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No. 138**

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Series on Testing and Assessment

No. 138

**Report of the Workshop on Using Mechanistic Information in
Forming Chemical Categories**



INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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or contact:

**OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This document is a report of the Workshop on Using Mechanistic Information in Forming Chemical Categories which was held on 8-10 December 2010 in Crystal City VA, USA. The workshop was held following the proposal from the 45th OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology in February 2010.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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REPORT OF THE WORKSHOP ON USING MECHANISTIC INFORMATION IN FORMING CHEMICAL CATEGORIES, 8-10 DECEMBER 2010, CRYSTAL CITY VA, USA

BACKGROUND

1. One of the aims of the OECD QSAR project is to build tools which allow the user to fill data gaps by using existing data for similar chemicals, i.e. to form chemical categories and fill data gaps by read-across or trend analysis. Using mechanistic characteristics to group similar chemicals has been shown to be very successful with the OECD QSAR Toolbox for some endpoints such as skin sensitisation or acute aquatic toxicity. A key aspect in forming toxicologically meaningful categories is identifying mechanistic characteristics (i.e. key events and processes) which relate to the risk assessment endpoint in question and can be measured or predicted.

2. In February 2010, the 45th Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to hold a *Workshop on Using Mechanistic Information in Forming Chemical Categories* with the purpose of acquiring scientific input which will guide further development and use of the concept of the adverse outcome pathway (AOP). An AOP delineates the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal and (when required) population levels of observation

WORKSHOP

3. The workshop was held on 8-10 December 2010 in Crystal City VA, hosted by the United States Environmental Protection Agency (USEPA). The agenda is outlined in Annex 1.

4. The workshop was attended by experts nominated by Australia, Canada, Denmark, France, Japan, Korea, the Netherlands, Norway, the United States, the European Commission, BIAC, ICAPO, invited experts and the OECD Secretariat. The list of the participants is attached to this document as Annex 2. The workshop was chaired by Vicki Dellarco of the USEPA.

Opening

5. Steve Bradbury (USEPA) welcomed the participants on behalf of the United States.

Purpose, Objectives and Specific Aims

6. The aims of the workshop were to:

- review the extent of the knowledge on mechanism or mode of action in the context of key events or processes that lead to specific adverse outcomes that are used in risk assessment;
- propose how scientific information on mechanism or mode of action can be organised as key events and processes within adverse outcome pathways to aid the formation of categories;
- examine a series of case studies using adverse outcome pathways;
- gather input on work flow(s) for using adverse outcome pathways to form chemical categories, and to

- gather input on the role of (Q)SAR methods in forming categories based on key events in an adverse outcome pathway.

Format of the Workshop

7. The format of the workshop was a series of case studies followed by breakout group discussions designed to address the list of question being posed to the participants. The five questions posed to the participants included:

Question 1

What are the major issues that need to be addressed to make the AOP concept usable in grouping chemicals or developing categories? And how exactly can the AOP concept be used in the: near term (i.e., next 2 years), intermediate term (i.e., the following 3 – 5 years), longer term (i. e., by the end of the next decade)?

Question 2

How can an AOP approach be used to inform an integrated approach to testing and assessment (e.g. a tiered approach including alternative methods as well as *in vivo* testing)?

Question 3

How can AOPs be used to guide (Q)SAR methods to predict key events?

Question 4

How can the AOP approach be used to better capture interspecies variability, and improve species extrapolation models? To what extent might AOPs help to identify pathways that are conserved, across taxa (from eco to human), and assist in identifying points where pathways diverge based on taxa?

Question 5

Considering the strengths and weaknesses of the case studies, what best principles can be defined for use in developing an AOP for use in chemical grouping?

Initial Thoughts on Using Mechanistic Information in the Toolbox to Form Categories

8. The Secretariat using skin sensitization as an example demonstrated the importance of using mechanistic information in forming categories. This included how this information is used in the current version of the Toolbox and how it may be used in a future version. This document is found in [Annex 2](#).

