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The potential of AOP networks for reproductive and developmental toxicity assay development

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Introduction

Historically, the prediction of reproductive and developmental toxicity has largely relied on the use of animals, for both human (primarily using rodents and rabbits) and for ecological (fish, amphibians and birds) risk assessments. Although animal tests are intrinsically capable of capturing complex biological processes, and often allow direct observation of adverse effects, there is widespread agreement that replacing animal tests with *in silico* (e.g., quantitative structure-activity relationship [QSAR] models, structural alerts), *in chemico* (e.g., receptor binding assays), *in vitro* (e.g., stem cell assays) and *in vivo* tests using non-protected taxa or life stages (e.g., fish embryo tests) is high on the priority list of 21st century toxicity assessment [1]. Many non-animal-based assays, or alternatives to current whole animal test designs that require fewer animals [2], focused on reproductive and developmental toxicity have already been developed (e.g. mouse whole embryo culture, limb micromass assay, mouse embryonic stem cell assay, fish embryo test, preantral follicle culture). However, the endpoints assessed by most of these assays are apical, and often do not provide mechanistic information on the underlying biological processes leading to observed effects (e.g., micromass assay: cell differentiation, proliferation and viability; whole embryo culture: embryo functionality, growth and morphology; fish embryo test: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat).

The Adverse Outcome Pathway (AOP) framework, introduced in 2010 [3], forms a basis for the development of new non-animal test methods. It also provides biological context for mechanistic information from existing assays, which can help increase confidence in, and utility of their results for risk assessment and regulatory decision-making. In summary, an AOP is a detailed description of a chain of events following a molecular initiating event (MIE, a direct interaction of a chemical with a molecular target) leading to an adverse outcome (AO) at the individual or population level through a series of intermediate key events (KE) spanning different levels of biological organization [4]. Within an AOP context, KEs help define relevant needs for assay improvement or development. A KE is generally defined as an observable change in biological state that is necessary (but not necessarily sufficient by itself) for the progression towards a specific AO [5]. Examples of KEs include changes in expression and/or function of genes, proteins, and metabolites, alterations in cellular or tissue morphology, physiological dysfunction, etc., along a causal pathway to an AO relevant to risk assessment. Since KEs must, by definition, be measurable [4], there is a clear linkage between the AOP framework and assay development, particularly with respect to development of alternatives to traditional whole organism tests focused on direct observation of apical outcomes. Further, since KEs should ideally be described in such a way that they can be reused in other AOPs [4,6], collective development of AOP descriptions by the scientific community can lead to de facto construction of sets of related or interconnected AOPs that have the potential to uncover new links among toxicological processes.

Although AOPs can support the development of non-animal test methods, newly defined AOPs, and assays that have been developed based on these AOPs, require validation before they can be used for regulatory purposes, which will often involve the use of animals. AOP development should, however, not be regarded as a source of additional animal testing since AOPs can help prioritize the investment of research efforts into validation of alternative tests, and future assessments based on validated AOPs will only occasionally require full validation experiments (for example, when moving to a new species, [7]).

Adverse Outcome Pathway Knowledge Base

To encourage AOP integration and data sharing among scientists and to allow systematic organization of AOP information for use in regulatory decision making, an online AOP Knowledge Base (AOP-KB, <http://aopkb.org>) has been established. The AOP-KB project is an Organisation for Economic Cooperation and Development (OECD) initiative, executed as a collaboration between the European Commission's Joint Research Centre, the United States Environmental Protection Agency, and the US Army Engineer Research & Development Center. The overall effort is coordinated with the activities of the OECD's Extended Advisory Group on Molecular Screening and Toxicogenomics (<http://www.oecd.org/env/ehs/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm>). The AOP-KB allows scientists to build and document AOPs by entering and then providing information about MIEs, KEs, and AOs and the biological plausibility and empirical evidence supporting their causal and/or predictive linkages (termed key event relationships; KERs). Since AOP elements are not necessarily unique to a single AOP, newly entered information is linked to existing knowledge by allowing the re-use of previously described MIE, KE, AO, and KER information. The AOP-KB is conceptualized as a central AOP knowledge hub which can be extended by adding modules (see <http://aopkb.org>). The core module of the AOP-KB is the AOP Wiki (<http://aopwiki.org>) which is used to describe AOPs in a manner that integrates them into the broader AOP-KB database. Other AOP-KB modules, such as Effectopedia and AOP Xplorer, can then be used to search and visualize AOPs, and to add quantitative toxicological information.

