

# Chemical Research in Toxicology<sup>®</sup>

Subscriber access provided by UNIV OF CAMBRIDGE

Perspective

## A History of the Molecular Initiating Event

Timothy Eric Harry Allen, Jonathan M. Goodman, Steve Gutsell, and Paul J Russell

*Chem. Res. Toxicol.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.6b00341 • Publication Date (Web): 16 Nov 2016Downloaded from <http://pubs.acs.org> on November 17, 2016

### Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.



ACS Publications

Chemical Research in Toxicology is published by the American Chemical Society.  
1155 Sixteenth Street N.W., Washington, DC 20036  
Published by American Chemical Society. Copyright © American Chemical Society.  
However, no copyright claim is made to original U.S. Government works, or works  
produced by employees of any Commonwealth realm Crown government in the course  
of their duties.

# A History of the Molecular Initiating Event

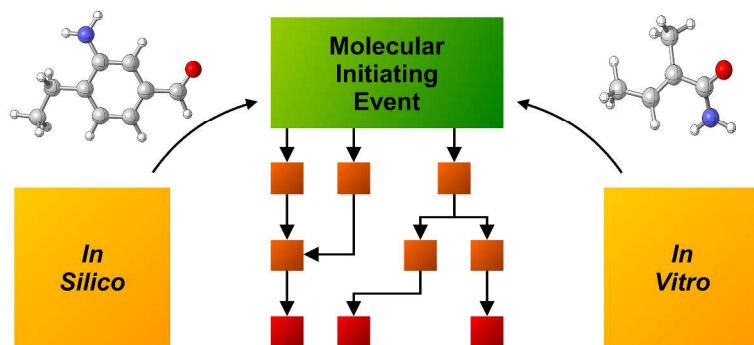
*Timothy E. H. Allen,<sup>†</sup> Jonathan M. Goodman,<sup>\*†</sup> Steve Gutsell,<sup>‡</sup> and Paul J. Russell<sup>‡</sup>*

<sup>†</sup>Centre for Molecular Informatics, Department of Chemistry, University of Cambridge,  
Lensfield Road, Cambridge, CB2 1EW, United Kingdom

<sup>‡</sup>Unilever Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook,  
Bedfordshire, MK44 1LQ, United Kingdom

KEYWORDS: Molecular Initiating Event (MIE), Adverse Outcome Pathway (AOP), Human  
Toxicology, Risk Assessment.

## Table of Contents Graphic



## ABSTRACT

The adverse outcome pathway (AOP) framework provides an alternative to traditional *in vivo* experiments for the risk assessment of chemicals. AOPs consist of a number of key events (KEs) linked by key event relationships (KERs) across a range of biological organization backed by scientific evidence. The first KE in the pathway is the molecular initiating event (MIE); the initial chemical trigger that starts an AOP. Over the last three years the AOP conceptual framework has gained a large amount of momentum in toxicology as an alternative to animal methods and so the MIE has come into the spotlight. What is an MIE? How can MIEs be measured or predicted? What research is currently contributing to our understanding of MIEs? With this review we outline answers to these key questions.

## INTRODUCTION

Toxicology risk assessment is undergoing a paradigm shift away from *in vivo* data and towards risk assessment frameworks that incorporate non-animal alternatives. Traditional *in vivo* toxicity studies form the basis of the majority of regulations globally that relate to assuring human and environmental safety. Increasingly however, there are societal, scientific and regulatory drivers to develop new ways of assuring safety that do not rely on data generation in animals. Rather than relying on apical toxicity endpoints in animals, new frameworks rely on an understanding of the mechanism of a chemical's toxicity in a relevant system which is thought to provide a more scientifically sound methodology on which to base risk assessment decisions.

1  
2  
3 The National Research Council of the United States (NRC) opened the field for discussion in  
4  
5 2007, in an attempt to make toxicity testing quicker, less expensive, and more relevant to human  
6  
7 exposures.<sup>1</sup> It pointed to a number of advances in the fields of biology and biotechnology that  
8  
9 make it possible to feed data into new risk assessment approaches. These include physiologically  
10  
11 based pharmacokinetic (PBPK) modelling methods, which provide a better understanding of how  
12  
13 a compound behaves inside the body, and how much of it is able to get to a site of action.<sup>2,3</sup>  
14  
15 Furthermore, additional understanding of biological processes and the holistic nature of biology  
16  
17 are being gained through -omics technologies<sup>4-6</sup> and systems biology.<sup>7,8</sup> *In silico* methods also  
18  
19 have their part to play in this paradigm shift. Informatics approaches such as (Quantitative)  
20  
21 Structure Activity Relationships ((Q)SARs) and Read-Across have the potential to make better  
22  
23 use of existing data and target required testing to bring down the volume of *in vivo* studies  
24  
25 required for a risk assessment.<sup>9,10</sup> In addition to these, the coordination of the international  
26  
27 scientific community, including collaboration between industry, academia and regulators, is of  
28  
29 great importance for the advance of toxicity testing.<sup>11,12</sup> These combined changes move  
30  
31 toxicology away from a predominantly observational craft towards a science based on  
32  
33 understanding.<sup>13</sup>  
34  
35  
36  
37  
38  
39  
40  
41

42 The adverse outcome pathway (AOP) framework for risk assessment is one approach to combine  
43  
44 *in silico*, *in vitro* and *in chemico* methods with existing data to offer an alternative to animal  
45  
46 experiments. Since it was first described in 2010, this framework has gained a lot of attention  
47  
48 within the toxicology community. The Molecular Initiating Event (MIE) is the initial chemical-  
49  
50 biological interaction that starts the AOP. Understanding, characterizing and predicting MIEs  
51  
52 will be of great importance to allow the AOP framework to realize its potential as a predictive  
53  
54 tool and this key interaction is the focus of this work. With this review we aim to cover key MIE  
55  
56  
57  
58  
59  
60

1  
2  
3 research between the description of the AOP framework in 2010 and the end of 2015, giving an  
4  
5 overview of how this concept has risen to prominence, and how it will aid toxicity risk  
6  
7 assessment of the future.  
8  
9

10  
11 This review is focused on the MIE within human health risk assessment, but MIEs are also  
12  
13 relevant to ecotoxicology. Many of the ideas presented are equally applicable to ecotoxicology  
14  
15 and human health, as the MIE occurs early in the AOP and can be common to a number of  
16  
17 species.  
18  
19

### 20 21 22 *Up to 2012* 23

24  
25 The first mention of a Molecular Initiating Event can be traced back to 2006. Schultz *et al*  
26  
27 identified plausible MIEs based on the covalent interaction of soft electrophiles and biological  
28  
29 molecules.<sup>14</sup> These interactions can lead to a number of toxicological endpoints including skin  
30  
31 sensitization, DNA damage and immunological responses. These MIEs are considered  
32  
33 appropriate targets for (Q)SAR modeling, and some models are presented linking molecular  
34  
35 reactivity at a thiol moiety to aquatic toxicity and respiratory irritation endpoints. These  
36  
37 reactivity-driven MIEs and their subsequent toxicity pathways were a key driver for the  
38  
39 development of the AOP framework.  
40  
41  
42  
43  
44

45  
46 The modelling of chemical interactions between electrophiles and biological macromolecules  
47  
48 was already established before the MIE. Work by Aptula and Roberts defined mechanistic  
49  
50 domains for reactive aquatic toxicants,<sup>15</sup> and the same mechanistic domains were later applied to  
51  
52 skin sensitizers.<sup>16</sup> This work due to a similarity in the MIE for these toxicological endpoints, the  
53  
54 covalent modification of proteins. This MIE can be broken down based on the type of  
55  
56 electrophilic chemistry that causes it. These categories were used as a basis for qualitative  
57  
58  
59  
60

1  
2  
3 mechanistic modelling (QMM), a type of local model based on linking toxic effects to  
4 physicochemical properties for a specific case rather than a global QSAR model encompassing a  
5 number of different reactivity domains and MIEs. These QMMs bring a transparent mechanistic  
6 basis to modelling, and a number have been developed since for different mechanistic categories  
7 leading to skin sensitization.<sup>17</sup> These models, based on the interpretation of mechanistic  
8 chemistry, can be thought of as the precursor to the MIE.  
9  
10  
11  
12  
13  
14  
15  
16  
17

18 The AOP framework was first outlined by Ankley *et al* in 2010.<sup>18</sup> Ankley presented the AOP as  
19 a conceptual framework, containing key information outlining the links between an MIE and an  
20 Adverse Outcome (AO) at a high level of biological organization relevant to risk assessment.  
21 Generalized examples of Key Events (KEs) along an AOP were presented by Ankley, including  
22 receptor/ligand interactions, DNA binding and protein oxidation. This conceptual framework is  
23 shown in Figure 1.  
24  
25  
26  
27  
28  
29  
30  
31  
32

33 A number of case examples from ecotoxicology were also presented, including pathways for  
34 narcosis, photo-activated toxicity, the aryl-hydrocarbon receptor, activation of the estrogen  
35 receptor and impaired vitellogenesis. It was noted that a complete AOP with full elucidation of  
36 all steps is not necessary for the AOP to be a useful tool. Gaps in an AOP can be filled using  
37 weight-of-evidence or statistical approaches to establish links between exposure and adverse  
38 outcomes. Much effort can be saved by specifically targeting areas of the AOP designated as  
39 important for the assessment of a specific endpoint. The AOP framework for risk assessment  
40 complies with the NRC's vision for the future of toxicity testing, allowing for the identification  
41 of specific endpoints of regulatory concern and providing understanding of the toxicity  
42 mechanisms that cause them.  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 This initial framework was extended by Villeneuve and Garcia-Reyero early in 2011,<sup>19</sup> who  
4 identified the importance of predictive methodology in the application of AOPs. MIEs are  
5 critical interactions that can be modelled to develop (Q)SARs for predicting the likelihood of a  
6 chemical interacting with a specific target. In an analogous manner the AOP highlights key  
7 toxicity pathway events that can be linked to *in vitro* tests, and so can be used to predict or test a  
8 pathway. These enable the AOP framework to build upon previous risk assessment approaches,  
9 as well as to bring new science to bear on the problems and challenges faced by toxicology.  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19