Working definitions of the Workshop

9. Kevin Crofton of the USEPA presented a document reviewing the terminology associated with pathways and proposed working definitions to be used during the Workshop. The Workshop participants agreed to use these working definitions for the purpose of the Workshop. These definitions along with examples are found in [Annex 3](#).

Simple Adverse Outcome Pathways

10. The Secretariat presented an example of a AOP (for weak acid respiratory uncouplers). This example served to introduce the concept of an AOP and the case study template adapted for the Workshop to the participants. This presentation and report are found in [Annex 4](#).

Effectopedia a web-based system for data gathering to develop AOPs

11. Hristo Aladjov of the Bulgarian Academy of Science described “Effectopedia”, a web-based application for collecting and storing pathway information. He presented several examples that related to the case studies presented at the Workshop. This presentation is found in [Annex 5](#).

BIAC

12. Hennie Kamp from BASF presented a project by BASF on how metabolomics can be used to form chemical categories and perform read-across for repeat dose toxicity. The participants agreed that this was a good example on how biological information can be used to group chemicals and perform read-across.

Case Study 1: AOP: ER-mediated Reproductive Impairment

13. Patricia Schmieder of the USEPA presented the first case study, the AOP for estrogen receptor (ER)-mediated reproductive impairment. In this example, the chemical categories are formed based on structure requirements for the molecular initiating event of ER-binding. However, the data gap is filled by read-across from *in vivo* data on reproductive impairment to fish. This presentation and report are found in [Annex 6](#).

Case Study 2: AOP: Voltage-Gated Sodium Channels (VGSC) mediated neural toxicity

13. Tim Shafer of the USEPA presented case study two, the AOP for voltage-gated sodium channel mediated neural toxicity. This type of toxicity is exhibited by pyrethroids which alter neuronal firing rates leading to two types of clinical signs. Since these clinical signs are difficult to measure, the chemical category is formed of short-lasting or long-lasting modifications of the cell action potential. This presentation and the report are found in [Annex 7](#).

Case Study 3: AOP: Hemolytic anemia and nephrotoxicity

14. Yuki Sakuratani of the National Institute of Technology and Evaluation in Japan presented case study three, the AOPs for hemolytic anemia induced by anilines and nephrotoxicity induced by 4-aminophenols in 28-day repeated dose testing in rats. The case study showed how liver metabolism differed for these two classes of chemicals and these differences lead to different pathways resulting in different organs being the site of the effect, hemolytic anemia with methemoglobinemia leading to extramedullary hematopoiesis in the case of anilines and renal toxicity (damage to the proximal convoluted tubules) in the case of aminophenol. This presentation and report are found in [Annex 8](#).

Breakout Groups Discussions and Feedback

15. After the presentations of the case studies the participants divided into breakout groups initially for a general discussion of AOPs with later discussions leading to the answers to the questions posed by the Workshop organizers.

Conclusions and Recommendations

18. The Workshop participants agreed on the following answers to the five questions outlined above.

Question 1: What are the major issues that need to be addressed to make the AOP concept usable in grouping chemicals or developing categories? And how exactly can the AOP concept be used in the: near term (i.e., next 2 years), intermediate term (i.e., the following 3 – 5 years), longer term (i. e., by the end of the next decade)?

Answer 1: In order to use AOPs in developing chemical categories there are three information libraries which must be collated, programmed, and integrated; 1) a library of effects used in hazard assessment, 2) a library of molecular initiating events, and 3) a library of AOPs. Also, there is the need to establish the work flow for integrating AOPs with the OECD QSAR Toolbox.

Question 2: How can an AOP approach be used to inform an integrated approach to testing and assessment (e.g. a tiered approach including alternative methods as well as *in vivo* testing)?

