

# Adverse Outcome Pathways: Will they deliver a superior alternative to animal testing?

By Dr Gill Langley on behalf of The Lush Prize

Animal testing is resource-intensive in terms of money, time and animal use, posing financial and ethical challenges. It is also highly fallible. Consequently it has become impossible to evaluate fully all the chemicals being developed or already on the market<sup>1</sup>.

Research to replace animal testing with non-animal alternatives, such as *in vitro*, *in chemico* and *in silico* approaches, has been underway for many years and has achieved considerable success. However, faster progress could be made with a transparent and systematic framework that provides an underlying rationale for developing and assessing non-animal models and tests, and reliably interpreting their data output. The Adverse Outcome Pathway (AOP) concept offers such a framework and, with it, the possibility of creating a genuinely 21st-century toxicology and risk assessment strategy that is fit for purpose.

Since 2012, the Lush Prize has awarded a range of annual prizes to encourage the replacement of animal tests in chemical toxicology. The research-based Lush Prizes focus on human toxicity pathways and 21st-century science. The Black Box Prize offers a £250,000 award, in any one year, for a key breakthrough in AOP research and application. It was awarded<sup>2</sup> for the first time in 2015, to leading scientists involved in developing and validating replacement tests applicable to the human AOP for skin sensitisation.

Developed from a briefing on AOPs to help guide the Lush Prize programme, this paper:

1. describes the AOP concept and how it helps replace animal tests in chemical toxicology;
2. discusses recent progress with AOPs;
3. assesses the confidence of the toxicology and regulatory communities in the AOP concept;
4. introduces the possibility of AOPs being applied to medical research and drug discovery;
5. considers what next steps are needed to progress the AOP approach.

## 1. The AOP concept and replacing animal tests in chemical toxicology

An AOP is a conceptual construct that describes a sequence of linked events in a toxicity pathway. The pathway starts at the molecular level and, via causal Key Events (KEs), progresses through different levels of biological organisation (i.e. cell, tissue, organ) to an adverse health outcome in an individual or population<sup>3</sup>.

The first step in an AOP is the Molecular Initiating Event (MIE), when a chemical first interacts with an organism. The AOP links the MIE to an Adverse Outcome (AO, e.g. illness, death) via a defined series of KEs that span the different scales of biological organisation. This progression through different biological levels is a notable feature of AOPs that distinguishes them from earlier toxicity pathway and mechanistic concepts.

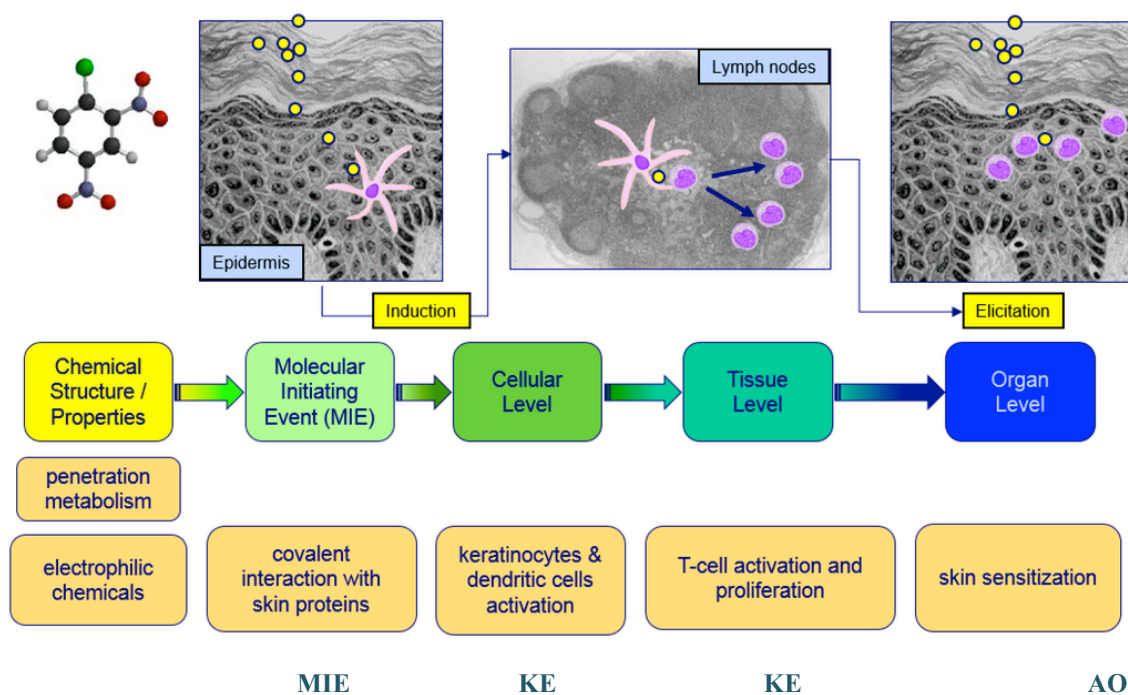
The skin sensitisation AOP was the first to receive endorsement by the Organisation for Economic Co-operation and Development (OECD) and is illustrated in Figure 1.

