Application of *in silico* and *in vitro* methods in the development of Adverse Outcome Pathway constructs in wildlife

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Short title: *In silico and in vitro methods in AOPs*

Abstract

There is a long history of using both *in silico* and *in vitro* methods to predict adverse effects in humans and environmental species where toxicity data are lacking. Currently there is a great deal of interest in applying these methods to the development of so-called adverse outcome pathway (AOP) constructs. The AOP approach provides a framework for organising information at the chemical and biological level, allowing evidence from both *in silico* and *in vitro* studies to be rationally combined to fill gaps in knowledge concerning toxicological events. Fundamental to this new paradigm is a greater understanding of the mechanisms of toxicity and in particular where these mechanisms may be conserved across *taxa*, such as between model animals and related wild species. This presents an opportunity to make predictions across diverse species, where empirical data are unlikely to become available as is the case for most species of wildlife.

Keywords: adverse outcome pathway, *in silico* modelling, *in vitro* techniques, predictive toxicology.

Introduction

The adverse effects of man-made chemicals (such as pesticides, industrial chemicals and intermediates) on the environment have long been established as a cause for concern in ecotoxicology. The potential effects of pharmaceuticals on wildlife species has been highlighted as an issue of particular concern since their initial detection in the environment. Whilst toxicity data are obtainable for certain species and endpoints, the effects of pharmaceuticals on the full diversity of wildlife cannot be readily determined, therefore predictive toxicological methods are essential. There is now greater understanding of the fundamental interactions between chemicals and biological systems enabling more rational, mechanistically-based predictions of toxicity to be proposed. In developing these mechanistically-based models, knowledge of *in vivo* outcomes is considered most useful. However, this information is usually unavailable, particularly for repeat, low dose exposure, characteristic of pharmaceuticals in the environment. Hence, knowledge acquired through the application of *in silico* and *in vitro* tools becomes invaluable.

The concept of toxicity pathways, whereby an initial perturbation of a biological system leads to a subsequent adverse effect in an organism, has been an important theory within toxicology for many years. In the last decade there has been an increased interest in this philosophy and recent developments have seen the concept evolve into the adverse outcome pathway (AOP) approach. This approach enables information from diverse sources to be organised within a logical framework facilitating greater understanding of the processes involved. Deconstruction of these pathways into a sequence of finite key events provides an opportunity to use mechanistic information obtained from *in vitro* and *in silico* analysis to fill gaps in current knowledge. Where key events within pathways are conserved across species, models that are applicable across a diverse range of organisms may be developed. In the present paper, we discuss the AOP framework and its application to the problem of predicting toxic effects in wildlife species as well as describing advances in both *in silico* and *in vitro* sciences which may help to resolve this complex issue.

Adverse outcome pathways in (eco)toxicology

Definition, structure and development of adverse outcome pathways

Mechanistic toxicology is a cornerstone of ecological and human risk assessment. AOPs play a major role in this paradigm providing a framework to organise events from the initial interaction of a chemical with a biological target, termed the molecular initiating event (MIE; for example ligand-receptor interactions or binding of the chemical to proteins or nucleic acids), and an adverse outcome at the organism or population level [1, 2]. Previous constructs, such as the source to outcome pathway (which includes consideration of the source of pollution and effects up to the level of community or ecosystem), and mechanism or mode-of-action pathways are well established in the area of environmental risk assessment. Uncertainty caused by differences in use and definition of the terms mode-of-action and mechanism-of-action have been overcome by the introduction of the AOP construct which requires both the MIE and the adverse outcome to be defining anchor points. Whilst AOPs are not conceptually new, they represent an evolution of previous constructs and are designed to be more appropriate to risk assessment [1]. An AOP consists of three blocks of information, namely (i) the MIE (ii) a set of key events and (iii) the adverse outcome [2]. For a given AOP, the MIE and the adverse outcome are the two defining anchor points. These are important as one MIE may result in a range of adverse outcomes, and similarly, a given adverse outcome may be a consequence of different MIEs. An AOP may involve as many key events as necessary; these are essential for generating the adverse outcome [2, 3]. An advantage of the AOP approach is its ability to incorporate data from a wide range of sources (*in silico*, *in vitro*, *in vivo* etc) and use this information to provide the linkages between the MIE and the adverse outcome [1]. Whilst mechanistic information is not essential *a priori* to develop an AOP, as this information becomes available it can be used as further supporting evidence. Greater understanding of the mechanisms driving the processes within the pathway can be used to provide justification in extrapolating from known to unknown conditions. One drawback of the AOP approach is the lack of explicit consideration of external and internal exposure which ultimately determines the concentration of a chemical at the target site. However, using the organisational framework of an AOP it is possible to determine what dosimetry information would be necessary to allow dose in an assay, or at a given level of biological organisation, to be related to