Answer 2: AOPs provide a:

- 1) Plausible and transparent means of linking molecular initiating events to the *in vivo* outcomes of regulatory interest and making uncertainties explicit.
- 2) Qualitative means of establishing causal linkages.
- 3) Conceptual framework for organising information at different levels of biological organisation, characterising the weight of evidence.
- 4) Evidence supporting robustness of chemical categories.
- 5) Means of forming categories based on both intrinsic chemical and biological activity.
- 6) Basis for testable hypotheses which in turn leads to the development and use of *in vitro* data bases for developing new profilers and to establish response-to-response relationships.
- 7) Means of developing and justifying targeted and efficient testing and assessment scenarios which save time and resources e.g. by identifying data gaps.
- 8) Means of supporting assessments of combined exposure to multiple chemicals within and across AOPs.
- 9) Greater biological context to what is currently a statistically based approach.

Question 3: How can AOPs be used to guide QSAR methods to predict key events?

Answer 3: QSAR methods can be used to predict chemical structures that can cause a molecular initiating event to trigger an AOP. Note: QSAR methods are important to capture chemistry associated with molecular initiating event, subsequently the focus is often biological events which may be modelled (including by QSAR).

Question 4: How can the AOP approach be used to better capture interspecies variability, and improve species extrapolation models? To what extent might AOPs help to identify pathways that are conserved, across taxa (from eco to human), and assist in identifying points where pathways diverge based on taxa?

Answer 4: The AOP approach could be used to:

- 1) Identify the key event(s) which establish species commonalities and differences.
- 2) Refine assessment factors.

It was agreed that the greater the level of understanding of the events in a pathway, the more likely it is to understand interspecies differences. And this can be explored through the use of discovery tools (e.g., comparing molecular initiating events by using comparative genomics). Note: While several examples of commonalities and differences were discussed many of these were metabolic differences that exist across species and these differences could be qualitative or quantitative.

Question 5: Considering the strengths and weaknesses of the case studies, what best principles can be defined for use in developing an AOP for use in chemical grouping?

Answer 5: An AOP should be based on:

- 1) A single, defined molecular initiating event and linked to a stated *in vivo* hazard outcome(s).
- 2) Any template used for AOP development should include a summary of the experimental support for the AOP and include a statement of the:
 - A) Level of qualitative understanding of the AOP.
 - B) Consistency of the experimental data.
 - C) Confidence in the AOP.
 - D) Level of quantitative understanding of the AOP.

The assessment of the qualitative understanding should include documented identification of:

- 1) The molecular initiating event and molecular site of action.
- 2) Key cellular responses.
- 3) Target tissue/organ(s) and key tissue or organ responses.
- 4) Key organism responses; both physiological and anatomical.
- 5) Key population responses (if required).

The assessment of the evidence in support of an AOP should include criteria based on the IPCS mode of action framework¹ specifically:

- 1) Concordance of dose-response relationships.
- 2) Temporal concordance among the key events and adverse outcome.
- 3) Strength, consistency, and specificity of association of adverse outcome and initiating event.
- 4) Biological plausibility, coherence and consistency of the experimental evidence.
- 5) Identification of any uncertainties, inconsistencies and/or data gaps.

The confidence in an AOP is ascertained by addressing the questions:

¹ Boobis, A.R., Doe, J.E., Heinrich-Hirsch, B., Meek, M.E., Munn, S., Ruchirawat, M., Schlatter, J., Seed, J. and Vickers, C. 2008. IPCS framework for analyzing the relevance of the non-cancer mode of action for humans. Crit. Rev. Toxicol. 38: 87-96.

- 1) How well characterized is the AOP?
- 2) How well are the initiating and other key events causally linked to the outcome?
- 3) What are the limitations in the evidence in support of the AOP?
- 4) Is the AOP specific to certain tissues, life stages / age classes?
- 5) Are the initiating and key events expected to be conserved across taxa?

The assessment of the quantitative understanding should include documented identification of:

- 1) The molecular initiating event.
- 2) Other key events.
- 3) Response-to-response relationships required to scale *in vitro* effect(s) to *in vivo* outcomes.

The identification of the chemical space is critical for forming categories and this is ascertained by addressing the questions:

- 1) What chemicals trigger and do not trigger the molecular initiating event in the AOP?
- 2) What chemical features increase / decrease the probability of a chemical being associated with an AOP?
- 3) Are there similar key events caused by the chemicals that could tie them to a common AOP?
- 4) Are there differences among the chemicals that could lead to sub-categorization?