<sup>1</sup> Tollefsen KE, Scholz S, Cronin MT et al. 2014. Applying Adverse Outcome Pathways (AOPs) to support Integrated Approaches to Testing and Assessment (IATA). *Regul Toxicol Pharmacol.* 70(3):629-640. doi: 10.1016/j.yrtph.2014.09.009

<sup>2</sup> <http://lushprize.org/2015-prize/2015-prize-winners/>

<sup>3</sup> Ankley GT, Bennett RS, Erickson RJ et al. (2010) Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* 29:730-741

**Figure 1. The skin sensitisation AOP: Covalent protein binding leading to skin sensitisation (human allergic contact dermatitis)**



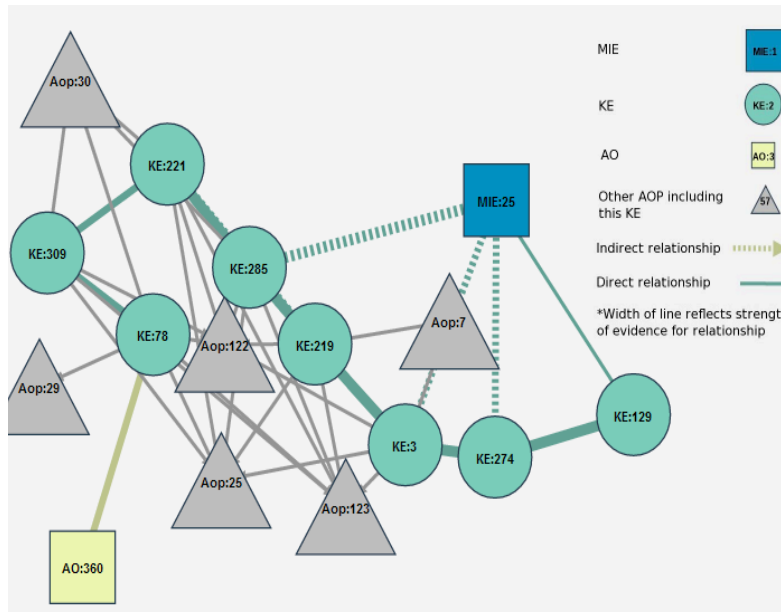
(Credit NC3Rs, based on a diagram in: OECD. 2012. The adverse outcome pathway for skin sensitisation initiated by covalent binding to proteins. OECD Series on Testing and Assessment No. 168. Part 1: Scientific Evidence. ENV/JM/MONO(2012)10/PART1. Paris: OECD.)