doses in other assays or at other levels of biological organisation. The dose at the target site (influenced *in vivo* by absorption, distribution, metabolism and excretion parameters and *in vitro* by binding to test system components) is a key determinant of the ultimate biological effect of a chemical [1]. The framework for an AOP, in contrast to a source to outcome pathway, is depicted in figure 1.

FIGURE 1 HERE

It is important to define the site of action of the MIE, as this directly determines the nature of the adverse outcome and can relate to chronic or acute toxicological effects at the local or systemic level. Note that whilst AOPs are usually presented linearly, whereby a graphical representation indicates the information blocks in a consecutive way, in reality many other factors may influence the progress from MIE to outcome, such as physiological feedback mechanisms and adaptive responses.

AOPs originate from the field of ecotoxicology, where the approach was proposed to enable the formation of toxicologically meaningful categories (TMCs) [4]. Grouping chemicals together into TMCs allows for read-across of activity between those chemicals with known activity and chemicals of unknown activity. Examples of applying read-across to toxicity of pharmaceuticals are discussed by Owen *et al* [5].

Detailed examples of AOPs in the area of ecotoxicology have previously been reported wherein the key events are identified and their potential use in regulatory toxicology is discussed. Schultz describes three AOPs relating to fish acute toxicity, including weak acid respiratory uncoupling, respiratory irritation and acetylcholinesterase inhibition in addition to AOPs for receptor binding of oestrogen mimics and skin sensitisation [4]. Ankley *et al* reported AOPs for narcosis, photoactivated toxicity, activation of the aryl hydrocarbon receptor (AhR), oestrogen receptor activation and a set of AOPs associated with impaired vitellogenesis (an apposite example of different MIEs resulting in the same adverse outcome) [1]. More recently AOPs for human toxicological endpoints have been published, including drug-induced liver fibrosis, steatosis [6] and cholestasis [7]. At a global level the Organisation for Economic Cooperation and Development (OECD) has recognised the importance of

the AOP approach, launching its program for the development of AOPs in 2012. At time of writing, 18 AOPs and 3 case studies are in development with additional ongoing projects relating to documentation and knowledge management (http://www.oecd.org/env/ehs/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm#Documents). A guidance document and reporting template for the development and assessment of completeness of newly postulated AOPs have been published [2]. This document clearly states the criteria by which a proposed AOP should be evaluated. Consideration is given to the strength of the scientific data collated and the causal link between the steps in the pathway. AOP evaluation is carried out by meeting the Bradford-Hill criteria which define the minimal requirements for establishing a causal link between the different information blocks [2, 3, 8]. Overall confidence in the AOP is assessed by considering how well the MIE and adverse outcome are characterised and if there are any limitations (e.g. related to life-stages of organisms). Throughout AOP evaluation, the extent to which the MIE and key events are conserved across species is specifically considered. This is relevant in the context of this paper, as it demonstrates the utility of the AOP approach in developing predictive models applicable across species.

AOPs have potential use for a variety of purposes pertinent to ecological and human toxicology and risk assessment. At the theoretical level they can provide greater understanding of the mechanisms of toxicology and enable the establishment of (quantitative) structure-activity relationships. From a regulatory perspective improvements in predicting properties of related chemicals, the development of novel *in vitro* toxicity screening tests and the elaboration of prioritisation strategies [3] are also highly relevant applications. Such AOP-based approaches can be included in integrated testing strategies (ITS) which are considered tools of the future for regulatory risk assessment. The AOP framework provides an ideal opportunity for the synergistic use of data from a range of novel *in silico* and *in vitro* approaches, such as those described below.