20  
21 The European funded initiative SEURAT-1<sup>20</sup> started in January 2011, with the aim of developing  
22 a replacement for repeat-dose, systemic-toxicity *in vivo* tests. The strategy was to combine the  
23 use of *in vitro* and *in silico* methodologies, through a number of linked projects, to deliver results  
24 applicable in both the pharmaceutical, and consumer goods/cosmetics spheres. Of the seven  
25 projects, the most relevant to MIEs are COSMOS<sup>21</sup> and ToxBank<sup>22</sup>. COSMOS is focused on the  
26 development of *in silico* open access tools for the prediction of systemic toxicity endpoints for  
27 cosmetic products. The ToxBank project is developing a web-based warehouse for systemic  
28 toxicity data, a database and repository for test compounds, and tissue and cell banks for *in vitro*  
29 tests.  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42

43 A first approach at linking the MIE to chemistry came from Enoch *et al* in 2011.<sup>23</sup> Enoch related  
44 the MIE to chemical category formation, using the fact that genotoxic chemicals are often  
45 electrophilically reactive, leading to the covalent modification of DNA. A number of mechanistic  
46 categories were developed to describe such MIEs, including acylation, Michael addition, Schiff  
47 base formation, S<sub>N</sub>1, S<sub>N</sub>2, and S<sub>N</sub>Ar. For each category, mechanistic and structural alerts were  
48 developed, combined into models know as *in silico* profilers, then assessed and discussed  
49 through a number of examples. Profilers such as these have been used to construct predictive  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



1  
2  
3 tools such as the OECD QSAR Toolbox.<sup>24</sup> Enoch noted that, while the predictions made using  
4 these methods were only as good as the toxicological data that was sourced to build them,  
5  
6 chemical categories are useful because they are mechanistically driven, and help to provide an  
7  
8 understanding of why a chemical is able to undergo a specific interaction. This work aligns well  
9  
10 with the MIE, as it too is mechanistically driven. This approach builds upon earlier work linking  
11  
12 electrophilic reactivity to human health endpoints and brings it into an MIE perspective.  
13  
14 Originally, an electrophilic index was used to predict skin sensitization potential quantitatively  
15  
16 using Read-Across QSAR techniques.<sup>25</sup> This was followed by the formation of electrophilic  
17  
18 chemical groups for low molecular weight chemical compounds known to cause respiratory  
19  
20 sensitization,<sup>26</sup> and these rules were later developed to link respiratory sensitization mechanisms  
21  
22 to the electrophilic index.<sup>27</sup> These studies were published before the introduction of the MIE and  
23  
24 AOP framework, but fit well with the ideas presented since, and conform to similar ideals.  
25  
26  
27  
28  
29  
30  
31

32  
33 A key step in defining the AOP, and terms used within it, came from the OECD in 2012,<sup>28</sup> when  
34  
35 it collected relevant definitions of important terms from the AOP and toxicity pathway research  
36  
37 frameworks. The OECD defined the AOP as a linear sequence of events from the exposure of an  
38  
39 individual to a chemical substance through to an understanding of the adverse (toxic) effect at  
40  
41 the individual level (for human health) or population level (for ecotoxicological endpoints). An  
42  
43 AOP consists of a number of KEs that are intermediate between the MIE and an apical adverse  
44  
45 outcome. These KEs must be toxicologically relevant to the apical outcome and experimentally  
46  
47 quantifiable. A number of similar, but distinctly different, definitions for the MIE were presented  
48  
49 by the OECD, including:  
50  
51  
52

- 53  
54  
55 • The initial point of chemical-biological interaction within the organism that starts the  
56  
57 pathway.<sup>29</sup>  
58  
59  
60

- Direct interaction of a chemical with specific biomolecules.<sup>19</sup>
- The molecular level, chemical-induced perturbation of a biological system.
- Chemical interaction at a molecular target leading to a particular adverse outcome.
- The seminal interaction (e.g. DNA-binding, protein oxidation, or receptor/ligand interaction) of a chemical with a biological target.

The OECD recognized that the development and elucidation of AOPs will require contributions of multiple scientific fields, covering chemistry, biology and toxicology. Standardization of terms used in AOPs is necessary to allow these fields to communicate and develop the framework into the future.

### ***Developments in 2013***

In March 2013, the 7th amendment to the cosmetics directive 76/7678/EEC,<sup>30</sup> and subsequent cosmetics regulation 1223/2009<sup>31</sup> came into effect in Europe, enacting a ban on the use of animal testing for cosmetic ingredients. At this time, the availability of alternatives to *in vivo* methods appropriate for cosmetic product risk assessment varied greatly by endpoint. The commission noted that good progress had been made in the area of *in vitro* alternatives to measure basal cytotoxicity.<sup>32</sup> In other areas, such as eye irritation and skin sensitization, more work is required to provide adequate alternatives. This important milestone, in the transition from toxicology dominated by *in vivo* methods to one accepting the non-animal alternatives of *in vitro*, *in silico* and *in chemico*, provides a key industrial incentive to the development of these alternatives, including the MIE and AOP.

In a study analogous to an MIE approach to carcinogenicity, Benigni *et al* reviewed and combined a number of 2D SARs.<sup>33</sup> Carcinogenicity is broken down by mechanism of action to

1  
2  
3 yield structural alerts for receptor binding, oxidative stress, hormonal imbalance, and direct and  
4  
5 indirect alkylation of DNA. Of nine structural alert classes across genotoxic and non-genotoxic  
6  
7 carcinogens, a positive predictivity of greater than 70% was obtained in six cases. This  
8  
9 computational methodology shows the promise of the integration of theoretical knowledge with  
10  
11 *in vitro* assay information. Benigni notes that the addition of further structural alerts will allow  
12  
13 for an expansion of this approach, increasing its impact.  
14  
15  
16

17  
18 Casalegno and Sello published a similar study predicting mode of action of environmental  
19  
20 toxicants by structural similarity.<sup>34</sup> These models were based not on experimental data, but on  
21  
22 structural features alone. This approach relies on the fact that the interaction between well-  
23  
24 characterized molecules and an ill-defined biological target depends on the chemistry of both  
25  
26 parts. Casalegno and Sello note that a lack of understanding of the modes of action associated  
27  
28 with ecotoxicology makes this a difficult study, as poorly defined modes of action, such as  
29  
30 narcosis or electrophilicity, are often used. Both a better understanding of the complex  
31  
32 mechanisms behind ecotoxicity and a greater availability of data are required to make an  
33  
34 approach such as this practically useful.  
35  
36  
37  
38

39  
40 Gutsell and Russell's 2013 analysis,<sup>35</sup> shows that chemistry is key to understanding the MIE and  
41  
42 also has other important roles to play in the AOP framework. Linking a defined dose of a  
43  
44 chemical compound to an adverse effect requires the use of both chemical information, including  
45  
46 analytical experiments, and theoretical techniques, such as (Q)SARs. An understanding of the  
47  
48 chemical attributes that are required to generate an MIE can be used to filter the number of  
49  
50 pathways that need to be considered in risk assessment. The MIE can be boiled down to a  
51  
52 chemical interaction, and so a series of *in chemico* experiments coupled with *in silico* models  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 will be able to make risk assessment, based on chemistry alone, a reality. A visualization of this  
4  
5 wet/dry cycle is shown in Figure 2.  
6  
7

8  
9 AOP approaches were reviewed in mid-2013 by Vinken.<sup>36</sup> This review covered the development  
10  
11 of AOPs from key building blocks, and several well-developed examples were discussed,  
12  
13 including skin sensitization, cholestasis, liver fibrosis and liver steatosis. Vinken also discussed  
14  
15 the key roles of (Q)SARs and *in vitro* tests to predict and to inform pathways. Key challenges in  
16  
17 the field are presented, including compliance with the complexity of toxicology, the inclusion of  
18  
19 dose-response relationships in pathway development, the integration of exposure and  
20  
21 toxicokinetic data, and the transparent and objective evaluation of the outcomes.  
22  
23  
24  
25

26  
27 Martin *et al* presented the use of mode of action to categorize aquatic toxicants and build  
28  
29 models.<sup>37</sup> This study involved modes of action including receptor agonism, enzyme inhibition,  
30  
31 chemical reactivity and biosystem disturbance. Martin notes that while the models provide  
32  
33 promising results (overall prediction accuracy 85%), further categorization of the more general  
34  
35 modes of action, such as chemical reactivity, into smaller structural-mechanism groupings (as  
36  
37 may be expected with a more MIE-like analysis) should improve predictivity.  
38  
39  
40

41  
42 A key issue, particularly in ecotoxicology, is the extrapolation of data points between species  
43  
44 during chemical risk assessment. This also applies to KEs within the AOP – including the MIE.