As there are many different toxicity pathways in humans, leading to numerous adverse outcomes, there will be many thousands of AOPs. One adverse effect may be caused by one AOP or many different ones, as toxicity pathways often overlap. KEs may be thus be related within and across different AOPs. This requires a lot of research, analysis and theoretical calculations to cover all the endpoints that are currently tested in animals. However, this also means that as more AOPs are identified, knowledge will grow faster than linearly, probably in an exponential way, as each new AOP is related to existing ones and so helps to build the bigger picture.

KE relationships (KERs) on different pathways and links between AOPs themselves will allow them to be interlinked into networks. This can already be seen in the OECD's AOP-Wiki: for example with AOP 23<sup>4</sup> as shown in Figure 2, below.

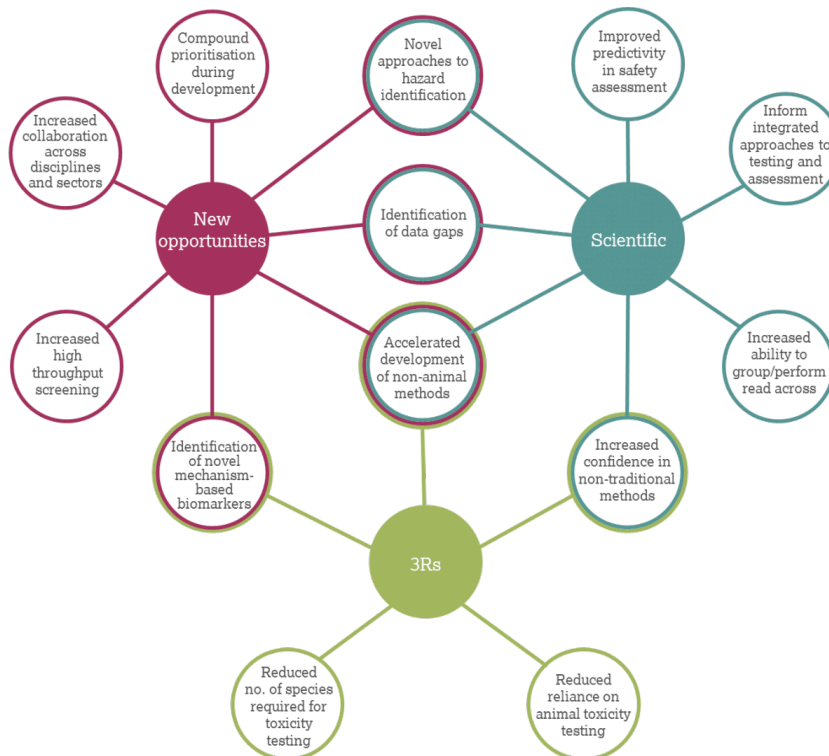
<sup>4</sup> [https://aopwiki.org/aops/23#network\\_view](https://aopwiki.org/aops/23#network_view)

**Figure 2. Network view of AOP 23 from the AOP-Wiki: Androgen receptor agonism leading to reproductive dysfunction**



The knowledge and understanding provided by AOPs have multiple applications that are expected to contribute to better safety evaluation and to replacing animal tests (Figure 3).

**Figure 3. Applications of AOPs in safety assessment and replacing animal tests**



(Credit NC3Rs)

These applications include:

- improving evidence-based hazard and risk assessments
- assisting the development of rational schemes (Integrated Approaches to Testing and Assessment, IATA) to assess hazard data from different tests, thereby maximising useful information from minimal testing
- increasing certainty of interpretation of existing as well as new information
- improving ability to predict toxicity of one chemical from data on another (with less/no need for testing), e.g. chemical read-across, structure/activity relationships
- identifying data gaps to better target human-relevant research
- accelerating the development and validation of non-animal methods
- providing a truly mechanistic basis for toxicity, allowing more computer predictions and fewer animal tests
- better and more rapid high-throughput screening for chemical prioritisation

It isn't necessary to wait for all the AOPs to be in place before those benefits start to be seen (see section 2, below). Even draft AOPs are useful, for example to assist in read-across. As each AOP is developed, independently peer-reviewed and iteratively improved, via the literature and the AOP Knowledge Base, it becomes reliable for and applicable to an expanding number of purposes. These range from focusing future research and developing new non-animal tests, right through to regulatory decision-making (which would require the highest levels of evidence and confidence).