Contribution of in silico modelling to adverse outcome pathway development

In silico (i.e. computational) methods to predict toxicity of chemicals have been widely used in environmental sciences for over 40 years. The science is based on the premise that the activity (e.g. toxicity) effected by a chemical is a consequence of its physico-chemical and structural properties. Hence, knowledge of a chemical's properties (or knowledge of related chemicals) can be used to make predictions on activity. Many (quantitative) structure-activity relationship ((Q)SAR) models have been developed for forecasting toxicity to aquatic and terrestrial species, with application to acute toxicity to aquatic species being most successful. (Q)SARs work well when the applied dose can be clearly linked to the dose at the site of action as occurs for continuous exposure that results in a steady-state concentration in the organism. In this case the external dose is proportional to the dose at the target site i.e. the dose that causes the effect. Knowledge and expertise in predictive eco(toxicology) have been translated into software packages for predicting both acute and chronic toxicity in a range of environmental species. Table 1 shows some of the packages currently available.

TABLE 1 HERE

Many *in silico* tools are available to predict acute toxicity, however, the prediction of adverse effects due to repeat, low dose exposure, particularly for complex endpoints such as reproductive toxicity, remains a challenging issue. *In silico* models, in common with other models, provide a simplistic representation of a system, hence the complexities of interactions and moderating processes within an organism cannot be fully characterised. Whilst there are limitations in the application of *in silico* models to predicting apical toxicity endpoints, these models can be usefully applied within an AOP construct. *In silico* tools can provide predictions relating to key events within an AOP, most notably relating to the first step, i.e. determining which chemicals may elicit an MIE.

Knowledge derived from mechanistic organic chemistry can be used to identify chemical features that may lead to an interaction with a biological macromolecule. For example, an electrophilic chemical may react with a biological nucleophile forming a covalent bond to proteins or nucleic acids. Figure 2 shows that the MIE associated with covalent protein binding may result in different effects in different

species, *in casu* respiratory irritation in fish and skin sensitisation in humans. Of key importance is that such interactions are predictable from chemical knowledge and can be used in the development of structural alerts. These alerts may indicate the potential of a chemical to elicit an MIE, such as protein binding. However, this may or may not lead to a downstream adverse effect. Alternatively, structural alerts may be developed that are directly associated with a given toxicity. Note that care must be taken in applying knowledge from structural alerts (i.e. the lack of an alert within a compound does not necessarily imply lack of toxicity). Reviews of alerts associated with covalent protein binding [9] and covalent DNA binding [10] have been published. Structural alerts associated with MIEs and the ability to elicit specific toxicities are continually being developed. Such alerts have been combined into so-called profilers (e.g. those used within the OECD QSAR Toolbox) which can be used to place chemicals into a category of related chemicals for the purpose of read-across (*vide supra*) [11]. The ability to derive justifiable groupings for chemicals and data being available for sufficient group members to enable confidence in the prediction are essential requirements for read-across. Data are available from a wide range of sources, although assessment of data quality is recommended prior to use [12].

FIGURE 2 HERE

In silico tools are also being applied to the question of interspecies correlation. Where a molecular target is associated with an MIE, conservation of this target across species may identify potentially susceptible organisms. Gunnarsson *et al.* predicted orthologs for 1318 human drug targets in 16 species. Vertebrates (fish and frogs) showed greater conservation of targets than Daphnia or green algae [13]. Whilst orthology is not necessarily associated with common function (similarly a functional interaction may occur in non-orthologous proteins), it is possible to use this approach to identify potential interactions between pharmaceuticals and protein targets (common MIEs or modes of action) that may be conserved across diverse species. LaLone *et al.* demonstrated the application of bioinformatics tools to identify where sequence similarity of molecular targets (associated with MIEs)

could be used to predict species sensitivity to pharmaceuticals (and other chemicals of known mode of action) within an AOP context. Case studies using targets for 17α-ethinyl estradiol, permethrin and trenbolone acetate were used to compare known toxicity to species susceptibility derived from sequence similarity [14]. Spironolactone acts as an antagonist of the androgen receptor in humans. This receptor is conserved across vertebrates but not invertebrates. It has been demonstrated that exposure to spironolactone reduced fecundity in fish species, but did not affect the reproduction of Daphnia [15]. This illustrates the potential of using this approach (i.e. identifying conserved molecular targets associated with an MIE in an AOP framework) to identify environmental species that may be sensitive to specific pharmaceuticals. This area is expanding rapidly and as more complete genomes for more taxa and more well-curated databases become available, the more widely applicable the approach will become.