16. 19. The Workshop participants agreed on the following recommendations on how to advance the use of the AOP concept.

Near Term (i.e., next 2 years):

- 1) Engage toxicologists and other scientists in discussions of AOPs in an effort to foster interactions by developing AOPs for well-established effects (e.g., skin sensitization).
- 2) Complete the proofs of concept began with this workshop by developing AOPs for the several different longer term health and ecotoxicological endpoints.
- 3) Establish, populate and maintain an accessible, electronic repository which allows for the qualitative description of the causal steps of pathways as well as the quantitative response linkages between biological effects in the AOPs, e.g. Effectopedia.
- 4) Develop a strategic plan for identifying, assessing and advancing AOPs and their integration into the Toolbox to include development of; A) an information template which can be used for developing and assessing AOPs, B) a set of guiding principles for assessing the completeness and acceptance of an AOP, and C) a format for attaining mutual acceptance of an AOP.
- 5) Harmonise the terminology associated with AOPs.
- 6) Establish the work flow for using AOPs in categorisation and read-across e.g. by integrating AOPs in the OECD QSAR Toolbox.
- 7) Complete the library of more comprehensive models of chemical conformation, speciation, and metabolic activation/detoxification currently be developed under phase 2 for the OECD QSAR Toolbox.

Intermediate Term (i.e., the following 3 – 5 years):

- 1) Complete the library of effects used in hazard assessment.
- 2) Develop AOPs where the key biological effects are common (i.e., at the cellular-level and conserved across species, sex and life stages).
- 3) Compile an initial series (a few 100) of molecular initiating events relevant to significant adverse outcomes in human health and the environment.
- 4) Review the literature and identify QSAR models and structural boundaries for the initial set of molecular initiating events.
- 5) Expand the library of profilers in the OECD QSAR Toolbox to include the initial series of molecular initiating events.

It is further recommended that specific work should focus on:

- 1) Establishing AOPs and the qualitative relationships between molecular initiating events and hazard assessment endpoints.
- 2) Assessment endpoints which by 2013 will have credible *in vivo* databases.
- 3) Developing profilers where there are relevant *in vitro* data to define applicability domains.
- 4) Integrating these efforts with other projects (e.g., ToxCast, Institute for Health and Consumer Protection, Advisory Group on Molecular Screening and Toxicogenomics etc.).

Longer Term (i. e., by the end of the next decade):

- 1) Compile an advanced series (500-1000) of additional molecular initiating events.
- 2) Compile harmonised *in vitro* data sets for molecular initiating events.
- 3) Compile harmonized data sets for complex *in vivo* endpoints.
- 4) Develop additional AOPs.

It was further recommended that specific work should focus on:

- 1) Establishing the quantitative relationships between molecular initiating events and hazard assessment endpoints.
- 2) Establishing a review procedure at the OECD level for acceptance of AOPs.

ANNEX 1

WORKSHOP AGENDA

OECD WORKSHOP ON USING MECHANISTIC INFORMATION IN FORMING CHEMICAL CATEGORIES TO FILL DATA GAPS FOR REGULATORY PURPOSES

**US-EPA
One Potomac Yard
2777 S. Crystal Drive
Arlington, VA 22202, USA**

8-10 December 2010

The meeting starts at 08h30 on Wednesday, 8 December and closes at 12h15 on Friday, 10 December 2010.

Wednesday, 8 December 2010

08h30 1 Opening and Welcome (15min)

The meeting will be opened by the Chair, Vicki Dellarco of the US Environmental Protection Agency. The Chair will explain housekeeping items. The meeting participants will briefly introduce themselves to the meeting (Tour de Table). Dr Steven P. Bradbury, Director of the Office of Pesticide Programs, US Environmental Protection Agency will provide the welcome.

08h45 2 Purpose, Objectives, and Specific Aims of the Workshop (15min)

The Chair will describe the purpose, objectives and specific aims of the Workshop. The participants are invited to comment as appropriate.

