systems of interacting AOPs with common KEs were defined as *AOP networks*. Perkins et al.[25] present approaches for the use of AOPs in the evaluation of the impacts of mixtures and networks of AOPs, providing rational ways to integrate data from multiple sources and tools for risk assessment. It is only logic to further put these concepts together, qAOPs, and AOP networks, and expect that they will soon become *qAOP networks* (Figure 1), which might eventually become the real functional units of prediction. These qAOPs and qAOP networks would have quantitative KER (qKER) and potentially and overall AO score (Figure 1).

One of the most exciting aspects of AOPs is the use of alternative species and the goal of eventually reducing and eliminating vertebrate animal testing (Figure 2). In order to accurately predict and relate chemical impacts across species, it is necessary to have a mechanistic understanding of the effects of pathway perturbation.[3] AOPs provide a framework to organize mechanistic and predictive relationships between MIEs, KEs and adverse outcomes. Thus, the framework allows the use of alternative models by informing the extrapolation of impacts across species. This extrapolation can happen at

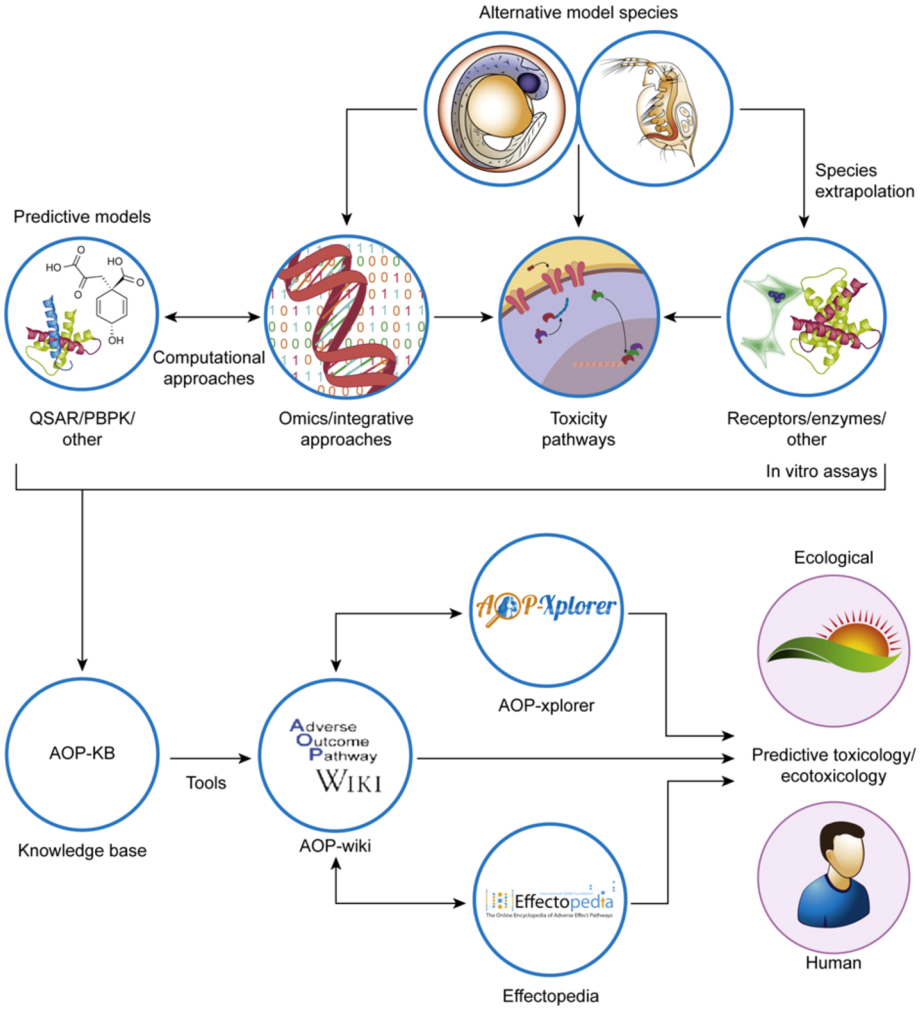

Figure 2. Use of alternative (model) species, such as fish embryo or Daphnia is one of the most exciting and challenging aspects of AOPs but does provide challenges, such as the need to accurately predict and relate chemical impacts across species, which requires a mechanistic understanding of the effects of pathway perturbation. Integrative approaches, including omics, the development and understanding of toxicity pathways, in vitro assays, and the development of predictive models will help inform and populate the AOP framework. The information will be used in the AOP-KB, which includes several modules, and will eventually inform predictive (eco)toxicology for both human health and ecological risk assessment.