Walker and McEldowney investigated the use of molecular docking techniques to predict the effects of three common pharmaceuticals (diclofenac, ibuprofen and leveonorgestrel) on environmental species. Sequence homology searching was performed for cyclooxygenase 2 (COX2) and the progesterone receptor, being the target receptor proteins for the drugs. Receptors in fish and frogs, but not daphnia, were shown to bind diclofenac in the same way as the human target, potentially leading to inhibition of COX2 function in these species. Similarly, levonogestrel was found to bind at the same site in fish and frogs as in humans [16]. Reduced fecundity in fish and frogs exposed to levonogestrel would therefore be predicted and is in-keeping with the known ecotoxicity profile for this compound.

Exposure to a chemical is also a key determinant of toxicity. Physiologically-based pharmacokinetic (PBPK) models can be used to determine the concentration of a chemical at a target organ (internal exposure). *In silico* tools are routinely applied in the development of these models, for example in predicting physico-chemical properties that are associated with uptake and distribution of chemicals. Interspecies differences can readily be accounted for in model development (e.g. differences in tissue blood flows, organ weights or metabolic capabilities) allowing extrapolation between species.

The research outlined above indicates *in silico* tools play a major role in several areas of predictive toxicology, namely during determination of concentrations at target site, upon defining mechanistic

interaction with biological targets (eliciting MIEs) and in identifying molecular targets that are conserved across species. When combined with information obtained by *in vitro* experimentation, these become powerful tools for informing AOPs.

Contribution of in vitro assays to adverse outcome pathway development

The identification of the different AOP building blocks may be based upon a literature survey or can be retrieved from experimental data. Following a given MIE, the establishment of a sequence of key events is the next step. As previously discussed any type of information can be fed into an AOP, including structural, *in chemico*, *in silico*, "omics-based", *in vitro* and *in vivo* data [2, 3]. *In vitro* tests are particularly suitable to reinforce the relevance of key events in AOP constructs as well as to identify new ones, which in turn may serve as the basis for the characterisation of biomarkers for hazard identification. This has been well exemplified in the case of the neurotoxicity induced by domoic acid, an algal toxin with adverse effects on humans and wildlife. Domoic acid is a potent agonist of kainate receptors, which increases intracellular calcium concentrations triggering excitotoxicity and cell death primarily in the hippocampus. This leads to seizures, impairs learning and memory and alters behaviour in some species. Altered neuronal calcium concentration is a key process in domoic acid toxicity, which is conserved among most species and that can be readily evaluated *in vitro* [17]. Indeed, several protocols exist to assess intracellular calcium concentration, all of which mainly use bioluminescent calcium indicators or chemical fluorescent indicators. Measurement of calcium concentrations as such can be accomplished with optical techniques, such as confocal or 2-photon excitation laser scanning microscopy, as well as with non-optical methods, including electrophysiology or calcium-selective electrodes [18].

Another example where *in vitro* methods can assist in ecological AOP development includes adverse effects mediated by AhR, which is a nuclear receptor that can be activated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related planar aromatic chemicals. AhR can be found in a variety of vertebrates, including species of mammals, birds, amphibians, bony fishes, cartilaginous fish and jawless fish [19]. AhR induces the expression of xenobiotic biotransformation

enzymes, and causes toxic effects, such as immunotoxicity, reproductive toxicity and cancer [20]. In embryonic zebrafish, TCDD is known to evoke cardiac malformation [21], an effect depending on AhR activation [22]. Vertebrates are more sensitive to the effects of TCDD and similar chemicals than invertebrates [23]. AhR-mediated toxicity can be considered as a key event in many AOPs that can be extrapolated across species. Induction of AhR is measurable *in vitro* using specific gene report assays, such as those based upon luciferase activity. It is hereby critical to consider the appropriate AhR isoform, as this might differ between species [19]. Furthermore, activation of AhR can be evaluated by studying the expression of classical AhR-responsive genes in mammals and fish, including cytochrome P450 1A, nicotinamide adenine dinucleotide (phosphate) dehydrogenase, aldehyde dehydrogenase 3, uridine diphosphate glucuronosyltransferase and selected glutathione transferases [22].