09h00 3 Format of the Workshop and Questions Posed to the Break out Groups (15 min)

The OECD Secretariat will describe the format of the workshop and go over the list of question being posed to the participants in the breakout group sessions. The participants are invited to comment as appropriate.

Part One: Overview of the Current Knowledge of the Adverse Outcome Pathway Concept

09h15 4 Initial thoughts about using the AOPs in forming categories (30min)

The OECD Secretariat will present a proposal of how AOPs may be used to form chemical categories. The participants are invited to discuss the proposal.

09h45 5 Working definitions for the workshop (30min)

Kevin Crofton of the US Environmental Protection Agency will review terminology associated with pathways and propose working definitions to be used during the workshop. The participants are invited to discuss the proposals.

10h15 Coffee break (15min)

10h30 6 An Adverse Outcome Pathway: Simple example (30min).

The OECD Secretariat will provide an example of how an AOP can be used to form chemical categories for weak acid respiratory uncouplers. The participants are invited to comment as appropriate.

11h00 7 Effectopedia a web-based system for data gathering to develop AOPs (45min)

Hristo Aladjov of the Bulgarian Academy of Science will describe “Effectopedia” a web-based application for collecting and storing pathway information and give some examples that relate to the workshop. The participants are invited to comment as appropriate.

11h45 8 Metabolomics – biologically based chemical grouping (30 min)

Dr. Hennie Kamp from BASF will present a project by BASF on how metabolomics can be used to form chemical categories for repeat dose toxicity.

12h15 *Lunch (75min)*

Part Two: Case Studies Presentation and Discussion in Breakout Groups

13h30 9 Guidance for the case study exercise (15min)

Chris Russom of the US Environmental Protection Agency will explain the format of the case study exercise. The participants are invited to comment as appropriate.

13h45 10 Case study 1: AOP: Estrogen receptor-mediated reproductive impairment (30 min)

Patricia Schmieder of the US Environmental Protection Agency will present this case study. The participants are invited to comment as appropriate.

14h15 11 Case study 2: AOP: Voltage gated sodium channel mediated neural toxicity (30 min)

Tim Shafer of the US Environmental Protection Agency will present this case study. The participants are invited to comment as appropriate.

15h15 *Coffee break (15 min)*

15h30 12 Case study 3: AOP: Hemolytic anemia and nephrotoxicity (30 min)

Yuki Sakuratani of NITE in Japan will present this case study. The participants are invited to comment as appropriate.

16h00 13 Breakout groups: Initial discussion (105min)

This initial discussion period in the breakout groups is designed to provide time for the group to have an open, general discussion of AOPs. It also provides an opportunity to ask the Secretariat for clarification on grouping chemicals or anything associated with the case studies.

17h45 **14 Poster session (60min)**

18h45 *Adjourn for the day*

Thursday, 9 December 2010

08h00 **15 Discussion Leaders' summary of first day discussions (10min)**

In plenary session the three discussion leaders will summarize the initial discussions of the breakout groups.

08h10 **16 Breakout groups: Questions 1 (80min)**

The breakout groups will be asked to discuss and answer the question: *What are the major issues that need to be addressed to make the AOP concept usable in grouping chemicals? And how exactly can the AOP concept be used in the near term (i.e., next 2 years)? the intermediate term (i.e., next 3 – 5 years)? and the longer term (i.e., by the end of the next decade)?*

09h30 **17 Plenary rapporteurs's answers to questions 1 (45min; 15 minutes per group)**

The rapporteurs will be asked to briefly present their breakout group's answer to Question 1.

10h15 *Coffee break (15min)*

10h30 **18 Breakout groups: Question 2 (45min)**

The breakout groups will be asked to discuss and answer the question: *How can an AOP approach be used to inform intelligent testing and assessment (i.e., data gap filling, category building, etc.)?*

11h45 **19 Plenary rapporteurs's answers to questions 2 (30min; 10 minutes per group)**

The rapporteurs will be asked to briefly present their breakout group answer to Question 2.