45  
46 LaLone *et al* described a quantitative tool for the extrapolation of MIEs between species using  
47  
48 bioinformatics approaches and an understanding of the target species biology.<sup>38</sup> The conservation  
49  
50 of KEs across several AOPs is an important simplification of biology within the AOP approach.  
51  
52  
53  
54 If some of these KEs can also be preserved across species, this will aid the difficult task of  
55  
56 ecotoxicological risk assessment, due to the diverse nature of the environment, and provide  
57  
58  
59  
60

1  
2  
3 additional data points in human toxicology, where results can be read across from existing  
4  
5 animal studies.  
6  
7

8  
9 An entirely quantitative risk assessment using the AOP framework was attempted by Maxwell *et*  
10  
11 *al* at the end of 2013.<sup>39</sup> The skin sensitization AOP was one of the first AOPs to be considered  
12  
13 well developed within the field.<sup>28</sup> Maxwell used KEs from early in the pathway to provide input  
14  
15 parameters for the “total haptenated protein” model, with the “CD8+ T cell response” model  
16  
17 providing predictions for KEs late in the pathway and for the overall adverse outcome (allergic  
18  
19 contact dermatitis).  
20  
21  
22

23  
24 Chemical dose applied to the skin is linked to the amount of total haptenated protein using  
25  
26 mathematical models, taking into account the pharmacokinetic steps as the chemical travels from  
27  
28 skin surface to the protein. These include clearance mechanisms, metabolism, and other chemical  
29  
30 transformations. The CD8+ T cell response model aims to predict the number of hapten-specific  
31  
32 human CD8+ central memory T cells generated following repeated exposure to a chemical  
33  
34 sensitizer, using inputs from the total haptenated protein model. Maxwell suggests that, once a  
35  
36 threshold of hapten-specific human CD8+ central memory T cells is exceeded, an inflammatory  
37  
38 response will manifest upon re-exposure to a sensitizer. These models were at the time in the  
39  
40 early stages of development. However, by benchmarking them against clinical data it should be  
41  
42 possible to predict whether a specific skin exposure will cause the required hapten-specific T cell  
43  
44 response required to cause an adverse effect upon re-exposure.  
45  
46  
47  
48  
49  
50

51 In November 2013, Wu *et al* published a decision tree to predict Developmental And  
52  
53 Reproductive Toxicity (DART) endpoints.<sup>40</sup> This *in silico* assessment was made without use of  
54  
55 an AOP framework and shows the complexity associated with predicting across multiple  
56  
57  
58  
59  
60

1  
2  
3 endpoints and toxicity pathways. Development of the decision tree identified a number of  
4  
5 important structural fragments that can enhance the understanding behind DART modes of  
6  
7 action using MIEs. The decision tree itself allows for rapid screening of potential DART.  
8  
9

### 10 11 *Developments in 2014*

12  
13  
14 In January 2014, Caldwell *et al* published an integrated approach for prioritizing pharmaceutical  
15  
16 ingredients for ecotoxicological risk assessment using an AOP-informed approach.<sup>41</sup> Mammalian  
17  
18 pharmacology data and pharmaceutical usage data were combined to provide a prioritization for  
19  
20 risk assessment and advanced research. Prioritization of assessment is particularly important in  
21  
22 ecotoxicology where the amount of data available to construct predictive models is often limited.  
23  
24  
25

26  
27 In 2014 the AOP Wiki was released as version 1.0.<sup>42</sup> This project represents a joint effort by the  
28  
29 European Commission - Joint Research Centre and the U.S. Environmental Protection Agency,  
30  
31 and serves as part of the OECD-sponsored AOP Knowledgebase. The AOP Wiki resource is  
32  
33 designed to allow toxicology scientists to share AOP-related knowledge in an appropriate format  
34  
35 in an open source forum. It also encourages the evaluation and acceptance of this research by the  
36  
37 AOP community. The AOP Wiki represents an important tool in data-sharing and peer-review.  
38  
39 Making research available through this wiki should be a priority for scientists working on AOPs  
40  
41 and MIEs.  
42  
43  
44  
45

46  
47 Computationally linking the reactivity of a chemical compound to its skin sensitization potential  
48  
49 has been a key goal of the AOP community as the skin sensitization AOP is one of the best  
50  
51 developed within the AOP Wiki. The MIE for skin sensitization is the covalent modification of a  
52  
53 skin protein by a toxicant. Building on their earlier work,<sup>15-17</sup> Roberts and Aptula linked  
54  
55 previously developed QMMs to local lymph node assay results for S<sub>N</sub>Ar electrophiles in 2014.<sup>43</sup>  
56  
57  
58  
59  
60

1  
2  
3 This resulted in the development of a linear relationship between reactivity parameters and EC3  
4 values measured experimentally. This analysis provides further evidence for the use of chemical  
5 reactivity as a descriptor for the analysis of the skin sensitization potential of novel molecules in  
6 the AOP framework.  
7  
8  
9  
10

11  
12  
13 Modern approaches to toxicology risk assessment must attempt to improve on existing *in vivo*  
14 methods. Leist *et al* highlighted this in 2014, pointing out that limited resources and high cost  
15 associated with animal methods have led to a large number of chemicals being untested, and  
16 mixtures are rarely evaluated at all.<sup>44</sup> Leist proposed a new vision, running chemicals and  
17 mixtures through *in silico* and *in vitro* approaches, using toxicity pathways, mode of action and  
18 AOP frameworks to gain a comprehensive evaluation. Only if no conclusive results can be  
19 obtained would animal testing be carried out. An understanding of the MIEs of these chemicals  
20 will aid in the generation of a comprehensive risk assessment of many chemicals without the use  
21 of *in vivo* experiments. A large number of under-tested chemicals and mixtures could be risk-  
22 assessed using this high-throughput method, as illustrated in Figure 3.  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37

38 Molecular modelling is one area of *in silico* science that shows much promise in its ability to  
39 study the MIE. One such approach was published by Tsakovska *et al* in 2014.<sup>45</sup> A model was  
40 developed for the PPAR $\gamma$  receptor as a target of interest for liver steatosis. The receptor binding  
41 pocket was analyzed using PPAR $\gamma$  complexes with full agonists from the Protein Data Bank  
42 (PDB),<sup>46</sup> and a pharmacophore was developed encompassing the most important features for  
43 binding and their role in PPAR $\gamma$  activation. As may be expected for a receptor binding MIE, the  
44 pharmacophore consists of hydrogen bonding, hydrophobic and aromatic interactions between  
45 the ligand and PPAR $\gamma$ . Models such as this provide *in silico* screens to identify potential  
46 steatogenic inducers early in risk assessment.  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 Following their previous work into reactivity-driven MIEs for DNA modification,<sup>23</sup> Nelms *et al*  
4 used a similar approach to develop a collection of structural alerts built into an *in silico* profiler  
5 for systemic toxicity of hair dyes.<sup>47</sup> Repeat dose toxicity studies for 94 hair dyes were used, and  
6 chemicals were grouped based on structural similarity. Four categories were identified for hair  
7 dyes containing a 2-nitroaminobenzene, a 4-nitroaminobenzene, an aromatic azo, or an  
8 anthraquinone moiety. Nelms went on to develop a mechanistic hypothesis for each of the four  
9 groups, and refined structural alerts were presented as an *in silico* profiler, covering pro-  
10 quinones, quinones, meta-substituted benzenes and aromatic azo compounds. This profiler  
11 assigned 56 of the 94 chemicals in the dataset to a mechanism-based chemical category. The  
12 reactivity-driven nature of these toxicity mechanisms may also allow the alerts to be used in  
13 mitochondrial toxicity, and this was also discussed by Nelms. This paper emphasizes that  
14 approaches like this do not attempt to predict oral repeat-dose toxicity, but instead a particular  
15 MIE that might be responsible for an AOP leading to chronic toxicity. Detailed studies such as  
16 this are required to develop models for MIEs, and to identify the MIEs themselves, as at this time  
17 they are generally poorly understood at a mechanistic level.

18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40 The workshop “Advancing AOPs for Integrated Toxicology and Regulatory Applications” took  
41 place in 2014. One report from this workshop by Tollefsen *et al* mentions the pivotal role of  
42 MIEs.<sup>48</sup> MIEs measured through *in silico*, *in vitro* and *in chemico* techniques were discussed and  
43 shown to have an important part to play in the AOP framework. MIEs cover a wide variety of  
44 chemical interactions, and these were highlighted through examples, including receptor binding  
45 MIEs leading to endocrine disruption and reproductive toxicity, as well as covalent binding to  
46 skin proteins leading to skin sensitization and allergic contact dermatitis, as has been discussed  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



1  
2  
3 above.<sup>28</sup> These discussions highlight the significance of the MIE and the important roles different  
4 areas of toxicology will play in gaining an understanding of these key interactions.  
5  
6

7  
8  
9 Chemistry's key role in MIE and AOP research was further highlighted by Allen *et al.*<sup>49</sup> AOP  
10 networks for a number of well-understood toxicants were developed using literature searches.  
11  
12 These case studies were used to unify existing definitions of the MIE and concluded that an MIE  
13 is the initial interaction between a molecule and a biomolecule or biosystem that can be causally  
14 linked to an outcome *via* a pathway. The role of the MIE in AOP research and toxicity risk  
15 assessment was also discussed, including a framework in which an *in silico* (Q)SAR relates  
16 molecular properties of a novel compound to an MIE, and any associated AOP can then infer an  
17 expected adverse outcome for the compound, as shown in Figure 4. By establishing an  
18 understanding of the chemistry behind interactions between molecules and biomolecules or  
19 biosystems (Q)SARs can be constructed, allowing the MIE to directly contribute to toxicity  
20 screening processes and, later, with further quantitative understanding, risk assessments.  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35

36 Madden *et al* published a report on the development of AOPs in ecotoxicology in late 2014,<sup>50</sup>  
37 noting that AOPs can receive criticism due to their simplistic nature which makes them poor  
38 reflections of complex toxicological processes. In response, they point out that an AOP should  
39 never be seen as a complete picture, but as a flexible tool that will improve over time as new data  
40 is constantly added. Even if an AOP is incomplete, it can still provide a large amount of useful  
41 information in a risk assessment. *In vitro* to *in vivo* extrapolation (IVIVE) of doses also presents  
42 a challenge due to the difference between *in vitro* experiments and *in vivo* situations. For  
43 example in an *in vitro* assay does not consider the metabolism of a chemical. Effective  
44 integration of data from *in chemico*, *in silico*, *in vitro* and *in vivo* will be required to overcome  
45 this, and the AOP Wiki,<sup>42</sup> among other resources,<sup>22,24,51,52</sup> provides a way to achieve this.  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 As has already been discussed,<sup>23,33,47</sup> structural alerts play a key part in predictive toxicology.  
4  
5 Chemotypes were introduced in 2014 as a new way of coding such alerts, and Chemotyper is  
6  
7 software that allows them to be searched against a dataset.<sup>53</sup> Chemical substructure mark-up  
8  
9 language (CSRML), an XML-based code, is used to capture chemotypes. This language allows  
10  
11 for the inclusion of physico-chemical properties and descriptors as well as 2D structure, allowing  
12  
13 greater flexibility of constructed models.  
14  
15  
16