An AOP case study that has gained particular attention the last decade includes (eco)toxicity driven by the oestrogen receptor, another nuclear receptor that dictates specific gene expression patterns. In fact, binding and activation of this receptor by agonists induces a variety of biological responses, such as modified reproduction, gonad alterations, changes in levels of sex steroids and the egg yolk protein, vitellogenin, as well as secondary sex characteristics [24]. Regarding the latter, a well-characterised example of endocrine disruption involved feminisation of male fish, reptiles, birds and mammals. This is caused by agonists of the oestrogen receptor, including 17β-estradiol and 17α-ethinylestradiol, present in relatively high concentrations, in discharges from municipal wastewater treatment plants [24, 25]. Effects of hormonally active pharmaceuticals are also discussed by Berg *et al* [26]. The potential of chemicals to activate the oestrogen receptor can be pinpointed *in vitro* directly by oestrogen receptor binding assays or indirectly by monitoring oestrogen receptor-dependent gene expression, including vitellogenin [1, 25, 27]. A more recently introduced and holistic approach includes the use of toxicogenomic approach to characterise a full transcriptional signature of endocrine disruption, *in casu* in fish [28, 29]. Wilson *et al.* [30] reviewed the application of toxicogenomic data (e.g. proteomics, metabolomics, transcriptomics) to providing a better understanding of mechanisms of toxicity and the role of these data in risk assessment. Such data can

be used also to identify biomarkers (e.g. by detecting changes in gene expression that precede a particular cellular reaction), to interpret interspecies differences in response and to corroborate existing mode-of-action hypotheses. Hence, advances in this technology will aid both definition of key events in an AOP and determination whether or not such effects can be extrapolated across species; whenever possible taking dose-related exposure effects into consideration.

In vitro tools can also be used to verify the domain of applicability of structural alerts, identified *in silico*, in addition to providing corroboration of proposed mechanisms. Perkins *et al.* evaluated data from high-throughput *in vitro* assays in the US Environmental Protection Agency ToxCast Phase I project many of which were designed to assess toxicity associated with known MIEs [31]. The authors provide examples of where MIEs are conserved across diverse species, such as toxicity arising from binding to the gamma-aminobutyric $acid_A$ receptor, interaction with hypothalamus-pituitary-gonad axis and inhibition of sex steroid synthesis. In certain cases toxicity may be extrapolated. However, in other cases, although the MIE is conserved, the apical endpoint may differ between species. Conversely, where there is a lack of conservation across, species extrapolation is not possible, as has been demonstrated for interactions with the oestrogen receptor, which is well conserved across vertebrates, but not invertebrates. Interspecies differences in route and amount of exposure and metabolism should also be considered when extrapolating toxicity data across species [31].

Conclusions and perspectives

Whilst the importance of AOPs has been recognised at the global level by the OECD [2], this area is still in its infancy. A criticism of AOPs is their simplicity and thereby their poor reflection of complex toxicological processes. In this context, AOPs must be envisaged as flexible tools that should be continuously refined by introducing new data. An increasing number of *in silico* tools are becoming available that may support AOP development. These include software to make predictions of apical endpoint toxicity in addition to those dedicated to providing more mechanistic understanding of the toxicological process, for example development of structural alerts and profilers (such as those used

within the OECD Toolbox; table 1). Furthermore, investigations into protein sequence similarity and molecular docking tools are helping to resolve the issue extrapolation to diverse species.

Iterative optimisation of AOPs should ideally consider concentration-time profiles giving a more quantitative prediction for external and internal exposure. Similarly, the search should not only be pursued for new biomarkers to identify hazard, but also for those that allow exposure assessment [3].

