National Centre for the Replacement Refinement & Reduction of Animals in Research

Adverse Outcome Pathway News

For scientists, risk assessors and regulators with an interest in pathways-based approaches

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NC3Rs programme on pathways-based approaches

When we began our programme of work on pathways-based approaches back in 2014, adverse outcome pathways (AOPs) had emerged from the field of ecotoxicology and were beginning to be expanded to other disciplines.

We recognised the potential 3Rs benefits of more mechanistic-based approaches to include the:

- Reduced reliance on animal toxicity testing and increased confidence in non-traditional methods (*in vitro* and *in silico*)
- Reduced number of species required for toxicity testing (e.g. through identification of conserved MIEs and key event relationships)
- Identification of novel approaches to hazard characterisation (e.g. development of mechanism-based biomarkers)

With the support of an expert Steering Group comprised of scientists from academia, industry and regulatory agencies, we aimed to raise awareness and support scientists in the development and application of pathways-based approaches to improve the identification and characterisation of hazards with reduced reliance on animals.

Highlights of our programme to date include two cross-industry workshops (the first held in 2014 to bring the scientific community together and raise awareness of pathways-based approaches, and the second held in 2016 with a focus on application), an AOP resource webpage, this 'AOP News' bulletin and two peer-review publications. We even attempted to develop an AOP ourselves, in collaboration with EURL ECVAM and an expert working group. This resulted in a strategic award to develop at AOP for 'L-type Ca^{2+} channel block leading to heart failure', which was won by Dr Luigi Margiotta-Casaluci, Brunel University. See the 'AOP Spotlight' on page 3 for more information on the development of this AOP.

Since 2014 there has been substantial progress in the science related to the area of pathways-based approaches and AOPs, and the continued establishment of frameworks designed to advance the application of pathways-based approaches. We are pleased to see that AOPs/mechanistic approaches are now becoming integrated into the science, rather than considered as a stand-alone topic. The focus now needs to be on how the information from AOPs can be applied in practice and used for decision-making. This is going to require consideration of AOP networks and incorporation of exposure considerations, as well as a move towards more quantitative AOPs. This is discussed by our AOP Steering Group in our article the "Future Trajectory of AOPs", published in *Archives of Toxicology* earlier this year. What is needed to increase the value of AOPs in toxicology is also discussed in "*Ask the experts*" on page 6.

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Links & Resources

- NC3Rs AOP resource page
- OECD's AOP framework
- AOP-Wiki

Pathway-Based Approaches to Chemical Risk Assessment: A Public Health Perspective

Dr Nicole Kleinsteuer, NICEATM

Establishing scientific confidence in new approach methodologies that are more human-relevant will require a shift away from animal data as the primary basis for comparison. Human biology-based AOPs represent a framework for developing integrated *in vitro* testing strategies that can be coupled with computational models to improve chemical risk assessment.

In early 2018, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), with representatives from 16 U.S. federal agencies, published a national strategic roadmap aimed at developing new approaches for evaluating the safety of chemicals and medical products in the United States (https://ntp.niehs.nih.gov/go/natl-strategy). One of the key principles of this document, and roadmaps published by individual agencies (e.g. FDA, EPA) or other regions (e.g. the Netherlands, UK) is to shift the focus from reliance on the "gold standard" of animal reference data to an approach that is driven by understanding of human biology and toxicity mechanisms.

As much of the legacy *in vivo* data we have relied upon for decades becomes digitized and computationally accessible (initially through arduous manual efforts and slowly *via* automated natural language processing systems under development), opportunities arise to assess the true variability and inherent uncertainty in the animal studies. These analyses, on bioassays such as the rodent uterotrophic and Hershberger endocrine-relevant tests, the murine local lymph node assay for skin sensitization, and the acute oral LD50 test for systemic toxicity, reveal that the agreement within the same type of high-quality guideline-like studies run independently on the same chemicals is only in the range of 70-80%. The inherent variability observed in these tests, despite controlling for study protocol factors, reflects the fact that animal studies should perhaps be considered more of a "bronze standard" and helps to appropriately set expectations for the performance of new non-animal approaches when compared to these reference data.