12h15 *Lunch (75min)*

13h30 **20 Breakout groups: Question 3 (45min)**

The breakout groups will be asked to discuss and answer the questions: *To what extent can (Q)SAR methods be used to predict key events in an AOP approach?*

14h15 **21 Plenary rapporteurs's answers to question 3 (30min; 10 minutes per group)**

The rapporteurs will be asked to briefly present their breakout group answer to Question 3.

14h45 *Coffee break (15min)*

15h00 **22 Breakout groups: Question 4 (45min)**

The breakout groups will be asked to discuss and answer the question: *How can the AOP approach be used to better capture interspecies variability, and improve species extrapolation models? To what extent might AOPs help to identify pathways that are conserved, across taxa (from eco to human), and assist in identifying points where pathways diverge based on taxa ?*

15h45 23 Plenary rapporteurs's answers to question 4 (30min; 10 minutes per group)

The rapporteurs will be asked to briefly present their breakout group answer to Question 4.

16h15 24 Breakout groups: Question 5 (45min)

The breakout groups will be asked to discuss and answer the question: *Considering the strengths and weaknesses of the case studies, what best principles can be defined for use in developing an AOP for use in chemical category grouping ?*

17h00 25 Plenary rapporteurs's answers to question 6 (30min; 10 minutes per group)

The rapporteurs will be asked to briefly present their breakout group answer to Question 5.

17h30 Adjourn for the day

Friday, 10 December 2010

08h30 26 Plenary discussion of question 1 culminating in conclusions and recommendations (60min)

09h30 27 Plenary discussion of question 2 culminating in conclusions and recommendations (30min)

10h00 Coffee break (15min)

10h15 28 Plenary discussion of questions 3 culminating in conclusions and recommendations (30min)

10h45 29 Plenary discussion of question 4 culminating in conclusions and recommendations (30min)

11h15 30 Plenary discussion of question 5 culminating in conclusions and recommendations (30min)

11h45 31 Any other business (30min)

12h15 32 Closure of the Workshop

ANNEX 2



Initial Thoughts on Using Mechanistic Information in the Toolbox to Form Categories

WORKSHOP ON USING MECHANISTIC
INFORMATION IN FORMING CHEMICAL
CATEGORIES TO FILL DATA GAPS FOR
REGULATORY PURPOSES
8-10 December 2010, Washington DC



The Problem

- For many hazards, especially long term *in vivo* effects endpoint information that is acquired under a test guideline is not in the scientific sense regarded as defined.
- Hence, it is very difficult to derive chemical categories directly from such information.

OECD  2 OCDE



The Proposed Solution

- is to make reliable predictions, especially for long-term adverse effects by:
 - forming categories based on
 - integrating knowledge of how chemicals interaction with biological systems (i.e., the molecular initiating events) with knowledge of the biological responses derived from
 - adverse outcome pathways.

OECD  3 OCDE



Adverse Outcome Pathways (AOPs)

- delineate the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal and (when required) population levels of observation.