Another challenge for future AOP development lies in the implementation of epigenetic mechanisms. Indeed, several drugs and environmental pollutants, including endocrine disruptors, perform their deleterious actions by interfering with chromatin remodelling processes [32]. Although *in vitro* assays are currently available to mechanistically investigate the different determinants of the epigenetic machinery, such as DNA methylation and histone modifications, a major point of debate presently relates to the design of such *in vitro* epigenetic studies in terms of timing and exposure in order to appropriately mimic the corresponding *in vivo* situation [33]. Their meaning in relation to identified and currently used biomarkers in risk assessment is often unknown.

In silico and *in vitro* models represent (parts of) the true, biological system; incorporating results from these approaches into an AOP requires confidence in the information that they provide. Results are subject to a degree of uncertainty relating to confidence in predictions or reliability / suitability of assays. It is also recognised that there are difficulties in extrapolating *in vitro* results to the *in vivo* situation, for example in converting *in vitro* doses to realistic *in vivo* exposure. Current limitations of the AOP approach and areas requiring further development have been identified previously [3]. Formal validation of methods can be a lengthy and controversial issue and more work is required in developing validation standards, however, pragmatic approaches to assessing reliability / suitability of models or assays are widely employed. For example, adherence to the OECD principles for the validation of (Q)SARs gives greater confidence to predictions obtained using *in silico* methods [34].

Effective integration of data from *in chemico*, *in silico*, *in vitro* and *in vivo* requires bespoke tools for recording and presenting data. Effectopedia (http://effectopedia.org/) is one such tool designed to address this problem, providing a freely available common space to collate and organise data relevant to AOP development. With the rapid evolution of *in vitro* and *in silico* methods and their integration to

support AOPs, it is anticipated that progress in this nascent area will proceed rapidly over the next few years.

Acknowledgements

This work was financially supported by the European Union (FP7) and Cosmetics Europe under grant agreement numbers 266835 (COSMOS project), 266777 (HeMiBio project) and 266838 (DETECTIVE project).

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Figure and table captions

Figure 1. Representation of the key features of a ‘source to outcome’ and ‘adverse outcome pathway’.

Figure 2. Binding of an electrophile to a nucleophilic group of a protein demonstrating that the same molecular initiating event can result in different toxicities in different species.

Table 1. Software for predicting environmental effects of chemicals.

Table 1

Software	URL	Notes
ADMET Predictor	http://www.simulations-plus.com/Products.aspx?pID=13	Estimates many properties relating to ADME and toxicity (including fish and daphnia acute toxicity) enables new models to be built from user's data
Discovery Studio (DS TOPKAT)	http://accelrys.com	Calculates molecular descriptors and generates (Q)SARs for several endpoints including daphnia and fish toxicity
ECOSAR	http://www.epa.gov/oppt/newchems/tools/21ecosar.htm	Predicts acute and chronic toxicity to algae, daphnia and fish using SARs and knowledge of chemical classes
CATALOGIC	http://oasis-lmc.org/products/software/catalogic.aspx	Includes models for predicting environmental fate and acute aquatic toxicity
ChemProp	http://www.ufz.de/index.php?en=6738	Chemical properties estimation software comprising a suite of modules for physico-chemical and toxicological endpoints
MCASE(MC4PC)	http://www.multicase.com/	Uses automated machine learning to identify structural alerts associated with a range of toxicity endpoints
oCHEM	https://ochem.eu/home/show.do	Screens chemicals against known structural alerts associated with different endpoints
OECD QSAR Toolbox	http://www.oecd.org/env/ehs/risk-assessment/theoecdqsartoolbox.htm	Tool for category formation and read-across; uses 'profilers' to enable grouping
TerraQSAR	http://www.terrabase-inc.com/	Modules predict toxicities using knowledge of molecular fragments and a neural network approach
T.E.S.T.	http://www.epa.gov/nrmrl/std/qsar/qsar.html#TEST	Uses seven different methods to predict acute toxicity in daphnia and fish (and other endpoints)
ToxPredict	http://apps.ideaconsult.net:8080/ToxP	Suite of programs capable of predicting over 50

	redict	(eco)toxicological endpoints and properties
VEGA	http://www.vega-qsar.eu/about-qsar.html	Provides access to a series of QSAR models for predicting a range of toxicities (including daphnia acute toxicity)
WEBICE	http://epa.gov/ceampubl/fchain/webice/	Estimates acute toxicity to fish, invertebrates, birds and mammals using knowledge from surrogate species