In fact, in areas such as skin sensitization where human clinical data are available as a basis for comparison, there is strong evidence that mechanistically driven testing strategies linked to the skin sensitization AOP outperform the animal tests in predicting human sensitization potential, leading one to hypothesize that other non-animal approaches designed to target human biology-based AOPs may in fact be superior to the animal tests even when their predictive performance against reference animal data appears sub-par. For example, a non-animal approach grounded in human biology could demonstrate only 75% predictive performance against animal reference data, but if the scientific rationale and mechanistic human relevance is sound, then perhaps the 25% discordance represents those chemicals missed by the animal test. A pertinent example of this concept occurs in the field of eye irritation testing, where the Draize rabbit eye test is notoriously irreproducible, and it stands to reason that rather than attempting to predict hazard categories based on rabbit eyes, effects observed *in vitro* on human corneal epithelial cells may present a more compelling case for human relevance.

Outside of topical toxicity applications, predicting systemic and internal organ toxicities also benefits from human-relevant pathway-based approaches. Often there are a multitude of mechanisms involved (e.g. in development and reproductive toxicology [DART], carcinogenicity), and this requires building AOP networks that can begin to represent complex interactions and provide insight into critical failure nodes or tipping points that may guide the development of testing strategies. In these cases, the proper interpretation of activity concentrations derived from non-animal methods in the context of real world exposures is paramount. This can be facilitated through computational approaches such

as *in vitro* to *in vivo* extrapolation using physiologically-based pharmacokinetic models to estimate external doses that would lead to internal concentrations predicted to cause toxicity. Modern translational toxicology pipelines are starting to couple such computational tools and *in vitro* models with select animal tests that are designed to be maximally informative, in terms of mechanism, population variability, genetic susceptibility, etc. Such animal tests would ideally be run only on chemicals highly prioritized based on exposure and human-relevant toxicity predictions.

A myriad of international efforts, operated through public-private partnerships, non-governmental organisations, the Organisation for Economic Co-operation and Development (OECD), and initiatives like the International Cooperation on Alternative Test Methods (ICATM), are ongoing to achieve a pivot towards pathway-based approaches rooted in human biology that will provide an equivalent or superior level of human health protection. Awareness is increasing that our current animal-based risk assessment system, which has provided adequate protection for decades, can be superseded by pathway-based approaches that achieve the 3Rs principles while yielding improved insight into human-relevant toxicity predictions.

AOP Spotlight: Dr Luigi Margiotta-Casaluci, Brunel University

Cardiotoxicity was identified as an area of potential interest for AOP development by a network of collaborators convened by the NC3Rs and the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) in 2015.

Since then, the NC3Rs has been coordinating a series of development activities, which culminated with funding via a dedicated Strategic Award in 2017 to accelerate the development efforts.

The award, won by Dr Luigi Margiotta-Casaluci, is now complete, and has resulted in not one, but three new AOPs. Blockade of L-type calcium channels (LTCC) leading to:

- heart failure via decrease in cardiac contractility (AOP-Wiki Project: 261)
- heart failure via disruption of cardiac electrophysiology (AOP-Wiki Project: 262)
- disruption of vascular tone maintenance (AOP-Wiki Project: TBC)

These will be published on the AOP-Wiki and available for comment shortly.

What were the initial reasons and drivers for developing this AOP?

The prediction of cardiovascular (CV) safety liabilities is a major challenge in drug development, and the development of novel CV safety biomarkers is still a pressing need. Currently, the hERG potassium channel represents one of the major CV safety pharmacology targets, as its inhibition can lead to alterations of cardiac electrophysiology. However, a number of stakeholders have called for dedicated inter-disciplinary efforts aimed at improving our understanding of the mechanistic basis of CV liabilities, beyond hERG current blockade. This project is an opportunity to contribute to those efforts, using the AOP concept to map the multi-scale effects triggered by the inhibition of another important ion channel, specifically LTCCs.

What approach was taken to develop this AOP?

We explicitly intended to prepare the ground for future development projects generating quantitative versions of the proposed AOPs. We extracted data from over 150 primary publications that investigated the effects of calcium channel blockers on different components of the CV system, and the effects of genetic manipulations of the Molecular Initiating Event (MIE). We generated a database containing over 1,100 *in vitro*, *ex vivo* and *in vivo* data points, which was used to identify potential Key Events (KEs). For each KE, we performed a quantitative analysis to determine effect direction and degree of responsiveness. We also assigned a database-specific confidence index to each KE, according to

the degree of reproducibility of the effect. This approach was used in combination with the assessment of biological plausibility to identify the final KEs characterized by the highest level of confidence.