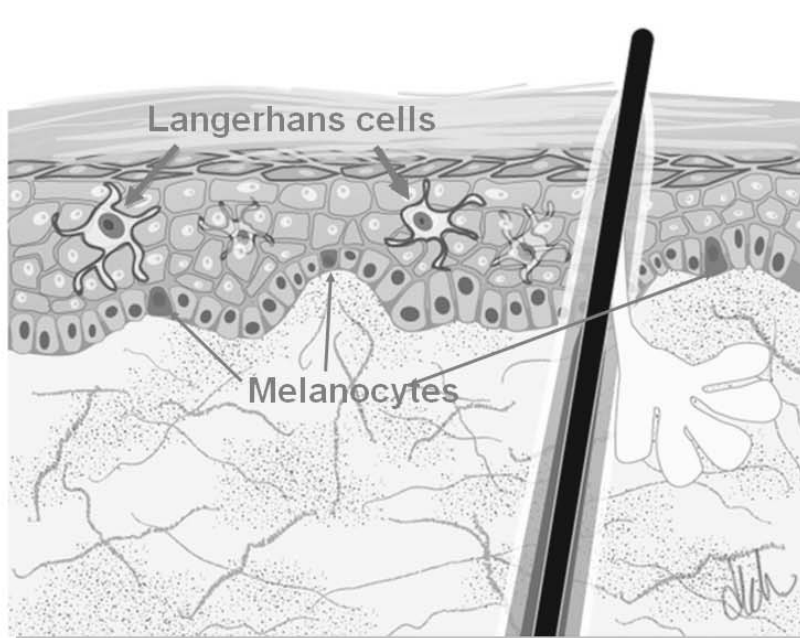
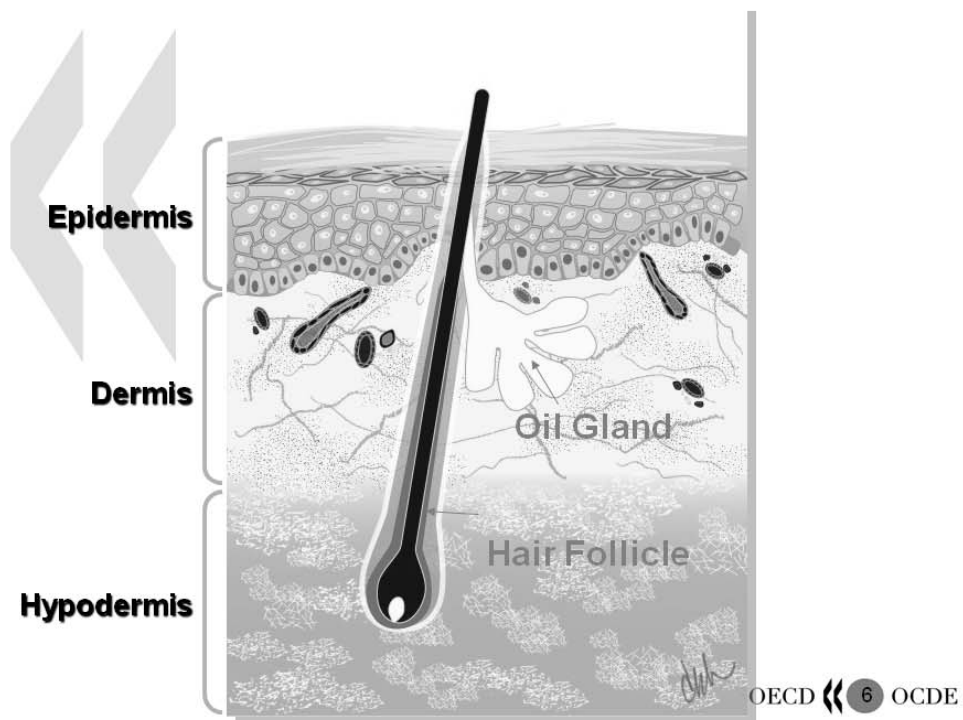
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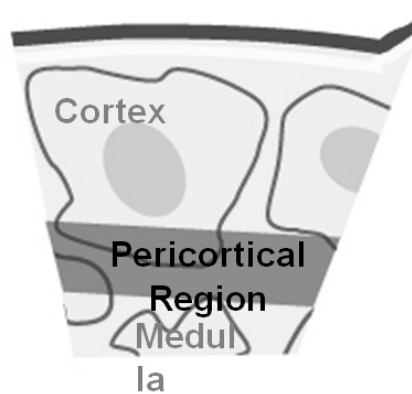
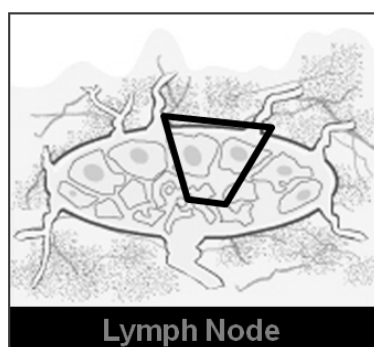
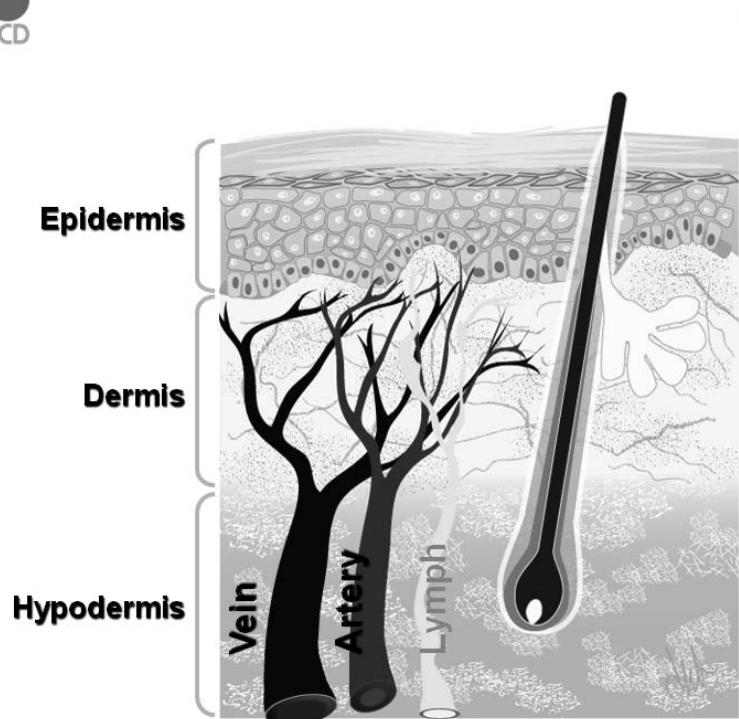