different levels of an AOP. For instance, at the MIE level, sequences or structures of proteins can be compared under the assumption that evolutionary conserved proteins may have conserved functions.[3] Many human drug targets are conserved among ecologically relevant vertebrate and nonvertebrate species.[26,27] At a pathway level, extrapolations might be more complex as one needs to consider the sequence of events and the dose or threshold required to activate these events. These pathways can be examined as discrete pathways or as networks using cross-species comparative genomics.[28,29] In a pathway context, alternative species including embryo tests can show similar effects to those found in mammals, although the concentration needed to have an effect and the potential mechanisms of compensation and recovery might be different. These similarities and differences can be explored and extrapolated using omics and integrative systems approaches to identify signaling pathways and genes that can be mapped to functional pathways conserved across species.[3,28] Many efforts are currently being applied toward developing pathway-based toxicology and linking it to the AOP framework, as well as toward exploring incorporation of systems biology approaches into risk assessment.[30−32] Ideally, the mapping of concentration-responsive genes to pathways would allow the use of pathways in a more traditional risk framework.[3] High-throughput screening (HTS) programs that assess toxicity are focused on MIEs relevant to human health.[33] When these MIEs are conserved among species, the HTS data can be informative for ecological risk assessment and to predict higher level effects across species.[3,34] High-throughput assays can be powerful and are certainly very promising in moving toward a reduction in animal testing. For instance, the ToxCast program from the U.S. Environmental Protection Agency demonstrated that assaying complex biological pathways in primary human cells identified potential chemical targets, toxicological liabilities, and mechanisms useful to elucidate AOPs.[35]

The AOP framework can undoubtedly benefit from the improvement and advance of many disciplines. For instance, omics technologies and integrated systems biology approaches are rapidly evolving and becoming increasingly efficient in extracting and mapping information. However, transforming these maps into efficient predictive models remains a challenge. A recent paper[36] proposes an innovative approach to move forward from genomic networks to multiscale models able to predict cellular phenotypes and answer biological questions

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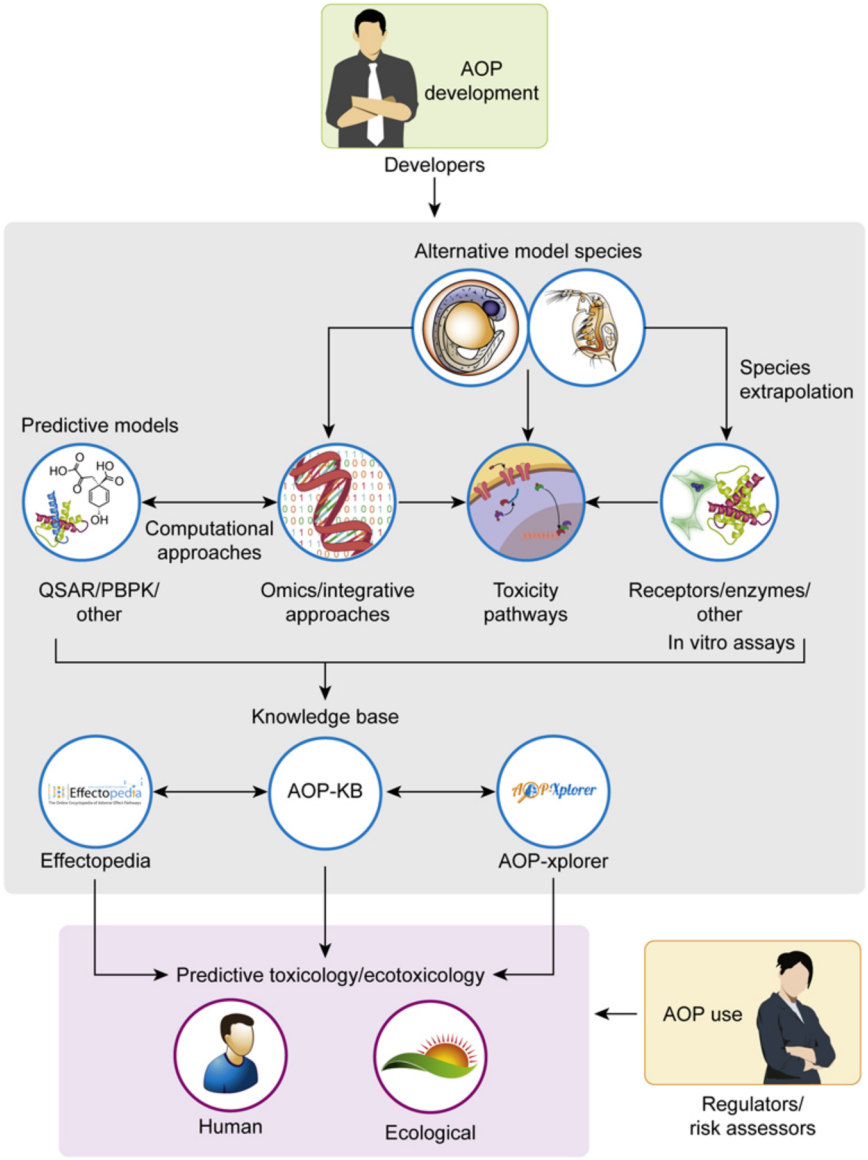