What is the intended application of your AOP?

The developed AOPs are mainly intended to support drug safety assessment. However, they could be applied to assess the hazard of any chemical that inhibits LTCCs. Several KEs of the AOPs we propose are already quantifiable using *in vitro* tests. We hope that, once fully developed, these AOPs can support the prediction of the *in vivo* implications of effects observed *in vitro*.

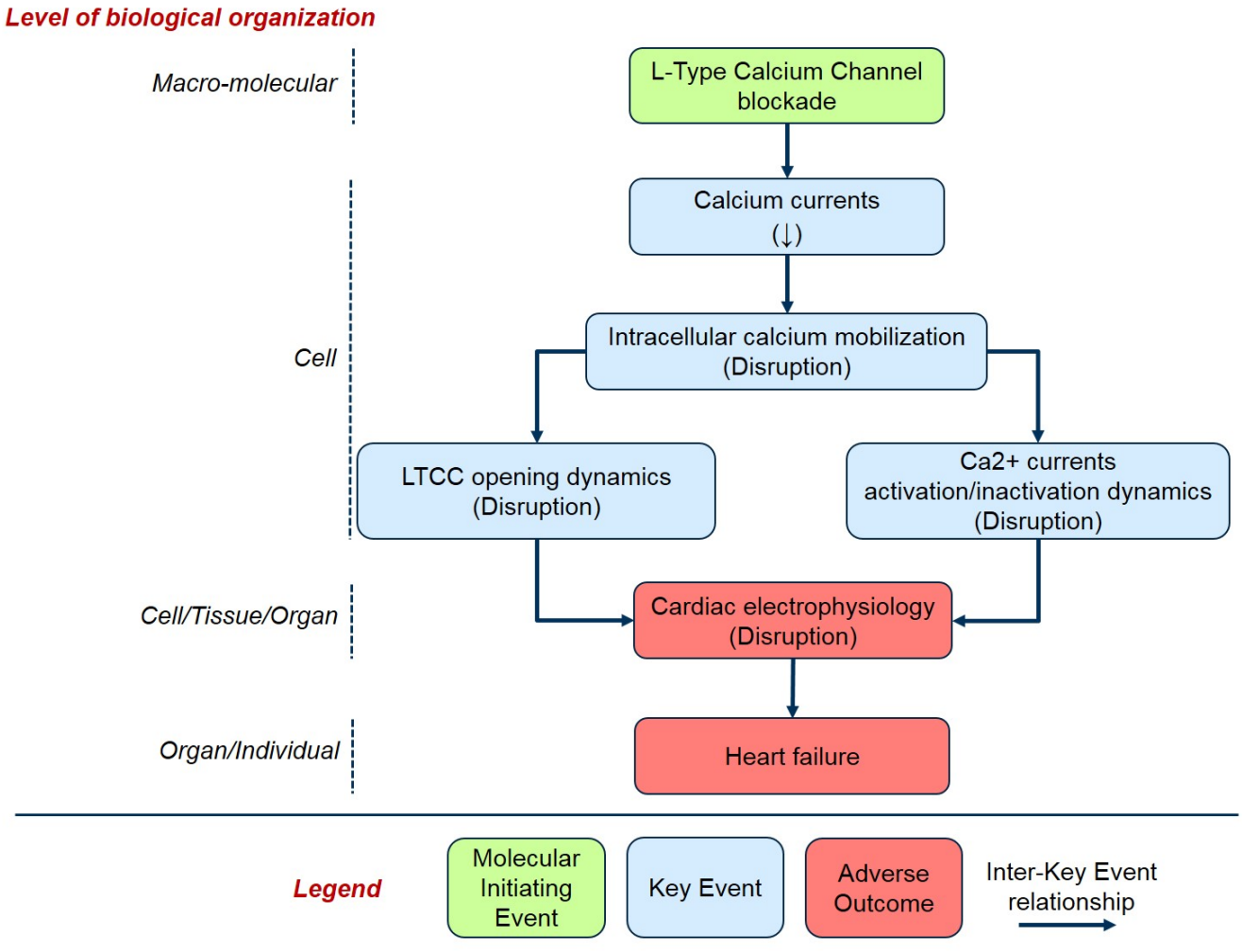
Can the AOP be currently be used for its intended application? What next steps are needed?