### 17 18 ***Developments in 2015*** 19

20  
21 In February 2015, a report from FutureTox II (held in January 2014) was published by Knudsen  
22  
23 *et al*<sup>54</sup> The goals of this meeting were to discuss the progress towards the NRC's vision for  
24  
25 toxicity testing in the 21st century<sup>1</sup> and *in silico* and *in vitro* methodologies featured heavily.  
26  
27 AOPs were discussed as a key concept. Despite OECD guidelines which state that an AOP links  
28  
29 a single MIE and an effect at a high level of biological organization, it is accepted that a single  
30  
31 MIE may lead to several AOPs and an adverse effect may be associated with several MIEs. AOP  
32  
33 networks can capture the complexity of the biological sphere far better; and the MIE is identified  
34  
35 as an important KE that can be used to screen compounds to identify the AOPs that are likely to  
36  
37 be of most interest.  
38  
39  
40  
41  
42

43  
44 The difficulty associated with IVIVE has already been mentioned.<sup>50</sup> Angrish *et al* presented an  
45  
46 approach to bridging the IVIVE gap in high throughput screening (HTS) assays.<sup>55</sup> Using an  
47  
48 ultra-sensitive gas-phase probe molecule, they aim to measure effects on metabolism that will  
49  
50 link through to understood *in vivo* outcomes. As an example, the pharmacokinetic parameters  
51  
52 associated with a cytochrome-2A6-driven metabolism were identified in order to measure the  
53  
54 effect of a toxicant indirectly by observing a metabolite: methyl tertiary-butyl ether.  
55  
56  
57  
58  
59  
60

1  
2  
3 Methodology such as this allows MIEs and other KEs to be directly linked to relevant *in vivo*  
4 data.  
5  
6

7  
8  
9 Steinmetz *et al* utilized open-source data to construct a screening tool for retinoic acid receptor  
10 binding using the MIE principle.<sup>56</sup> 2D fragments of binders were coded in SMARTS based on  
11 “rules” derived from the PDB. In addition to this, physicochemical properties (vertex adjacency  
12 information magnitude,<sup>57</sup> number of rotational bonds, molecular weight and logarithm of the  
13 water-octanol partition coefficient) were used to give an insight into the physico-chemical  
14 applicability domain for retinoic acid receptor binding. These early approaches towards MIE  
15 tools are designed not to replace *in vitro* testing or be a complete *in silico* model, but rather to  
16 provide a rapid screen and prioritization methodology to assist in risk assessment.  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27

28  
29 The importance and potential impact of AOPs as a framework in non-animal risk assessment was  
30 reviewed by Burden *et al* in March 2015.<sup>58</sup> Key to the recent paradigm shift in toxicity testing is  
31 the Replacement, Refinement and Reduction of animal methods - also known as the 3Rs.<sup>59</sup> The  
32 AOP framework provides an important scientific basis on which new risk assessment practices  
33 can be built - as no single alternative testing method will replace an *in vivo* model like-for-like.  
34 One such combined approach could involve the prediction of adverse effects based on a pre-  
35 determined MIE. Concerted efforts to collect, integrate and organize data from relevant sources  
36 across scientific disciplines will be required to reap the benefits of such a coherent framework.  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48 Regulatory input is also required, to guide the use of AOPs in risk assessment decision-making.  
49  
50

51 While single AOPs are considered the building blocks of AOP development, they are not a  
52 complete representation of complex biological systems when considered in isolation. AOP  
53 networks would seem to provide a solution to this, as described by Knapen *et al*.<sup>60</sup> AOPs are  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 constructed from MIEs, KEs and AOs that can be shared across multiple pathways. Where these  
4  
5 common elements are shared, AOPs can be combined into AOP networks. This principle is  
6  
7 demonstrated in the case of DART, where aryl hydrocarbon receptor, estrogen receptor,  
8  
9 aromatase and androgen receptor-based MIEs lead to pathways that converge in DART  
10  
11 endpoints. This AOP network is shown in Figure 5. The analysis of networks such as this  
12  
13 conveys a number of advantages over considering AOPs in isolation. Not only are AOPs in  
14  
15 isolation unrealistic, by combining them into networks of assays, measurement of a single MIE  
16  
17 or KE can provide information on a number of different endpoints and pathways, improving  
18  
19 testing efficiency. The more understanding that can be gained about the MIEs, KEs, and their  
20  
21 networks the more potential combinations will come to light, further benefitting risk assessment.  
22  
23  
24  
25  
26

27  
28 Judging the quality of AOPs will be an important step in their development to become a staple of  
29  
30 the chemical risk assessment procedure. This is discussed by Becker *et al* who present a number  
31  
32 of examples using the OECD approved Bradford-Hill considerations for the assessment of  
33  
34 confidence in an AOP.<sup>61</sup> These considerations include the biological plausibility and empirical  
35  
36 evidence (i.e. dose-response) for KE relationships (KERs), and the essentiality of KEs (i.e. are  
37  
38 downstream events prevented if an upstream event is blocked?). Each consideration is broken  
39  
40 down, with the aim being to assign a high, moderate or low confidence in each KE or KER.  
41  
42  
43

44 Several case studies are given and the importance of using weight-of-evidence-based approaches  
45  
46 to analyze AOPs discussed. To ensure transparency and promote consistency across AOP  
47  
48 research, these kinds of confidence assessments must be carried out and analyzed. This  
49  
50 ultimately will lead to enhanced rigor, transparency and reproducibility for AOP confidence  
51  
52 assessment, improving confidence in AOPs themselves.  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 This particular area of AOP research has been further explored by Yauk *et al*<sup>62</sup> The AOP for  
4 alkylation of DNA in male premeiotic germ cells leading to heritable mutations was examined in  
5 depth in order to establish its biological plausibility and the empirical evidence that supports it.  
6  
7

8  
9  
10 In turn each KE was considered, examining how the KE works and how it was measured or  
11 detected experimentally. KERs are then examined, looking at how the biological processes work,  
12 the weight of evidence that supports them, any uncertainties or inconsistencies in their  
13 supporting data, and the level of quantitative understanding of the linkage they provide.  
14  
15

16  
17  
18 Following this analysis the KEs and KERs are assessed as having strong, moderate, or low levels  
19 of confidence. A particular challenge, noted by Yauk, in this AOP development was the  
20 gathering of appropriate dose-response data for assessment of the Bradford-Hill considerations  
21 from historical studies that were not conducted with this type of research in mind and which are  
22 time-consuming to interpret. The AOP framework should be able to overcome this hurdle, since  
23 the more KEs and KERs are isolated, the more likely it is that new AOPs will incorporate KEs  
24 and KERs which have already been characterized, and so the process will speed up.  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36

37 Dent *et al* discussed the AOP for anti-androgenic activity in humans in a similar level of detail.<sup>63</sup>

38  
39 The aim was to establish the status of the tools and approaches being put towards a non-animal  
40 risk assessment for this AOP. A particular point noted here was the high level of uncertainty  
41 associated with the extrapolation of data from effects seen in high dose animal studies to the  
42 much lower exposures anticipated for humans. Therefore, the exposure of the human to the  
43 chemical will be key. If human exposure is not significant, is a detailed risk assessment  
44 necessary at all?  
45  
46  
47  
48  
49  
50  
51  
52

53  
54  
55 Risk assessments for metals pose a number of different issues compared to those for organic  
56 chemicals. Von Stackelberg *et al* used an AOP approach to analyze the neurodegeneration  
57  
58  
59  
60

1  
2  
3 endpoint caused by the exposure of humans to metals and mixtures of metals, specifically lead,  
4  
5 arsenic, cadmium and manganese.<sup>64</sup> An AOP was developed and assessed using the Bradford-  
6  
7 Hill considerations. This AOP linked the activation of extracellular-signal-related kinase by  
8  
9 metals to an increase in levels of cellular calcium ions and neurological disorders including  
10  
11 deficits in learning and cognition. It is important that MIE and AOP approaches can be utilized  
12  
13 in these different situations to increase their impact in toxicology.  
14  
15  
16

17  
18 Following on from previous work into *in silico* models for reactivity driven MIEs for DNA  
19  
20 modification<sup>23</sup> and skin sensitisation<sup>47</sup>, Nelms *et al* published the development of an *in silico*  
21  
22 profiler for analyzing mitochondrial toxicity in June 2015.<sup>65</sup> Category formation based on  
23  
24 structural similarity was performed on a set of pharmaceutical drugs with mitochondrial toxicity  
25  
26 data. Once categories had been decided, a literature search was undertaken to elucidate  
27  
28 mechanistic information behind the MIE, and other KEs in AOPs associated with mitochondrial  
29  
30 disruption. Structural alerts were defined and coded in SMARTS using information from the  
31  
32 literature and substructures from the category formation. The structural alerts highlight  
33  
34 molecules that exhibit toxicity as protonophores, redox cyclers, and inhibitors of the complexes  
35  
36 of the electron transport chain. With a small amount of data available, this study has been able to  
37  
38 gain insight into several key MIEs for mitochondrial dysfunction, and provide a profiler that can  
39  
40 be utilized to screen large data sets to identify chemicals with the potential to induce  
41  
42 mitochondrial toxicity.  
43  
44  
45  
46  
47  
48

49  
50 A number of AOPs leading to hepatic toxicity were compiled by Mellor *et al* in late 2015.<sup>66</sup> This  
51  
52 work links the agonism and/or antagonism of several nuclear receptors to hepatic steatosis *via* a  
53  
54 number of converging pathways. These pathways are currently reasonably well understood from  
55  
56  
57  
58  
59  
60

1  
2  
3 a biological standpoint, but further research is required to elucidate key information about the  
4  
5 MIE and build structural alerts or other models to be able to predict this endpoint.  
6  
7

8  
9 Difficulty in understanding MIEs can be aided by finding new ways to measure them, and this  
10  
11 was demonstrated by Sanderson *et al* using NMR spectroscopy.<sup>67</sup> The binding of molecules to  
12  
13 protein residues features in MIEs for skin sensitization and hepatotoxicity among others. The rate  
14  
15 of this reaction could be used to inform toxicological risk assessments quantitatively, providing  
16  
17 an *in chemico* link between molecule and extent of protein modification. NMR spectroscopy was  
18  
19 used to measure these rates for the reactions of electrophilic organic chemicals (representing  
20  
21 different mechanistic classes) and simple amines and thiols (representing lysine and cysteine  
22  
23 protein side chains respectively). *In chemico* assays, such as this, will be important in informing  
24  
25 MIE and AOP driven toxicological risk assessment, providing vital quantitative information and  
26  
27 experimental validation.  
28  
29  
30  
31  
32