The AOP Wiki was made publically available in September 2014, and the amount of information contained was rather limited at that point in time. In January 2015, the AOP Wiki contained 42 AOPs, of which seven were indicated as “ready for commenting” (i.e., well developed and properly described in accordance with OECD guidance; https://aopkb.org/common/AOP_Handbook.pdf, [6]) while the remaining 35 were marked as “under development”. Of these 42 AOPs, 11 are related to reproductive or developmental toxicity, covering nine different MIEs.

Table 1 summarizes these 11 reproductive/developmental effects-related AOPs. The majority are focused on fish, and although multiple efforts are ongoing to add additional mammalian AOPs, these remain underrepresented for the time being.

Table 1. Overview of AOPs related to reproductive and developmental toxicity available in the AOP Wiki (Jan. 2015)

AOP	OldID	MIE	AO	Taxon	Status
22	19259	AHR activation	Developmental effects, embryotoxicity	Birds	RFC
23	19261	AR agonism	Reproductive dysfunction	Fish	RFC
25	19262	ARO inhibition	Reproductive dysfunction	Fish	RFC
30	19265	ER antagonism	Reproductive dysfunction	Fish	RFC
7	19556	PPAR γ activation	Decreased female fertility	Rodents	UD
18	19271	PPAR α activation	Decreased male fertility	Rodents	UD
19	19263	AR antagonism	Impaired male reproductive capacity	Mammals	UD
21	19260	AHR activation	Embryotoxicity	Fish	UD
28	18011	COX inhibition	Reproductive failure	Birds	UD
29	19266	ER agonism	Reproductive dysfunction	Fish, Birds, Amphibia	UD
24	19264	AR antagonism	Reproductive dysfunction	n.s.	UD/INI

AOP: identification number of the AOP in the AOP Wiki; OldID: version number of the AOP. To retrieve the version of an AOP as it has been used and cited in this article, use the following URL pattern: <https://aopkb.org/aopwiki/index.php?title=aop:XX&oldid=YYYYY>, in which XX = AOP number and YYYYY = OldID. AHR: aryl hydrocarbon receptor; AR: androgen receptor; ARO: aromatase; ER: estrogen receptor; PPAR: peroxisome proliferator-activated receptor; COX: cyclooxygenase; n.s.: not specified; RFC: ready for commenting; UD: under development; INI: initiated (Wiki entry created but no data available).

In addition to the AOPs listed in the AOP Wiki, several AOPs related to mammalian reproductive and developmental toxicity are included in the OECD AOP development programme workplan and are currently being described and evaluated (e.g., AOPs related to embryonic vascular disruption, thyroid hormone metabolism in the context of neurodevelopment, heritable germ cell-derived disease;

<http://www.oecd.org/chemicalsafety/testing/listsofprojectsontheaopdevelopmentprogrammeworkplan.htm>).

The AOP network approach

A single AOP is considered as a pragmatic unit for AOP development and not as a complete biological representation of toxicological processes encompassing all possible molecular, biochemical and physiological components involved. Consequently, individual AOPs are generally conceptualized as a “linear” construct, without converging or diverging pathways connected to it. However, it is recognized that a single AOP may not capture all events that could contribute to any relevant toxic effect. For example, Villeneuve et al. outlined approximately 12 separate AOPs which could plausibly converge at the key event of impaired swim bladder inflation and lead to reduced young of year survival in fish [5]. Chemical inhibition of any one of seven different enzymes or three important developmental signaling cascades could either independently, or collectively contribute to the AO. Rather than attempt to describe every possible means through which an event like swim bladder inflation or reproduction could plausibly be impaired as a single AOP, it is pragmatic to describe one or a few of those means at a time, and allow the a more realistic representation of the complex webs of molecular and biochemical perturbation that can contribute to any particular AO, or the diversity of AOs a single MIE may trigger, emerge through the description of multiple AOPs. Multiple AOPs are therefore probably required to describe most toxicologically relevant processes and to predict AOs in most real-world scenarios, especially when considering mixtures [8] or multigenerational effects of chemicals [7]. Since MIEs, KEs and AOs can be shared among different AOPs, it is relatively easy to link different AOPs into an AOP network (an AOP network is defined as a set of AOPs sharing at least one common element [8]). AOP networks are therefore capable of more realistically representing potential chemical effects. They provide information on interactions between AOPs and have the potential to reveal previously unknown links between biological pathways.

