Adverse Outcome Pathways (AOPs): A Means of Linking Alternative Methods to *in Vivo* Outcomes

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Adverse Outcome Pathways (AOPs) describe the linkages between a chemical interaction with a biological system at the molecular level and the biological effects at the subcellular, cellular, tissue, organ, and whole animal and population levels. An AOP for any given hazard endpoint can be the basis for developing integrated testing strategies for that hazard endpoint.

The AOP approach is a bottom up approach where events measured at the *in chemico* and *in vitro* level are linked to events measured at the *in vivo* level. For example in fish, estrogen agonists bind to the estrogen receptor, which can be measured *in chemico*, and set off a cascade of responses including the up regulations of vitellogenin production in the liver, which can be measured *in vitro*, the conversion of testes to ova and the feminization of males observed *in vivo* leading to reproductive impairment and a decrease in the population.

An AOP should be based on a single, defined molecular initiating event and linked to a stated *in vivo* hazard outcome. To establish an AOP three blocks of information are used. The first block is the chemical-induced perturbations of biological systems at the molecular level (anchor one). While a number of biochemical steps are required for a toxic response to be realised, the molecular initiating event is a prerequisite for all subsequent steps. The last block is typically the *in vivo* outcome of regulatory interest (anchor 2). These are often the reported endpoints from standard test guidelines.

These two anchors can establish an AOP much like two points determine a straight line. While AOPs may be depicted as linear frameworks, in that feedback mechanisms are not considered, the amount of detail and linear character of the pathway between a molecular initiating event and adverse outcome can vary significantly. This is especially true for human health endpoints, where effects are the result of multiple organ interactions (e.g., skin sensitisation), multiple events (e.g., repeat dose toxicity), accumulation over time (e.g., neural toxicity), or are related to a specific life stage of an organism (e.g., developmental toxicity).

To develop an AOP, different types of data can be utilised. These data include: structural alerts that are reflective of the types of chemicals that can initiate a pathway, *in chemico* methods that measure the relative reactivity or other chemical-biological interactions, *in vitro* assays that confirm the subsequent cellular responses (e.g. molecular screening data) and, ultimately, *in vivo* tests that measure endpoints that are directly relevant to the adverse outcome that drives current regulatory decision making. This information can be used to identify key steps in the AOP and provide scientific evidence supporting the AOP.

Many molecular initiating events are defined in the form of “receptor binding”; others are based on the principles of organic chemistry (electrophile-nucleophile reactivity). The understanding of the molecular initiating event allows for the definition of the properties of chemicals inducing the perturbation, such as bioavailability, structural requirements (especially for receptor binding) and metabolic transformation. The understanding of the chemistry of potential inducers helps to define the applicability domain for the AOP. In the ideal scenario, when the initiating event is well-defined, not only should the potential inducer of that event be recognised but also the site of action, which implies the type of biological macromolecule that interacts with the target chemical.

During the identification of intermediate events, a review of the existing literature is required to find out as much information as possible about the plausible mechanism and the steps leading to the apical outcome. This aspect is crucial for the development of the AOP. Usually, multiple intermediate events are identified. Therefore, the assembled knowledge has to be filtered and selected to match the single AOP.

It is necessary to understand the basis of normal physiology (e.g., nervous system function, reproductive processes, differentiation of tissues) of the AOP. The identified AOPs must not contradict any steps of normal biological processes, since they need to be biologically plausible. Even if some steps are not known with certainty, the overall process must agree with what is known about the particular biology being considered.

At the beginning, the collected information should be used to present the whole adverse pathway step-by-step starting from the general characterisation of the route of exposure (e.g., dermal) and related chemical properties, to the identification of the molecular initiating event and site of action, if possible. After that, the responses at the molecular, cellular/tissue, organ, organism, and population\ecosystem levels should be identified ; the final stage depends on the level of biological organisation of the adverse outcome. This report on the knowledge relating to the AOP is often based on one of a few well-studied toxicants. Following this, a concise summary of the qualitative understanding of the AOP has to be undertaken. For this purpose, the key events, documentation of the experimental support for each event, and an evaluation of the strength of the scientific evidence for that event need to be summarised.

Key events are seminal to the AOP approach. They are intermediate steps along the pathway that represent pivotal events, usually at the different levels of biological organisation. To be a key event, the intermediate step must be able to be evaluated experimentally. That is to say, the event must be able to be used in a hypothesis which can then be tested. There are no rules as to which types of data have to, or can be used to support a key event. However, such data should be reliable and relevant to the specific adverse outcome.

It is considered critical to be able to gauge the reliability and robustness of an AOP. This should be done by evaluating the experimental support of the AOP. In such an assessment, the understanding of the AOP has to be analysed. This means that key steps should be clearly identified and scientifically proven, both qualitatively and (if possible) quantitatively.

A well-identified AOP, with an accurately described sequence of events through the different levels of biological organisation in organisms, provides valuable pieces of mechanistic information which can be used for many purposes. By identifying and describing the key events, the AOPs could inform the work of the OECD Test Guideline Programme. In addition, an AOP, for any given hazard endpoint can be the basis for developing an integrated approach to testing and assessment or an integrated testing strategy for that hazard endpoint. The application of alternative approaches, like the OECD QSAR Toolbox where categories are first formed and data gaps filled within the category, will lead to the refinement, reduction and/or replacement of conventional *in vivo* testing.

While in the end it will be important to understand the linkages and scaling factors as the pathway moves up the level of biological organisation, especially for events which depend on potency in the *in vivo* outcome, initially good qualitative understanding of the AOP will allow it to be used for a variety of purposes including read-across. However, without transparent descriptions of a plausible progression of adverse effects at the different levels of biological organization provided by AOPs, it will be difficult to provide solid mechanistic reasoning to use an alternative method rather than the traditional *in vivo* test.