As the OECD says<sup>5</sup>:

“Assessment of AOPs and evaluation of their suitability for application in different regulatory contexts relies in part on (1) the confidence and precision with which the KEs can be measured, (2) the level of confidence in the relationships between the KEs linked in an AOP based on biological plausibility, empirical support for the KER and consistency of supporting data and among different biological contexts, and (3) weight of evidence for the overall hypothesised pathway, taking into account a number of additional considerations”.

AOPs are more than just toxicity pathways. The AOP framework allows a new, systematic approach to collecting, organising and evaluating toxicity information. It enables toxic effects (adverse outcomes) to be understood as processes, in contrast to animal testing which reveals toxic effects in a species but explains nothing about underlying mechanisms. AOPs support non-animal methods by focusing on predicting adverse outcomes on the basis of the MIE and KEs, using mechanistic data generated by *in vitro*, *in chemico* and *in silico* techniques.

The validation and regulatory use of alternatives, whether they be lung-on-a-chip, human stem cells, or high-throughput assays, depend on the AOP framework to support the regulatory relevance and application of these new methods. Regulators have said that with an AOP behind it, methods will be more easily accepted as valid.

## 2. Recent progress with AOPs

The AOP concept was first articulated in 2010<sup>3</sup> and by 2012 only two to three scientific papers per year had referred to it. But by June 2017 the OECD had fully endorsed six AOPs, five of them with relevance to human health, as shown below in Table 1. 18 other AOPs are now under review at the OECD, indicating they're at a late stage in the endorsement process. These AOPs are for a range of

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<sup>5</sup> OECD. 2016. Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways, OECD Series on Adverse Outcome Pathways, No. 1, OECD Publishing, Paris. <http://dx.doi.org/10.1787/5jlv1m9d1g32-en>

toxic endpoints including embryo toxicity, epilepsy, learning and memory deficits, impaired fertility, liver tumours and others.

The AOP-Wiki, the global online resource launched in 2014, where AOPs are entered, freely shared and updated, already has a list of more than 241 AOPs<sup>6</sup>. These range from the simplest with only two KEs; to complex pathways with KEs that also feature in 13 related AOPs – close to a network of toxicity pathways.

**Table 1. OECD-endorsed AOPs (July 2017)**

<b>AOP title and its current/potential uses</b>	<b>AOP- Wiki no.</b>	<b>MIE</b>	<b>Adverse Outcome</b>	<b>Species data on which AOP based</b>	<b>OECD Project no.</b>
Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations Likely future application to humans	AOP no. 15	DNA alkylation	Offspring inherit mutations via father's sperm	Mouse, rat, hamster, fish	1.11
Aromatase inhibition leading to reproductive dysfunction (and population decrease) A high-confidence AOP suitable for use in regulatory and risk assessment applications for fish, & future applications for amphibians, reptiles and birds (i.e. ecotoxicology)	AOP no. 25	Aromatase Inhibition	Reproductive dysfunction	Fish	1.12
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. Can be used for <i>in vitro</i> assay development allowing screening of chemicals toxic to adult brain	AOP no. 48	Glutamate receptor agonism	Learning and memory impairment	Human, mouse, rat	1.23
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities Will assist development of new <i>in vitro</i> assays to assess chemicals that might impair children's cognitive function	AOP no. 13	N-methyl-D-aspartate receptor antagonism	Learning and memory impairment	Human, monkey, rat, mouse	1.22
Covalent Protein binding leading to Skin Sensitisation Has supported the development of methods for assessing chemical sensitisation hazard or potency in humans without animal testing	AOP no. 40	Covalent binding of protein	Skin sensitisation	Human, mouse	1.1
Protein Alkylation leading to Liver Fibrosis (caused by chemicals) Will aid identification/selection/development of <i>in vitro</i> methods and chemical read-across for untested	AOP no. 38	Protein alkylation	Liver fibrosis	Human, rat	1.14