During the development phases, we dedicated particular attention to maximise the potential of each AOP to effectively inform the development of suitable testing strategies. For example, we generated a responsiveness analysis of the various methods and endpoints that can be used to quantify each KE, highlighting how some measurement are more likely than others to detect LTCC-blockade-induced perturbations.

The volume of evidence collected so far is probably enough to inform the development of specific testing strategies, but more work is needed to unlock the full applicative potential of these AOPs.

What next steps are needed?

The transition from qualitative to quantitative AOPs is the major factor that determines the applicative potential of AOPs in risk assessment and decision-making. The two AOPs related to the cardiac compartment (261 and 262) share the same MIE and two different KEs, and can be considered either individually or as a functional network. Interestingly, several *in silico* methods are currently available to determine the quantitative relationship between various KEs in the two proposed AOPs in humans. Future development efforts will be aimed at combining those different methods to develop a fully quantitative AOP network.



What challenges did you encounter whilst developing this AOP?

The unexpected challenge was related to data and vocabulary harmonization. We encountered a significant diversity of terminology used to indicate endpoints, techniques, exposure conditions, etc. This diversity required an extensive harmonization effort, which took much longer than expected.

How do you think use of this AOP in practice could impact on the 3Rs?

AOP-related research consists of two aspects: development and application. The development of an AOP alone, has no applicative value (and hence, no 3Rs value) as AOPs are chemically-agnostic and non-quantitative. Nonetheless, the application of AOPs to specific toxicology scenarios can be used to effectively guide the implementation of the 3Rs in present and future testing strategies. Although our project was focused on AOP development, we intentionally prepared the ground for future application projects that may have potential 3Rs implications. Specifically, we would like to highlight the following points:

a) **Identification of specific AOP sub-regions that can be modelled *in silico***. As previously mentioned, several *in silico* methods are currently available to determine the quantitative relationship between various KEs in the two proposed AOPs in humans. We are very interested in the possibility of incorporating those models in future AOP development projects.

b) **Effect database containing quantitative information relevant to PK/PD modelling.** The effect database generated contains quantitative data that may be readily used as input for existing *in silico* models (i.e. effect concentrations for each drug/endpoint, species, test system, exposure duration, quantification method, drug log K_{ow} and log *D*7.4). Additionally, our database can be readily expanded to incorporate additional relevant information such as effect size and study quality assessment.

c) **Novel data visualisation strategies to identify the most reliable endpoints/measurements**. Often, a given KE can be quantified using many different methods; an aspect generally overlooked by current AOP development. For each KE, we provided a graphic representation of the responsiveness analysis of each method. The analysis revealed that whereas some measurements are highly sensitive to LTCC blockade, others are poorly responsive. This information can be used to guide the selection of the most responsive quantification method for each KE, and the quantification methods best aligned with the 3Rs.

What advice would you give to researchers about to embark on developing an AOP?

Set the bar before starting. Each AOP development project may have different expectations in terms of ambition, timeline, and complexity. The ultimate goal is to develop an AOP that is robust enough to be submitted to the OECD for review and approval. However, reaching this goal within the first development attempt is not always possible (due to time and resource availability), and multiple follow up efforts may be required. It is important to set realistic expectations at the very beginning of the project to identify and implement the most appropriate/efficient development strategy. If time is limited, completing a few development modules with high quality information may be more efficient than uploading low quality information in all modules.

Don't underestimate the complexity of multi-disciplinary evidence assessment. Although the final AOPs may look like simple models, the development process involves the consideration of data generated by many different sub-disciplines of biology. Interpreting all available evidence and establishing their relevance/quality can be a complex task as it goes beyond the expertise of the individual AOP developer. Ask advice of experts whenever possible. This can be a great opportunity to expand the network of collaborators.