33  
34 Constant development of new AOPs has led to the discovery and characterization of new MIEs,  
35  
36 for example, the chemicals binding to tubulin identified by Marchetti *et al* in late 2015.<sup>68</sup> This  
37  
38 MIE leads to aneuploidy offspring – a teratogenic disorder causing an abnormal number of  
39  
40 chromosomes. The majority of data for the MIE and KEs of this AOP come from rodent studies.  
41  
42 They are thought to be conserved in humans, allowing the extrapolation of the existing *in vivo*  
43  
44 data for use in human toxicology. Similarities in the mechanism of action across several phyla  
45  
46 and the high homology between mouse and human tubulin provide evidence for this. This MIE  
47  
48 presents an interesting case, in which *in silico*, *in chemico*, *in vitro* and *in vivo* studies may all be  
49  
50 able to be used to predict the impact of an MIE in humans.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 The main drive in the development of AOPs up to this point has been for the risk assessment of  
4  
5 cosmetic products and industrial chemicals. AOPs can also find use in drug development, as was  
6  
7 discussed by Patlewicz and Fitzpatrick in December 2015.<sup>69</sup> They focused mainly on the use of  
8  
9 *in silico* tools, particularly (Q)SARs, for the prediction of KEs along an AOP. While a number of  
10  
11 tools, some of which we have already discussed, have been developed previously, the number of  
12  
13 KEs requiring prediction will continue to demand more from (Q)SAR scientists. It is particularly  
14  
15 highlighted that AOPs must be kept in mind during the development of new (Q)SARs,  
16  
17 promoting the development of tools which model small steps between KEs and not large leaps  
18  
19 over many complex levels of biological organization.  
20  
21  
22  
23  
24

25  
26 At the end of 2015 the SEURAT-1 initiative closed and the EU-ToxRisk program,<sup>70</sup> was initiated  
27  
28 early in 2016 to continue driving mechanism-based toxicity testing for risk assessment in the  
29  
30 21st century.  
31  
32

## 33 CONCLUSIONS

34  
35  
36  
37 As toxicology moves away from animal experiments and towards *in silico*, *in vitro* and *in*  
38  
39 *chemico* methods, as well as combinations of these, deeper understanding and new tools are  
40  
41 required. The AOP framework provides a powerful method to do this. As arguably the most  
42  
43 important key event within the AOP, a greater knowledge of MIEs will be vital to the success of  
44  
45 this framework. A greater understanding of the MIE must be developed, and tools must be  
46  
47 constructed to allow MIEs to be predicted, and measured for novel compounds.  
48  
49  
50

51  
52 The MIE sits on the boundary between biology and chemistry, and as such both these fields have  
53  
54 important roles to play in its development. This has been shown in this review, with a number of  
55  
56  
57  
58  
59  
60



1  
2  
3 publications coming from both a chemical and biological background. *In silico*, *in vitro* and *in*  
4  
5 *chemico* methods all have a part to play, and this is also represented.  
6  
7

8  
9 As the first KE in an AOP, the MIE has a key role to play in the understanding of toxicity in both  
10  
11 humans and animals. As a result the MIE is applicable in both human health and ecotoxicology  
12  
13 risk assessment. The MIE and AOP provide a template for the coming together of these fields, as  
14  
15 it is expected that some, but not all, of the KEs and KERs will be shared across species. One of  
16  
17 the main drives of the AOP framework is for better risk assessment procedures, and this  
18  
19 collaboration can help by providing additional data and understanding previously confined to  
20  
21 their fields.  
22  
23

24  
25 As outlined above a number of MIEs have now been characterized. This allows us to begin  
26  
27 grouping these interactions based on their chemistry. The earliest studied MIEs were covalent  
28  
29 bond forming reactions between chemicals and biological molecules. These MIEs include the  
30  
31 reactions of electrophilic chemicals with DNA molecules,<sup>23</sup> and the covalent modification of  
32  
33 skin proteins.<sup>43</sup> MIEs such as these can lead to toxicological endpoints such as allergic contact  
34  
35 dermatitis,<sup>39</sup> genotoxicity, and immunological disorders.<sup>39</sup>  
36  
37  
38  
39  
40

41  
42 A second category of MIEs is those which involve non-covalent binding of a chemical to a  
43  
44 biological target such as a receptor or enzyme. These can include the activation of PPAR $\gamma$ ,<sup>45</sup> or  
45  
46 the agonism or antagonism of nuclear receptors,<sup>66</sup> both leading to liver steatosis, the inhibition  
47  
48 of complexes of the mitochondrial electron transport chain leading to mitochondrial  
49  
50 dysfunction,<sup>65</sup> and the binding of chemicals to tubulin leading to teratogenic endpoints.<sup>68</sup>  
51  
52  
53

54  
55 A final category of MIEs is for those chemicals that do not directly interact with a specific  
56  
57 biomolecule, but rather cause a disturbance in cellular or organelle biosystems. Biosystem  
58  
59  
60

1  
2  
3 disturbance has been alluded to in several cases<sup>37,49</sup> and provides an MIE category for the  
4  
5 narcosis mode of action often used in ecotoxicology which is associated with membrane  
6  
7 disruption.<sup>34</sup> Other biosystem disturbance MIEs include chemicals which act as protonophores or  
8  
9 redox cyclers leading to mitochondrial toxicity endpoints.<sup>65</sup>  
10  
11

12  
13  
14 The impact of the MIE and AOP framework has undoubtedly increased over the last three years,  
15  
16 with the number of publications contributing to the area increasing each year. The understanding  
17  
18 gained, and tools developed so far, represent an important platform for future development, with  
19  
20 the ultimate aim being a tool for quantitatively predicting the impact of a chemical on a human  
21  
22 or ecotoxicological target. The emergence of the MIE highlights the idea that chemical  
23  
24 understanding is critical in modelling, and that mechanistic local modelling is key to providing  
25  
26 the best predictions. Feeding an MIE prediction tool into a combined AOP framework,  
27  
28 incorporating exposure, absorption, distribution, metabolism and excretion (ADME), and an  
29  
30 understanding of downstream effects along the AOP will be able to provide a genuine alternative  
31  
32 for toxicology: reliable risk assessment entirely free of *in vivo* experiments.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 AUTHOR INFORMATION  
4  
5

6 **Corresponding Author**  
7

8  
9 \*Centre for Molecular Informatics, Department of Chemistry, University of Cambridge,  
10  
11 Lensfield Road, Cambridge, CB2 1EW, United Kingdom. [Tel: +441223 336434](tel:+441223336434). E-mail:  
12  
13 [jmg11@cam.ac.uk](mailto:jmg11@cam.ac.uk).  
14  
15

16  
17 **Author Contributions**  
18

19  
20 The manuscript was written through contributions of all authors. All authors have given approval  
21  
22 to the final version of the manuscript.  
23  
24

25 **Funding Sources**  
26

27  
28 The authors acknowledge the financial support of Unilever.  
29  
30

31 **Data Statement**  
32

33  
34 According to the University of Cambridge data management policy, all the data used in this  
35  
36 paper is available either in the paper or in the SI. A copy of the data is also available in the  
37  
38 University of Cambridge repository at: <https://www.repository.cam.ac.uk/>  
39  
40  
41

42 **ABBREVIATIONS**  
43

44 AHR, aryl hydrocarbon receptor; AOP, adverse outcome pathway; CRSML, chemical  
45  
46 substructure mark-up language; DART, developmental and reproductive toxicity; E2, estradiol ;  
47  
48 GtH, gonadotrope hormone; HTS, high throughput screening; IVIVE, *in vivo in vitro*  
49  
50 extrapolation; KE, key event; KER, key event relationship; MIE, molecular initiating event;  
51  
52 NRC, national research council; PDB, protein data bank; QMM, quantitative mechanistic model;  
53  
54 (Q)SAR, (quantitative) structure activity relationship; T, testosterone; VTG, vitellogenin.  
55  
56  
57  
58  
59  
60

## REFERENCES

- (1) Committee on Toxicity Testing and Assessment of Environmental Agents and National Research Council. (2007) Toxicology Testing in the 21st Century: A Vision and a Strategy.
- (2) Andersen, M. E., Clewell, H. J., Gargas, M. L., Smith, F. A., and Reitz, R. H. (1987) Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.* 87, 185–205.
- (3) Sexton, K., Reiter, L. W., and Zenick, H. (1995) Research to strengthen the scientific basis for health risk assessment: a survey of the context and rationale for mechanistically based methods and models. *Toxicology* 102, 3–20.
- (4) Aardema, M. J., and MacGregor, J. T. (2002) Toxicology and genetic toxicology in the new era of “toxicogenomics”: impact of “-omics” technologies. *Mutat. Res.* 499, 13–25.
- (5) MacGregor, J. T. (2003) The future of regulatory toxicology: impact of the biotechnology revolution. *Toxicol. Sci.* 75, 236–248.
- (6) Pognan, F. (2004) Genomics, proteomics and metabonomics in toxicology: hopefully not “fashionomics.” *Pharmacogenomics* 5, 879–893.
- (7) Kitano, H. (2002) Systems biology: a brief overview. *Science.* 295, 1662–1664.
- (8) Henry, C. J. (2003) Evolution of Toxicology for Risk Assessment. *Int. J. Toxicol.* 22, 3–7.
- (9) Auer, C. M., Nabholz, J. V., and Baetcke, K. P. (1990) Mode of Action and the assessment of chemical hazards in the presence of limited data: Use of Structure-Activity Relationships (SAR) under TSCA, Section 5. *Environ. Health Perspect.* 87, 183–197.
- (10) Höfer, T., Gerner, I., Gundert-Remy, U., Liebsch, M., Schulte, A., Spielmann, H., Vogel, R., and Wettig, K. (2004) Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation. *Arch. Toxicol.* 78, 549–564.
- (11) Hartung, T. (2009) A Toxicology for the 21st Century—Mapping the Road Ahead. *Toxicol. Sci.* 109, 18–23.