<sup>6</sup> <https://aopwiki.org/>

chemicals, applicable to humans					
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Table 1 illustrates that AOPs for human health endpoints often include animal test data. This is partly because for many toxicity endpoints, sufficient mechanistic human data has not been available. In the past there's been very little biomonitoring of human populations to see how they've been affected by toxic exposures. However, newer research techniques such as genomic analysis in humans, as well as new human species-specific *in vitro* and *in silico* studies, will increasingly confirm whether or not the animal data incorporated in AOPs proves relevant for human health endpoints. If animal data is used in AOPs for human health endpoints, it is essential that it's proved to be valid and is used critically.

### **Two recent AOP success stories**

The skin sensitisation AOP (no. 40 in the AOP-Wiki, Table 1) was the first to be endorsed at OECD level and three non-animal tests based on KEs have been fully accepted for regulatory use in the OECD test guidelines<sup>7</sup>. Through its example, scientists and regulators are beginning to see how an AOP can lead to an IATA that they can have confidence in.

Can we point to other breakthroughs yet? Let's look at OECD-endorsed AOP 25, (see Table 1), which deals with aromatase inhibition leading to reproductive dysfunction. The normal function of aromatase is to convert testosterone to estradiol. Its inhibition by certain endocrine disrupter chemicals leads to reproductive dysfunction in female fish and consequent population crashes. The AOP has seven KEs involving different biological levels of the reproductive system. A series of computational simulation models, some of them reflecting dose/response aspects, have now been aligned with the published AOP<sup>8</sup>. One of the computational models accounts for feedback and adaptation/compensation in the system, in order to simulate complex relationships among the KEs as a function of dose and time.

The linked models could be used for quantitative risk assessment (i.e. understanding dose/response relationships), with the initial input based upon the degree of inhibition of aromatase by a test chemical, and a direct measurement of the MIE made using an *in vitro* test system. This is a relevant application of an AOP in the context of international endocrine disrupter screening and testing programmes such as at the OECD and US Environmental Protection Agency. The approach was recently used successfully to make quantitative predictions of fecundity of white sucker fish at a site impacted by a pulp and paper mill. The scientists who published this example in 2017 said<sup>8</sup>:

“We argue that the systematic organization of knowledge into AOP frameworks can inform and help direct the design and development of computational prediction models that can further enhance the utility of mechanistic and *in silico* data for chemical safety assessment.”

Another success in the field of endocrine disrupter testing based on AOPs involves AOP 42 in the AOP-Wiki, Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals, currently under review at the OECD. This AOP has already led to the development of a high-throughput screening assay to detect potential thyroperoxidase inhibition, based on a KE<sup>9</sup>. The new assay (so far not a replacement as it uses rat thyroid tissue *in vitro*) drastically reduces animal use, requiring one-tenth of the rat thyroid protein used for the standard assay whilst also giving dose/response information. It can be used to screen large numbers of chemicals as an integral component of a tiered endocrine-disrupter screening approach. AOP 42 will also inform quantitative structure/activity relationships and read-across models. It's expected to lead to systems

<sup>7</sup> [http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects\\_20745788](http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788)

<sup>8</sup> Wittwehr C, Aladjov H, Ankley G et al. 2017. How Adverse Outcome Pathways Can Aid the Development and Use of Computational Prediction Models for Regulatory Toxicology. *Toxicol Sci.* 155(2):326-336. doi: 10.1093/toxsci/kfw207