Figure 1

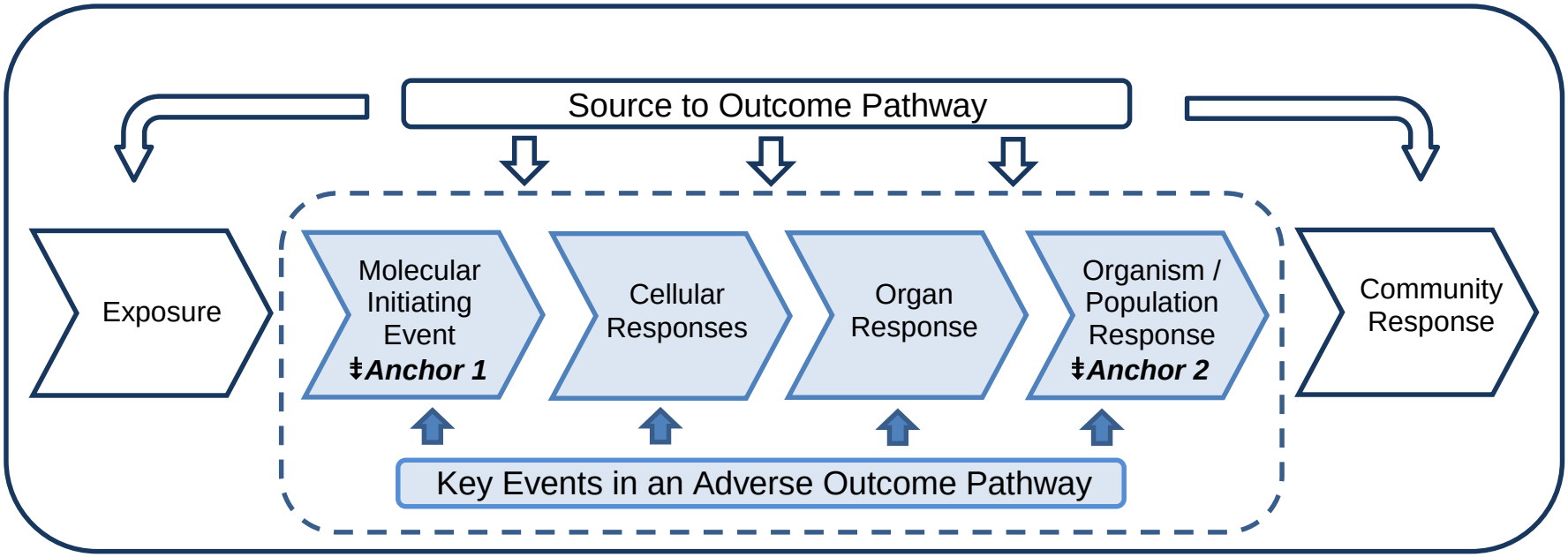

Source to Outcome Pathway
Exposure
Molecular Initiating Event
‡Anchor 1
Cellular Responses
Organ Response
Organism / Population Response
‡Anchor 2
Community Response
Key Events in an Adverse Outcome Pathway

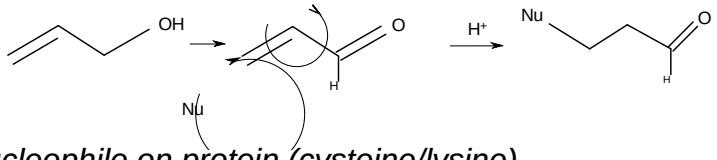
Figure 2

Mechanism

Outcome

Covalent protein binding of chemical

Electrophile



Nucleophile on protein (cysteine/lysine)

➡ Respiratory irritation in gill of fish

➡ Skin sensitisation (allergic contact dermatitis) in humans