Figure 3. For the AOP framework to be successful, there is a need to keep the AOP development independent of the potential use of AOPs by regulators, which should start exploring the use of AOPs, even if they are not completely developed.

using recent advances in computer science embodied by intelligent agents such as Siri: as they call it, *the Siri of the cell*. Similarly, and following this very visionary approach, computational biology and artificial intelligence could be used in the future to predict KE linkages and adverse outcomes, maybe even extrapolate among species, and help inform risk assessment (*the AOP Siri* anyone?).

■ CONCLUDING REMARKS

While the AOP framework has been rapidly embraced all over the world, it has unquestionably received criticisms. Some of the criticisms involve concerns about overpromising what AOPs can be used for. For the AOP framework to be truly accepted, it must be demonstrated without reasonable doubt that it is efficient in predicting adverse outcomes. That, on the other hand, leaves us with the challenge of not being able to use the framework until it is completely developed, which might not be the best use of the available resources. There is a need to keep the AOP development independent of the potential use of AOPs by regulators (Figure 3), which should start exploring the usefulness and effectiveness of available AOPs without waiting for the framework to be completely developed. Their experience would be extremely valuable for AOP developers to detect deficiencies and correct them. Other criticisms point to the fact that the AOP framework implies that effects at the individual level will result in impacts at the population level, which is certainly not straightforward. Sometimes effects at the individual level do not translate to population because of compensatory processes at several levels of organization which create a complex and nonlinear linkage.[37,38] These criticisms are undoubtedly all very valid points, and as it is always the case in science, constructive criticisms that point to deficiencies will challenge developers and scientists and help improve the system. Indeed, these criticisms have helped spur the development of qAOPs and eventually qAOP networks (Figure 1).

Furthermore, in order for the AOP framework to be successful, it is crucial that multidisciplinary teams learn to work together. To do so, a common language must be achieved

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among not only people from different disciplines but also with different roles, such as scientists, government agencies, industry, and regulators. The same applies to risk assessment. While the AOP concept is being explored for both human health and ecological risk assessment, the two disciplines although with equivalent goals are intrinsically different. For instance, human health and ecological exposures are very different concepts (ie water versus organ/organism), further emphasizing the need for a common language so each discipline can help, inform, and learn from the other.

In conclusion, while AOPs are still facing many challenges, the amount of effort and resources being designated to their development worldwide is definitely impressive. AOPs might not be the magic bullet, but they are certainly providing us with a framework to organize and link information in a logic manner. So until the next revolution comes by, it seems that AOPs are here to stay, to evolve, to improve, and to eventually serve their purpose.

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Notes

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