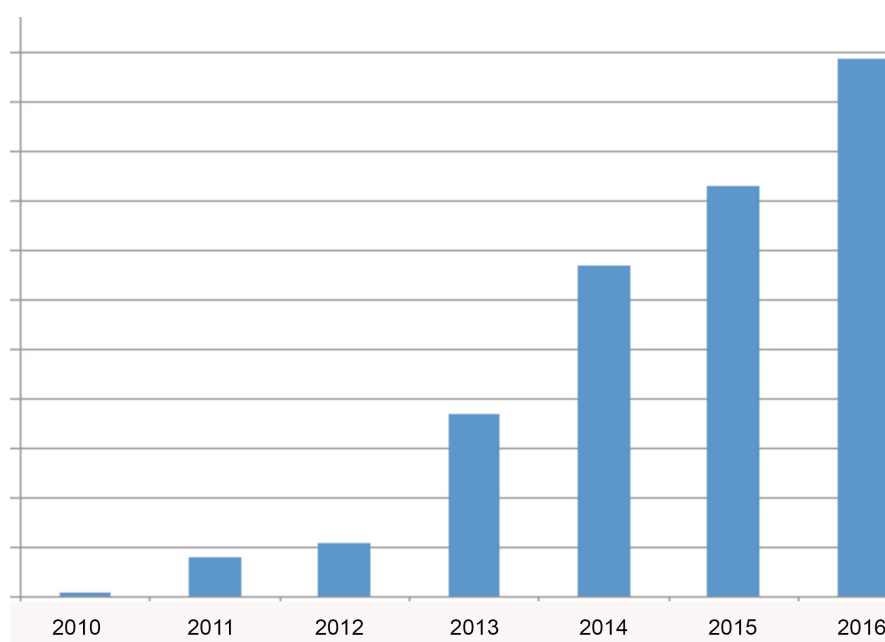
<sup>9</sup> Paul KB, Hedge JM, Rotroff DM et al. 2014. Development of a thyroperoxidase inhibition assay for high-throughput screening. *Chem. Res. Toxicol.* 27(3):387-99. doi: 10.1021/tx400310w

biology (computational) models that quantitatively simulate and predict cognitive outcomes from *in vitro* thyroperoxidase data.

### **Number of published papers on AOPs**

Another way to look at progress is to assess the number of scientific papers published year by year since the AOP concept was first described in 2010. PubMed was searched for published papers containing the text words “adverse outcome pathway” and “adverse outcome pathways”. The resulting Graph 1 shows exponential growth, suggesting that the AOP concept is gaining widespread traction.

**Graph 1. Scientific papers on AOPs published each year, 2010-2016**



In a recent paper, Burden and colleagues wrote<sup>10</sup>:

“It is clear that the well-considered use of AOPs has the potential to drive positive change in toxicology towards a reduced reliance on predictions made using vertebrate animal models and on the measurement of apical toxicity endpoints per se. The concept itself, and the widespread international uptake, is already stimulating the development of novel tools and screening methods and driving real innovation within human toxicology and ecotoxicology.”

Now that there are several AOPs for some related pathways (e.g. in endocrine and reproductive toxicity), people can begin to see how these pathways are becoming networks that have real biological relevance. More tangible examples are in process, including IATA for carcinogenicity and developmental toxicity that are in development by the OECD, the European Commission’s Joint Research Centre and others.

<sup>10</sup> Burden N, Sewell F, Andersen ME et al. 2015. Adverse Outcome Pathways can drive non-animal approaches for safety assessment. *J Appl Toxicol.* 35(9):971-975. doi: 10.1002/jat.3165

### 3. Do the toxicology and regulatory communities have confidence in AOPs?

In general, the AOP framework has been taken up by much of the toxicology community. This buy-in represents the thinking that AOPs are the first real concept of how to replace animals with a non-animal predictive toxicity system. The OECD is very much on board and resources are being dedicated to the AOP-Wiki. The US National Institutes of Health (NIH), the EPA and to some extent the Food and Drugs Administration (FDA) are active in the area: the EPA is working on AOPs and making updates to the Wiki. There's growing interest/support in other countries like China, Korea and Japan. There is very strong support for this kind of knowledge within the EU.