- 1  
2  
3 (12) Hartung, T. (2009) Toxicology for the twenty-first century. *Nature* 460, 208–212.  
4  
5  
6 (13) Leist, M., Hartung, T., and Nicotera, P. (2008) The Dawning of a New Age of Toxicology.  
7  
8 *ALTEX* 25, 103–114.  
9  
10 (14) Schultz, T. W., Carlson, R. E., Cronin, M. T. D., Hermens, J. L. M., Johnson, R., O'Brien,  
11  
12 P. J., Roberts, D. W., Siraki, A., Wallace, K. B., and Veith, G. D. (2006) A conceptual  
13  
14 framework for predicting the toxicity of reactive chemicals: modeling soft electrophilicity. *SAR*  
15  
16 *QSAR Environ. Res.* 17, 413–428.  
17  
18 (15) Aptula, A. O., and Roberts, D. W. (2006) Mechanistic applicability domains for nonanimal-  
19  
20 based prediction of toxicological end points: general principles and application to reactive  
21  
22 toxicity. *Chem. Res. Toxicol.* 19, 1097–1105.  
23  
24 (16) Roberts, D. W., Patlewicz, G., Kern, P. S., Gerberick, F., Kimber, I., Dearman, R. J., Ryan,  
25  
26 C. A., Basketter, D. A., and Aptula, A. O. (2007) Mechanistic applicability domain classification  
27  
28 of a local lymph node assay dataset for skin sensitization. *Chem. Res. Toxicol.* 20, 1019–1030.  
29  
30 (17) Roberts, D. W., Aptula, A. O., and Patlewicz, G. Y. (2011) Chemistry-based risk  
31  
32 assessment for skin sensitization: quantitative mechanistic modeling for the S(N)Ar domain.  
33  
34 *Chem. Res. Toxicol.* 24, 1003–1011.  
35  
36 (18) Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D.,  
37  
38 Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrano, J. A., Tietge, J. E., and  
39  
40 Villeneuve, D. L. (2010) Adverse outcome pathways: A conceptual framework to support  
41  
42 ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* 29, 730–741.  
43  
44 (19) Villeneuve, D. L., and Garcia-Reyero, N. (2011) Vision & strategy: Predictive  
45  
46 Ecotoxicology in the 21st century. *Environ. Toxicol. Chem.* 30, 1–8.  
47  
48 (20) SEURAT-1 initiative, <http://www.seurat-1.eu/>  
49  
50 (21) COSMOS Project, <http://www.cosmostox.eu/home/welcome/>  
51  
52 (22) ToxBank, [http://wiki.toxbank.net/w/index.php/Main\\_Page](http://wiki.toxbank.net/w/index.php/Main_Page)  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 (23) Enoch, S. J., Cronin, M. T. D., and Ellison, C. M. (2011) The use of a chemistry-based  
4 profiler for covalent DNA binding in the development of chemical categories for read-across for  
5 genotoxicity. *ATLA* 39, 131–145.  
6  
7

8  
9  
10 (24) The OECD QSAR Toolbox,  
11 <http://www.oecd.org/chemicalsafety/riskassessment/theoecdqsartoolbox.htm>  
12

13  
14 (25) Enoch, S. J., Cronin, M. T. D., Schultz, T. W., and Madden, J. C. (2008) Quantitative and  
15 mechanistic read across for predicting the skin sensitization potential of alkenes acting via  
16 Michael addition. *Chem. Res. Toxicol.* 21, 513–520.  
17

18  
19  
20 (26) Enoch, S. J., Roberts, D. W., and Cronin, M. T. D. (2009) Electrophilic reaction chemistry  
21 of low molecular weight respiratory sensitizers. *Chem. Res. Toxicol.* 22, 1447–1453.  
22

23  
24  
25 (27) Enoch, S. J., Roberts, D. W., and Cronin, M. T. D. (2010) Mechanistic category formation  
26 for the prediction of respiratory sensitization. *Chem. Res. Toxicol.* 23, 1547–1555.  
27

28  
29  
30 (28) OECD. Proposal for a Template, and Guidance on Developing and Assessing the  
31 Completeness of Adverse Outcome Pathways, Appendix I, Collection of Working Definitions.  
32 <http://www.oecd.org/chemicalsafety/testingofchemicals/49963576.pdf>.  
33

34  
35  
36 (29) OECD. (2011) Report of the Workshop on Using Mechanistic information in Forming  
37 Chemical Categories. OECD Environment, Health and Safety Publications Series on Testing and  
38 Assessment No.138.  
39

40  
41  
42 (30) The 7th Amendment to the Cosmetics Directive (2003),  
43 [http://ec.europa.eu/consumers/sectors/cosmetics/files/doc/antest/\(2\)\\_executive\\_summary\\_en.pdf](http://ec.europa.eu/consumers/sectors/cosmetics/files/doc/antest/(2)_executive_summary_en.pdf)  
44

45  
46  
47 (31) Regulation 1223/2009 Cosmetics Regulation, Regulation (EC) No 1223/2009 on cosmetic  
48 products (2009),  
49 <http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:342:0059:0209:en:PDF>  
50

51  
52  
53 (32) Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K., and Walum, E. (1989)  
54 MEIC-A new international multicenter project to evaluate the relevance to human toxicity of in  
55 vitro cytotoxicity tests. *Cell Biol. Toxicol.* 5, 331–347.  
56  
57  
58  
59  
60

1  
2  
3 (33) Benigni, R., Bossa, C., and Tcheremenskaia, O. (2013) Nongenotoxic carcinogenicity of  
4 chemicals: Mechanisms of action and early recognition through a new set of structural alerts.  
5 *Chem. Rev.* 113, 2940–2957.  
6  
7

8  
9  
10 (34) Casalegno, M., and Sello, G. (2013) Determination of toxicant mode of action by  
11 augmented top priority fragment class. *J. Chem. Inf. Model.* 53, 1113–1126.  
12

13  
14 (35) Gutsell, S., and Russell, P. (2013) The role of chemistry in developing understanding of  
15 adverse outcome pathways and their application in risk assessment. *Toxicol. Res.* 2, 299–307.  
16

17  
18 (36) Vinken, M. (2013) The adverse outcome pathway concept: A pragmatic tool in toxicology.  
19 *Toxicology* 312, 158–165.  
20  
21

22  
23 (37) Martin, T. M., Grulke, C. M., Young, D. M., Russom, C. L., Wang, N. Y., Jackson, C. R.,  
24 and Barron, M. G. (2013) Prediction of aquatic toxicity mode of action using linear discriminant  
25 and random forest models. *J. Chem. Inf. Model.* 53, 2229–2239.  
26  
27

28  
29 (38) LaLone, C. A., Villeneuve, D. L., Burgoon, L. D., Russom, C. L., Helgen, H. W.,  
30 Berninger, J. P., Tietge, J. E., Severson, M. N., Cavallin, J. E., and Ankley, G. T. (2013)  
31 Molecular target sequence similarity as a basis for species extrapolation to assess the ecological  
32 risk of chemicals with known modes of action. *Aquat. Toxicol.* 144–145, 141–154.  
33  
34

35  
36 (39) Maxwell, G., MacKay, C., Cubberley, R., Davies, M., Gellatly, N., Glavin, S., Gouin, T.,  
37 Jacquilleot, S., Moore, C., Pendlington, R., Saib, O., Sheffield, D., Stark, R., and Summerfield,  
38 V. (2014) Applying the skin sensitisation adverse outcome pathway (AOP) to quantitative risk  
39 assessment. *Toxicol. Vitro.* 28, 8–12.  
40  
41

42  
43 (40) Wu, S., Fisher, J., Naciff, J., Laufersweiler, M., Lester, C., Daston, G., and Blackburn, K.  
44 (2013) Framework for identifying chemicals with structural features associated with the potential  
45 to act as developmental or reproductive toxicants. *Chem. Res. Toxicol.* 26, 1840–1861.  
46  
47

48  
49 (41) Caldwell, D. J., Mastrocco, F., Margiotta-Casaluci, L., and Brooks, B. W. (2014) An  
50 integrated approach for prioritizing pharmaceuticals found in the environment for risk  
51 assessment, monitoring and advanced research. *Chemosphere* 115, 4–12.  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 (42) European Commission Institute for Health and Consumer Protection. AOP Wiki,  
4 [https://aopkb.org/aopwiki/index.php/Main\\_Page](https://aopkb.org/aopwiki/index.php/Main_Page)  
5  
6

7  
8 (43) Roberts, D. W., and Aptula, A. O. (2014) Electrophilic reactivity and skin sensitization  
9 potency of SnAr electrophiles. *Chem. Res. Toxicol.* 27, 240–246.  
10

11  
12 (44) Leist, M., Hasiwa, N., Rovida, C., Daneshian, M., Basketter, D., Kimber, I., Clewell, H.,  
13 Gocht, T., Goldberg, A., Busquet, F., Rossi, A. M., Schwarz, M., Stephens, M., Taalman, R.,  
14 Knudsen, T. B., McKim, J., Harris, G., Pamies, D., and Hartung, T. (2014) Consensus report on  
15 the future of animal-free systemic toxicity testing. *ALTEX* 31, 341–356.  
16  
17

18  
19 (45) Tsakovska, I., Al Sharif, M., Alov, P., Diukendjieva, A., Fioravanzo, E., Cronin, M. T. D.,  
20 and Pajeva, I. (2014) Molecular modelling study of the PPAR $\gamma$  receptor in relation to the mode  
21 of action/adverse outcome pathway framework for liver steatosis. *Int. J. Mol. Sci.* 15, 7651–  
22 7666.  
23  
24

25  
26 (46) Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov,  
27 I. N., and Bourne, P. E. (2000) The Protein Data Bank. *Nucleic Acids Res.* 28, 235–242.  
28  
29

30  
31 (47) Nelms, M. D., Ates, G., Madden, J. C., Vinken, M., Cronin, M. T. D., Rogiers, V., and  
32 Enoch, S. J. (2014) Proposal of an in silico profiler for categorisation of repeat dose toxicity data  
33 of hair dyes. *Arch. Toxicol.* 89, 733–741.  
34  
35