Think early about the potential applications of the AOP and its quantitative aspects. Identifying potential real-world applications of the AOP under development can be a great way to accelerate and shape the development process, establish links with potential end users (e.g. industry), and support the 3Rs vision. Keeping in mind the potential quantitative aspects of the AOP can also help to prioritise the sub-regions of the AOP that should be considered in subsequent development projects.

Ask the experts

We asked experts in the field of AOPs to describe the one innovation that will increase the value of AOPs in toxicology.

Abbreviations: Adverse Outcome (**AO**); Molecular Initiating Event (**MIE**); Key Events (**KE**); Key Event Relationships (**KER**), Integrated Approaches to Testing and Assessment (**IATA**).

Professor Ian Kimber, University of Manchester

Incorporate consideration of exposure metrics and thresholds

There are many developments that could, to greater or lesser extent, have the potential to increase the utility of AOPs. However, I believe there is one in particular that could have a transformative effect. It has been argued previously that to allow AOPs to evolve from a vehicle for effectively describing the key features of mode of action pathways, to a tool that could inform risk assessment, there is a need to incorporate consideration of exposure metrics. This is clearly the case, but the question is how best to achieve this. One innovation I would like to see is the alignment of AOPs with thresholds; that is an understanding of the level of exposure that is required to trigger the MIE, and subsequently the degree of change that is necessary in each instance for downstream KEs to be triggered. This might be described as bringing a quantitative element to KERs. This vision is not new, and others are also working on this, so let's hope that this research will bear fruit and that we can look forward to the development and application of quantitative AOPs.

Dr Brigitte Landesmann, JRC

Better training and recruitment to facilitate AOP development

I doubt there is THE one innovation that will increase utility. The further development of the AOP framework is an evolutionary process, shaped by experiences, with small changes continuously on-going. What I see as major problem is the low uptake by the "crowd"; more people need to be "recruited" for active collaboration. Efforts are increasing to disseminate the message, to give more targeted training and to facilitate further AOP development. Special emphasis need to be given to the training of students. Alternative methods and AOP thinking is still not included in many (most) curricula for studies in toxicology/life sciences. I think increased value will currently rather depend on increased uptake and exchange than on innovations of the framework itself.

Dr Annamaria Carusi, University of Sheffield

Greater awareness and support for AOP development

It may be obvious to say that the AOP approach will be more valuable as it gains a critical mass of researchers and other stakeholders using it, and as there is a substantial increase in the number of AOPs that are easily accessible and shareable. But to achieve this, there needs to be greater awareness of the AOP Framework as both a scientific and a social tool, as it organises scientific information and knowledge in such a way as to make it shareable with others, that is, it fulfils a social function that needs organisational and institutional support. So that AOPs can be populated as extensively as they need to be for the different uses to be made of them, they need to be unbound from the traditional publishing system. For there to be a groundswell of support for the approach from individual researchers and labs that translates into uploading and sharing AOPs, it is crucial that the approach be 'owned' by an organisation that is capable of setting in place a sustainable long-term strategy that promotes open access data publishing, community led standardisation, and engagement with core stakeholders beyond academic research.

Dr Daniel Villeneuve, EPA

Response-response modelling

Response-response modelling is an innovation that will increase the value of AOPs in toxicology. The field of toxicology was largely founded on the concept of dose-response. We tend to frame our experimental designs, our risk assessments, and our thinking around the identification of the concentration of a chemical (or stressor) that elicits a biological response. However, one of the major aims of the AOP framework is to help us take the measurements we can make easily, cost-effectively, rapidly, and ideally in higher throughput with the use of fewer animals, and use them to predict the outcomes we care about from a regulatory or management perspective (i.e. traditional apical adverse outcomes).

Consequently, when developing AOPs, what we are aiming to define is a reproducible and generalisable relationship between one measurable biological change (e.g., reduction in an enzyme activity) and another (e.g., reproductive success), not between a concentration of a chemical and a biological change. However, we generally don't design experiments in which we specifically vary the endogenous activity of an enzyme or the expression of a gene and then measure the associated effect on the apical endpoint of concern. We may capture that kind of information in our toxicological studies, but rarely conduct analyses in which, rather than placing dose/concentration on the X-axis, we instead place an upstream biological response variable on the X-axis and a downstream biological response on the Y-axis and evaluate how robust that relationship is across a range of different stressors and scenarios. Consequently, the basis for extrapolation among KEs along an AOP are generally qualitative and descriptive in nature, but lack the quantitative rigor that we have generally put into defining dose-response relationships. Developing the scientific mindset, experimental designs, and response-response modelling approaches needed to define and describe biological response-response relationships in mathematical terms would represent a major leap forward in the utility of AOPs in toxicology and for their ultimate application in risk assessment and supporting the broader use of alternatives to traditional whole animal guideline toxicity testing.