For example, an expert Panel of the European Food Safety Authority (EFSA) recently looked at data linking pesticide exposure to childhood leukaemia and Parkinson's disease, and developed some AOPs. They concluded<sup>11</sup>:

“The Panel supports the use of the AOP framework to scientifically and transparently explore the biological plausibility of the association between pesticide exposure and human health outcomes, identify data gaps, define a tailored testing strategy and suggests an AOP's informed Integrated Approach for Testing and Assessment (IATA).”

The NC3Rs recently surveyed academia, industry and regulators on the issue of AOPs<sup>12</sup>. Over 70% of respondents indicated that they were comfortable using AOP terminology. 86% said it was possible or likely they or their organisation would contribute to the OECD's AOP programme. The three key issues that would most encourage respondents to invest in AOP work were increased confidence in the potential benefits; regulatory buy-in and increased funding.

The following list shows just some of the organisations and projects in Europe, North America and internationally which are actively working on and supporting AOPs, through research, funding, holding workshops, organising training courses, developing and publishing AOPs, holding webinars, and more.

#### ***Some organisations and projects (in Europe, North America and internationally) supporting the AOP concept***

American Chemistry Council  
BioDetection Systems  
Center for Alternatives to Animal Testing (US and EU)  
Dow Chemical Company  
Environment Canada  
European Centre for Ecotoxicology and Toxicology of Chemicals  
European Food Safety Authority  
European Commission Directorate General Joint Research Centre  
EU-ToxRisk  
Evidence-based Toxicology  
Human Toxicology Project Consortium  
Humane Society International  
Humane Society of the United States  
International Life Sciences Institute - Health and Environmental Science Institute  
Norwegian Institute for Water Research  
Organisation for Economic Co-operation and Development (OECD)

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<sup>11</sup> EFSA Panel on Plant Protection Products and their residues (PPR). 2017. Investigation into experimental toxicological properties of plant protection products having a potential link to Parkinson's disease and childhood leukaemia. EFSA Journal 15(3). doi: 10.2903/j.efsa.2017.4691

<sup>12</sup> NC3Rs. 2016. Adverse Outcome Pathway News. Issue 4 December 2016



People for the Ethical Treatment of Animals  
Physicians Committee for Responsible Medicine  
Proctor and Gamble  
Society for the Advancement of Adverse Outcome Pathways  
Society of Environmental Toxicology and Chemistry  
Society of Toxicology  
ToxCast  
Toxicity Testing in the 21st Century (TT21C)  
UK National Centre for the Three Rs  
Unilever  
US National Center for Advancing Translational Sciences  
US National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)  
US Army Engineer Research and Development Center  
US Environmental Protection Agency  
US National Institutes of Health  
WHO International Programme on Chemical Safety

#### **4. Will AOPs make an impact in medical research and drug discovery?**

This is the next logical step and is an initiative being promoted by Humane Society International with its BioMed21 project, which has now held three scientific workshops (in Brussels, Brazil and the USA, the latter with US National Institutes of Health engagement). Senior regulatory and academic scientists have participated in these and the first workshop has already been published<sup>13</sup>. Although medical research is moving towards capturing mechanistic information about diseases – information which could inform an AOP-like approach – the medical research community is not yet familiar enough with the new concept. However, papers on this are starting to be published<sup>14,15,16</sup>, putting the developmental stage of this field roughly equivalent to 2010-2011 for toxicology AOPs (Graph 1).