36  
37 (48) Tollefsen, K. E., Scholz, S., Cronin, M. T., Edwards, S. W., de Knecht, J., Crofton, K.,  
38 Garcia-Reyero, N., Hartung, T., Worth, A., and Patlewicz, G. (2014) Applying Adverse  
39 Outcome Pathways (AOPs) to support Integrated Approaches to Testing and Assessment  
40 (IATA). *Regul. Toxicol. Pharmacol.* 70, 629–640.  
41  
42

43  
44 (49) Allen, T. E. H., Goodman, J. M., Gutsell, S., and Russell, P. J. (2014) Defining Molecular  
45 Initiating Events in the Adverse Outcome Pathway framework for risk assessment. *Chem. Res.*  
46 *Toxicol.* 27, 2100–2112.  
47  
48

49  
50 (50) Madden, J. C., Rogiers, V., and Vinken, M. (2014) Application of in silico and in vitro  
51 methods in the development of adverse outcome pathway constructs in wildlife. *Philos. Trans. R.*  
52 *Soc. B Biol. Sci.* 369, 1–7.  
53  
54  
55  
56  
57  
58  
59  
60



- 1  
2  
3 (51) Effectopedia, <http://www.effectopedia.org/>  
4  
5  
6 (52) Kohonen, P., Benfenati, E., Bower, D., Ceder, R., Crump, M., Cross, K., Grafström, R. C.,  
7 Healy, L., Helma, C., Jeliaskova, N., Jeliaskov, V., Maggioni, S., Miller, S., Myatt, G.,  
8 Rautenberg, M., Stacey, G., Willighagen, E., Wiseman, J., and Hardy, B. (2013) The ToxBank  
9 data warehouse: Supporting the replacement of in vivo repeated dose systemic toxicity testing.  
10 *Mol. Inform.* 32, 47–63.  
11  
12 (53) Yang, C., Arvidson, K., Richard, A., Worth, A., Tarkhov, A., Ringeissen, S., Marusczyk, J.,  
13 Gasteiger, J., Rathman, J., and Schwab, C. (2014) Chemotypes and chemotyper: A new  
14 structural representation standard to include atomic/bond properties into structural alerts for  
15 toxicity effects and mechanisms.  
16  
17 (54) Knudsen, T. B., Keller, D. A., Sander, M., Carney, E. W., Doerrer, N. G., Eaton, D. L.,  
18 Fitzpatrick, S. C., Hastings, K. L., Mendrick, D. L., Tice, R. R., Watkins, P. B., and Whelan, M.  
19 (2015) FutureTox II: In vitro data and in silico models for predictive toxicology. *Toxicol. Sci.*  
20 *143*, 256–267.  
21  
22 (55) Angrish, M. M., Madden, M. C., and Pleil, J. D. (2015) Probe Molecule (PrM) approach in  
23 Adverse Outcome Pathway (AOP) based High-Throughput Screening (HTS): In vivo discovery  
24 for developing in vitro target methods. *Chem. Res. Toxicol.* 28, 551–559.  
25  
26 (56) Steinmetz, F. P., Mellor, C. L., Meinel, T., and Cronin, M. T. D. (2015) Screening chemicals  
27 for receptor-mediated toxicological and pharmacological endpoints: Using public data to build  
28 screening tools within a KNIME workflow. *Mol. Inform.* 34, 171–178.  
29  
30 (57) Yin, Y., Chang, D. T., Grulke, C. M., Tan, Y.-M., Goldsmith, M.-R., and Tornero-Velez, R.  
31 (2014) Essential set of molecular descriptors for ADME prediction in drug and environmental  
32 chemical space. *Research 1*, 996.  
33  
34 (58) Burden, N., Sewell, F., Andersen, M. E., Boobis, A., Chipman, J. K., Cronin, M. T. D.,  
35 Hutchinson, T. H., Kimber, I., and Whelan, M. (2015) Adverse Outcome Pathways can drive  
36 non-animal approaches for safety assessment. *J. Appl. Toxicol.* 35, 971–975.  
37  
38 (59) Russell, W. M. S., and Burch, R. L. (1959) The Principles of Humane Experimental  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Technique.

(60) Knapen, D., Vergauwen, L., Villeneuve, D. L., and Ankley, G. T. (2015) The potential of AOP networks for reproductive and developmental toxicity assay development. *Reprod. Toxicol.* 56, 52–55.

(61) Becker, R. A., Ankley, G. T., Edwards, S. W., Kennedy, S., Linkov, I., Meek, B., Sachana, M., Segner, H., Burg, B. Van Der, Villeneuve, D. L., Watanabe, H., and Barton-Maclaren, T. S. (2015) Increasing scientific confidence in Adverse Outcome Pathways: Application of tailored Bradford-Hill considerations for evaluating weight of evidence. *Regul. Toxicol. Pharmacol.* 72, 514–537.

(62) Yauk, C. L., Lambert, I. B., Meet, M. E., Douglas, G. R., and Marchetti, F. (2015) Development of the Adverse Outcome Pathway “Alkylation of DNA in male premeiotic germ cells leading to heritable mutations” Using the OECD’s users’ handbook supplement. *Environ. Mol. Mutagen.* 56, 724-750.

(63) Dent, M. P., Carmichael, P. L., Jones, K. C., and Martin, F. L. (2015) Towards a non-animal risk assessment for anti-androgenic effects in humans. *Environ. Int.* 83, 94–106.

(64) von Stackelberg, K., Guzy, E., Chu, T., and Henn, B. C. (2015) Exposure to mixtures of metals and neurodevelopmental outcomes: A multidisciplinary review using an Adverse Outcome Pathway framework. *Risk Anal.* 35, 971–1016.

(65) Nelms, M. D., Mellor, C. L., Cronin, M. T. D., Madden, J. C., and Enoch, S. J. (2015) Development of an in silico profiler for mitochondrial toxicity. *Chem. Res. Toxicol.* 28, 1891–1902.

(66) Mellor, C. L., Steinmetz, F. P., and Cronin, M. T. D. (2015) The identification of nuclear receptors associated with hepatic steatosis to develop and extend adverse outcome pathways. *Crit. Rev. Toxicol.* 8444, 1–15.

(67) Sanderson, P. N., Simpson, W., Cubberley, R., Aleksic, M., Gutsell, S., and Russell, P. J. (2016) Mechanistic understanding of molecular initiating events (MIEs) using NMR spectroscopy. *Toxicol. Res.* 5, 34–44.

1  
2  
3 (68) Marchetti, F., Massarotti, A., Yauk, C. L., Pacchierotti, F., and Russo, A. (2015) The  
4 adverse outcome pathway (AOP) for chemical binding to tubulin in oocytes leading to aneuploid  
5 offspring. *Environ. Mol. Mutagen.* 57, 87-113.  
6  
7

8  
9  
10 (69) Patlewicz, G. Y., and Fitzpatrick, J. (2015) Current and future perspectives on the  
11 development, evaluation and application of in silico approaches for predicting toxicity. *Chem.*  
12 *Res. Toxicol.* 29, 438-451.  
13

14  
15  
16 (70) EU-ToxRisk, <http://www.eu-toxrisk.eu/>  
17

#### 18 19 AUTHOR BIOGRAPHIES

20  
21 Dr Timothy E. H. Allen is a post-doctoral research associate in the group of Professor Jonathan  
22 Goodman at the Department of Chemistry, University of Cambridge having completed his Ph.D.  
23 in Chemistry in the same group in 2016. His research interests include studying the MIE from a  
24 chemical perspective, identifying the chemical characteristics that allow such interactions to  
25 occur and constructing computational models based on them.  
26  
27  
28

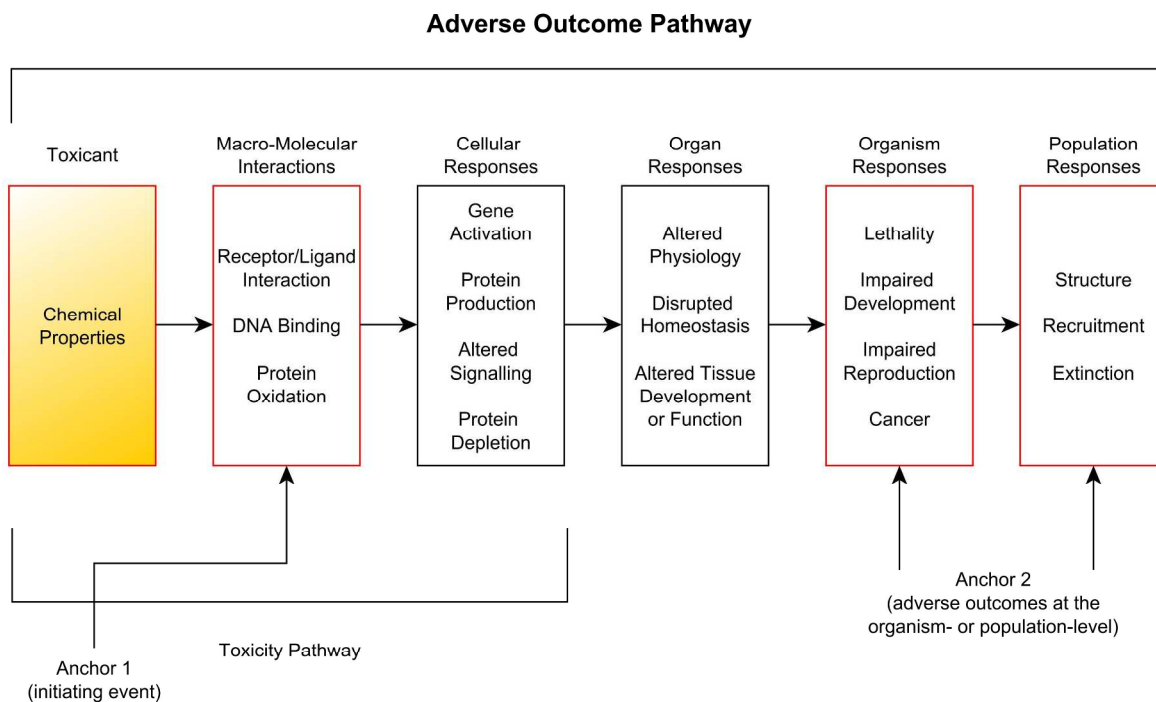
29  
30  
31 Professor Jonathan M. Goodman did experiments on aldol reactions during his PhD with Ian  
32 Paterson at the University of Cambridge, and then was a post-doc with Clark Still at Columbia  
33 University, before returning to Cambridge, where he is now Professor of Chemistry and is  
34 investigating how knowledge of chemistry and molecular structures can contribute to issues in  
35 toxicology.  
36  
37  
38