From the perspective of developing assays to predict an AO, AOP networks offer the advantage of being able to target KEs that are either uniquely connected to a specific MIE and that are therefore indicative of a very specific toxic mechanism,

or KEs that are shared among multiple MIEs. Figure 1 gives an example of an AOP network for reproductive and developmental toxicity in fish that has been built based on the five AOPs that are currently available for fish in the AOP Wiki (Table 1, AOP Nos. 21, 23, 25, 29 and 30). In this example, reduced estradiol synthesis in granulosa cells is linked to two different MIEs, while reduced vitellogenin synthesis in hepatocytes is linked to three different MIEs and reduced testosterone concentration in theca cells is uniquely linked to androgen receptor agonism. While all three KEs lead to, and can potentially be used to predict, the same AO of decreased female fecundity in terms either of egg production or embryonic survival, they have varying specificity with respect to the MIE triggering the chain of events.

In general, AOP networks therefore offer the potential to guide the development of assays with different degrees of specificity for toxicological mode(s) of action, being indicative of either a very specific MIE or, alternatively, of clusters of mechanistically related MIEs. This type of assay development logic may be particularly useful for differential screening of compounds with unknown molecular targets, e.g. in the context of Integrated Approaches to Testing and Assessment (IATA, [9]), in which sequential elimination of possible mechanisms may be quickly achieved using assays probing strategically chosen KEs in an AOP network. Such an approach has, for example, already been implicitly implemented to some extent in the Fish Sexual Developmental Test (OECD Test Guideline [TG] 234, [10]), which uses vitellogenin measurements in males and females as well as determination of sex ratio to differentiate among estrogen receptor activation, androgen receptor antagonism, and inhibition of sex steroid synthesis (e.g., aromatase inhibition). Positioning TG 234 within an AOP network such as depicted in Figure 1 would allow the addition of assays based on KEs (e.g. testosterone and estradiol measurements), possibly resulting in an increased resolution to differentiate among potentially perturbed pathways. Furthermore, although single endpoint assays may be sufficient to predict AOs in some cases, measuring multiple, related endpoints are often needed to reliably assess pathway perturbation [11], for example, in cases where the combined occurrence of effects on different KEs builds a weight-of-evidence linkage to a

given AO. In such scenarios, AOP networks allow one to select a specific combination of KEs that is likely to yield reliable results. Similarly, AOP networks allow targeted stepwise selection of KEs at different levels of biological organization for supporting tiered testing strategies.

Figure 1. Example of an AOP network based on the five reproductive and developmental toxicity-related AOPs that are available for fish in the AOP Wiki (Jan. 2015). MIEs are indicated in green, KEs in orange, and AOs in red, as per the AOP Wiki template. The dotted squares indicate KEs that are defined as changes in opposite direction (increase versus decrease) of the same biological component. AHR: aryl hydrocarbon receptor; GtH: gonadotrope hormone; T: testosterone; VTG: vitellogenin; E2: estradiol. KE descriptions have been directly derived from the AOP Wiki whenever possible. In some cases, slight modifications of descriptions were necessary to generate a re-usable KE in this specific network. This figure illustrates the AOP network approach but does not make any assumptions about the scientific validity of the underlying AOPs. AOPs, and hence the depicted AOP network, may be subject to change before they are formally finalized. See Table 1 for how to view the exact version of the AOPs that were used in this network.

Conclusions

In conclusion, the AOP framework, and an AOP network approach in particular, offers great potential for improvement of existing assays by increasing confidence in their results or by extending them with additional targeted endpoints, for allowing selection of assay specificity relative to the MIE(s), or mechanism of action, and for informing and guiding the development and application of new assays (although KEs are defined to be measurable, they can be theoretical at the time of AOP development – delineating an AOP therefore does not necessarily imply that assays for all KEs have already been developed). AOP networks can then provide justification and increased confidence for applying these assays to predict toxicity. As an example, Martin et al. linked ToxCast™ Phase I *in vitro* data of 256 chemicals to rat multigenerational reproductive toxicity studies and built a predictive model capable of identifying rodent reproductive toxicants with an accuracy of around 75% [12] (ToxCast™ is a US Environmental Protection Agency research program that uses automated chemical screening technologies to screen for changes in biological activity in living cells or isolated proteins after exposure to chemicals). ToxCast™ assays important to the model include peroxisome proliferator-activated receptor α and γ , androgen receptor and estrogen receptor agonist and antagonist assays, as well as cytochrome P450 enzyme inhibition, G protein-coupled receptor and cell signaling pathway assays. Since it is clear that specific mechanistic information that is directly relevant to reproductive toxicity is being used by this model, in addition to more general biological processes, positioning these assays within well-described AOP networks could allow further improvement of such models by providing biological context for interpreting the results, as well as by providing directions for building the models, e.g. by implementing supervised learning methods focused on AOP-annotated groups of KEs. Such an integrated approach could result in a more defined model capable of distinguishing among different types of reproductive toxicity.

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