An important step would be to develop ‘disease research roadmaps’ that focus on human biology-based models<sup>13</sup>. This would involve a gap analysis to identify what human pathophysiological knowledge is currently lacking in each disease area, and whether we have the technologies and models to acquire it. The AOP concept provides a framework for assembling existing knowledge, identifying those gaps and developing/adapting new techniques to fill them.

The obvious overlaps between toxicology AOPs and ‘disease AOPs’ will increasingly become familiar to medical researchers. For example, an AOP for drug-induced cholestasis (liver disease) – strictly speaking a toxicology AOP – was published in 2013. However, this AOP is highly relevant both to toxicology and liver disease research and illustrates the potential for the AOP concept to assist in understanding disease processes and identifying new drug targets.

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<sup>13</sup> Langley GR, Adcock IM, Busquet F et al. 2017. Towards a 21st-century roadmap for biomedical research and drug discovery: consensus report and recommendations. *Drug Discov Today*. 22(2):327-339. doi: 10.1016/j.drudis.2016.10.011

<sup>14</sup> Langley GR. 2014. Considering a new paradigm for Alzheimer's disease research. *Drug Discov Today*. 19(8):1114-24. doi: 10.1016/j.drudis.2014.03.013. Epub 2014 Mar 21

<sup>15</sup> Pistollato F, Ohayon EL, Lam A, et al. 2016. Alzheimer disease research in the 21st century: past and current failures, new perspectives and funding priorities. *Oncotarget*. 7(26):38999-39016. doi: 10.18632/oncotarget.9175

<sup>16</sup> Langley G, Austin CP, Balapure AK et al. 2015. Lessons from Toxicology: Developing a 21st-Century Paradigm for Medical Research. *Environ Health Perspect*. 123(11):A268-A272. doi: 10.1289/ehp.1510345

## 5. What next steps are needed to progress the AOP approach?

The ideal goal for the AOP concept is that it should facilitate the complete replacement of animal testing with advanced, non-animal approaches. While we're still a long way from that, there are clear directions that can be taken to bring this goal closer.

Views were solicited internationally from scientists and regulators as to the challenges that must be addressed to realise the full potential of the AOP framework in research and regulatory decision-making<sup>17</sup>. The main issues identified were AOP networks; quantitative AOPs; collaboration on and communication of AOP knowledge; AOP discovery and development; chemical and cross-species extrapolation; exposure/toxicokinetics considerations; and AOP applications.

There are some very practical questions relevant to the use of AOPs in a regulatory context that must be answered, including<sup>10</sup>: What are the weight-of-evidence considerations necessary before an AOP can be accepted? Could an AOP based solely on *in vitro* or computational data be acceptable as a basis for chemical risk assessments?

None of these challenges needs prove impossible to overcome: some are already being addressed, although others will be difficult. There are often precedents that can be adapted from related fields. One example is the proposed scientific confidence framework for evaluating AOPs for specific toxicology and regulatory purposes<sup>18</sup>. This recent proposal was based on a similar confidence framework developed for a prediction model for high-throughput screening for endocrine endpoints. In this way, developments in toxicology – and in medical research and drug development – interact and influence each other, aided by increased multi-disciplinary collaboration.

The traction already gained by the AOP concept is impressive. It could be that this is the step-change that toxicology has needed to make it truly fit for the 21st century.

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### *Acknowledgements*

Thanks to Troy Seidle, Gilly Stoddart, Kristie Sullivan and Kate Willett for their input.

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<sup>17</sup> LaLone CA, Ankley GT, Belanger SE et al. 2017. Advancing the adverse outcome pathway framework - An international horizon scanning approach. *Environ Toxicol Chem.* 2017 36(6):1411-1421. doi: 10.1002/etc.3805

<sup>18</sup> Patlewicz G, Simon TW, Rowlands JC et al. 2015. Proposing a scientific confidence framework to help support the application of adverse outcome pathways for regulatory purposes. *Regul Toxicol Pharmacol.* 71(3):463-477. doi: 10.1016/j.yrtph.2015.02.011