39  
40 Dr Steve Gutsell obtained his PhD in Organic Chemistry from the University of Wales, Swansea  
41 and is currently a Computational Chemist at Unilever's Safety and Environmental Assurance  
42 Centre. His research is concerned with using predictive methods such as (Quantitative)  
43 Structure-Activity Relationships ((Q)SAR), Read Across and other techniques to predict both  
44 toxicological and ecotoxicological activities from chemical structure. Recent areas of interest  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

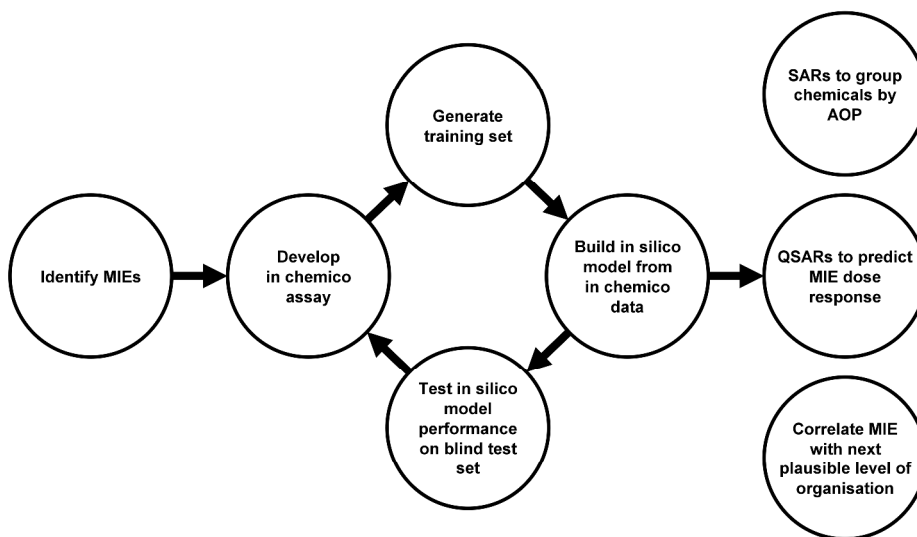
1  
2  
3 include how pathways-based approaches can be used to create novel risk assessments for  
4  
5  
6 consumer products.  
7  
8  
9  
10

11  
12 Dr Paul Russell obtained his PhD in Pharmaceutical Science from King's College London and is  
13  
14 currently Chemistry Strategic Science Leader at Unilever's Safety and Environmental Assurance  
15  
16 Centre. His research interests are the development of non-animal tools and approaches to define  
17  
18 toxicity pathways from Molecular Initiating Events in support of the safety assessment of  
19  
20 chemicals in consumer goods.  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

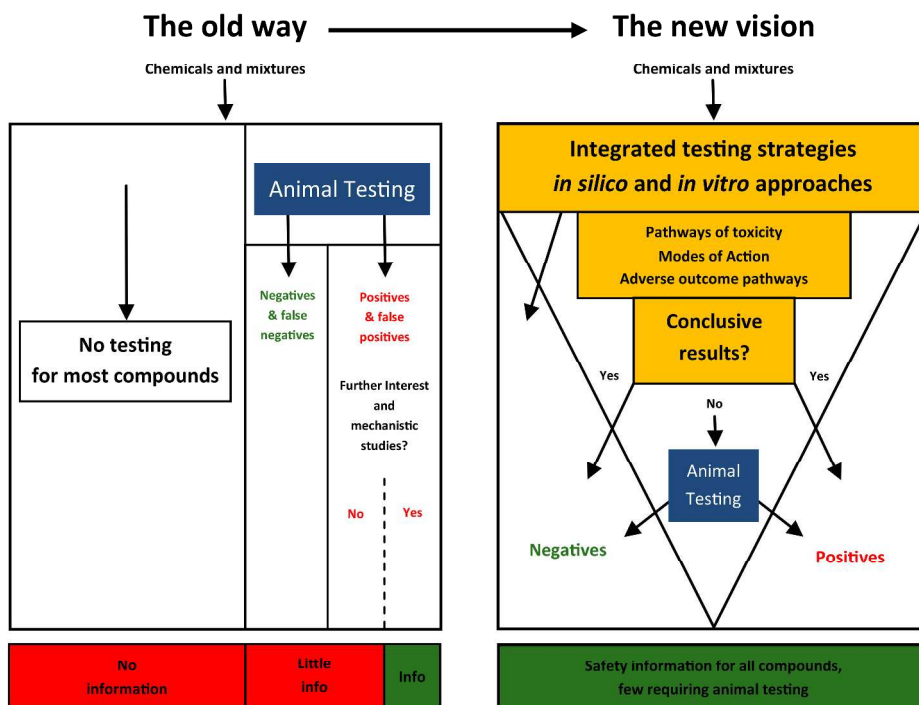
## FIGURES



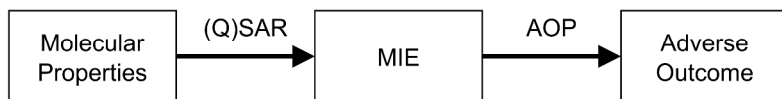
**Figure 1.** Ankley's conceptual diagram of an AOP, including the MIE (Anchor 1). Adapted with permission from Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrano, J. A., Tietge, J. E., and Villeneuve, D. L. (2010) Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.*, Vol. 29, 730–741,<sup>18</sup> from John Wiley & Sons Inc.



**Figure 2.** A wet/dry cycle for the development of in silico models. Adapted from Gutsell, S., and Russell, P. (2013) The role of chemistry in developing understanding of adverse outcome pathways and their application in risk assessment. *Toxicol. Res.*, Vol. 2, 299–307,<sup>35</sup> with permission from The Royal Society of Chemistry.



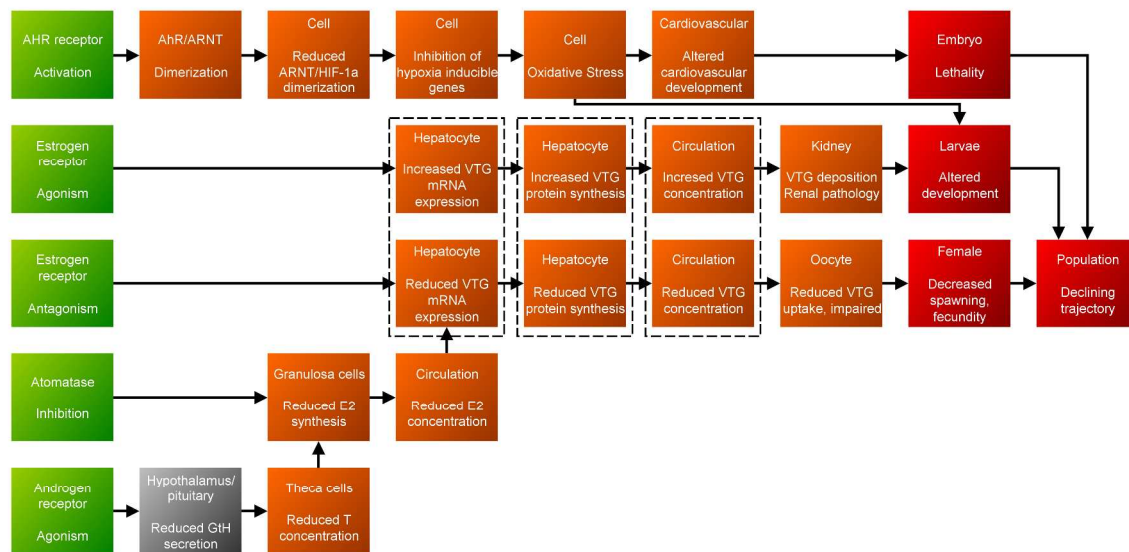
**Figure 3.** Leist's new vision for the future of toxicity testing. Adapted with permission from Leist, M., Hasiwa, N., Rovida, C., Daneshian, M., Basketter, D., Kimber, I., Clewell, H., Gocht, T., Goldberg, A., Busquet, F., Rossi, A. M., Schwarz, M., Stephens, M., Taalman, R., Knudsen, T. B., McKim, J., Harris, G., Pamies, D., and Hartung, T. (2014) Consensus report on the future of animal-free systemic toxicity testing. *ALTEX* 31, 341–356.<sup>44</sup>



**Figure 4.** Framework for a (Q)SAR approach based around MIEs. The (Q)SAR relates molecular characteristics to the MIE and the AOP infers an adverse outcome from the MIE.

Adapted from Allen, T. E. H., Goodman, J. M., Gutsell, S., and Russell, P. J. Defining Molecular Initiating Events in the Adverse Outcome Pathway framework for risk assessment. *Chem. Res. Toxicol.* 27, 2100–2112. Copyright 2014 American Chemical Society.<sup>49</sup>





**Figure 5.** An example of an AOP network based on five reproductive and developmental toxicity-related AOPs in fish available on the AOP wiki. MIEs are indicated in green, KEs in orange and adverse outcomes in red, as per the AOP wiki template. The grey box represents a KE with a “weak” weight of evidence. The dotted squares indicate KEs that are defined as changes in the opposite direction of the same biological component. AHR: aryl hydrocarbon receptor, E2: estradiol, GtH: gonadotrope hormone, T: testosterone, VTG: vitellogenin.

Reprinted from *Reproductive Toxicology*, Vol. 56, Dries Knapen, Lucia Vergauwen, Daniel L. Villeneuve, Gerald T. Ankley; The potential AOP networks for reproductive and developmental toxicity assay development; pp 52-55; Copyright 2015;<sup>60</sup> with permission from Elsevier.