Note, the author's response does not constitute nor reflect U.S. EPA policy.

Dr Robert Landseidel, BASF

Move away from AO (the hazard) and focus on the 'P' (the mechanistic pathway)

Currently, toxicological assessment and regulation of substances is based on hazard, which is mostly characterised by the results of animal studies. AOPs facilitate the use of mechanistic information (the pathway, P) in predicting the AO – which is the hazard. In other words: AOPs assist hazard-based toxicological assessments by describing KEs leading to the hazardous effect. Any new description of an AOP, any new method addressing a KE of an AOP and any IATA, based on an AOP, can improve the value of AOPs in toxicology – yet all this is still bound to the hazard.

The innovation which will increase the value of AOPs in toxicology, is the actual use of P (the mechanistic data) instead of AO (hazard) as key data for toxicological assessments. This would rather ask which physiological processes must not be disturbed to a certain extent and for a certain time, rather than which hazardous effect is observed in animals. It is easily of more relevance to us humans, but it will require knowledge of our network of critical physiological processes and their dose- and time-responses to substance exposure. Turning from AO to P will certainly improve human toxicology – but it might not always require the actual mapping of the complete pathway, P, and a link to a specific AO. Eventually, it may therefore not even be named "AOPs" in toxicology anymore.

Professor Mark Cronin, Liverpool John Moores University

AOP networks with quantification and definition of KERs

AOPs are widely acknowledged to organise the mechanistic jigsaw puzzle for toxicology; the KEs are the jigsaw pieces and their inter-relationships join the pieces together. This relatively simplistic approach may be adequate for a simple linear representation of an AOP and indeed has been helpful for endpoints such as skin sensitisation. However, to model toxicology AOPs will need to embrace the growing concept of networks and that not all "connections" (i.e. KERs) are equal. We are already seeing AOP networks for organ level adverse effects and beyond, but innovation must go further than this to define and quantify the KERs in terms of response, species, dose, time, feedback, adaptation and all other relevant effects. With this innovation our AOP "jigsaw puzzle" turns into something which is functional and applicable to toxicology, whilst maintaining its mechanistic heritage, flexibility and transparency.

Events

- **EUROTOX**, 2 September 2018, Brussels, Belgium
  - CE course (Sunday 2 Sept) Creating quantitative adverse outcome pathways (AOPs)
  - SOT/EUROTOX debate (Monday 3 Sept) Adverse outcome pathways are the future for regulatory toxicology
  - Session 17 (Tuesday 4 Sept) Adverse outcome pathways and development of alternative methods

- **ESTIV**, 14 October 2018, Berlin, Germany: *AOPs – an essential tool for in vitro toxicology*
  - Preconference workshop: 'AOPs: an essential tool for *in vitro* toxicology' (Sunday 14 October)

- **4th International Congress on Toxicity Testing Alternatives and Translational Toxicology / 2nd Asian Congress on Alternatives**, 9 October, Guangdong, China
  - CE course (Tuesday 9 October) Introduction to AOP and hands-on training on AOP-wiki
  - Session 6 (Thursday 11 October) Mode of action and Adverse Outcome Pathways (AOPs)

Publication Highlights

Sakuratani Y, Horie M, Leinala E (2018). **Integrated Approaches to Testing and Assessment: OECD Activities on the Development and Use of Adverse Outcome Pathways and Case Studies.** *Basic & Clinical Pharmacology & Toxicology* Jan 9. doi: 10.1111/bcpt.12955.

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Leist M, Ghallab A, Graepel R *et al.* (2017). **Adverse outcome pathways: opportunities, limitations and open questions.** *Archives of Toxicology* 91:3477-3505. doi: 10.1007/s00204-017-2045-3.

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