Skin Sensitization: An Example

- Expression of the sensitization requires a high degree of coordination between two organs, the skin and lymph node coupled by the associated vasculature.
- It also requires the coordination of events between at minimum two cell types, the Langerhans dendritic cell and the T-lymphocyte.

OECD  5 OCDE



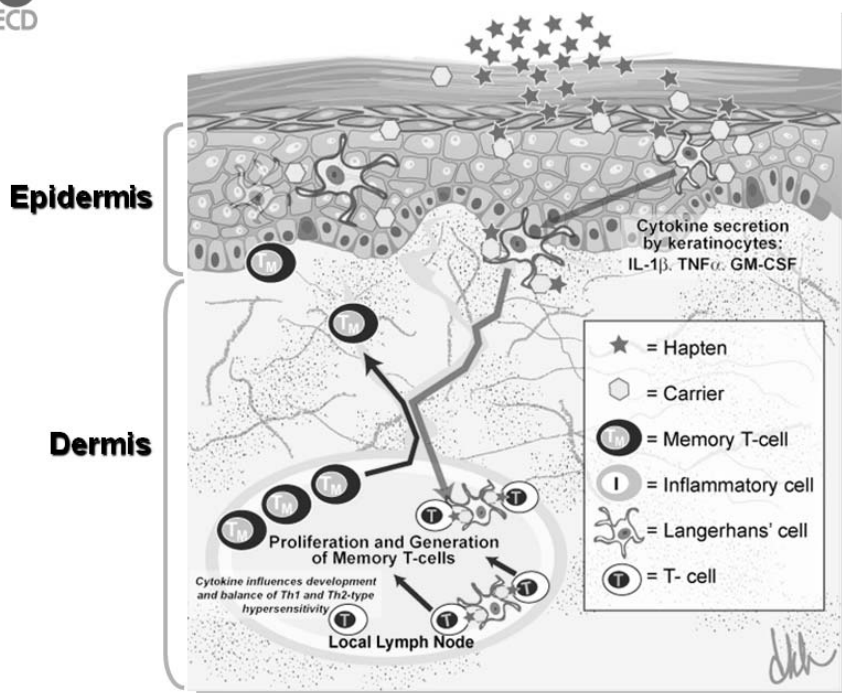


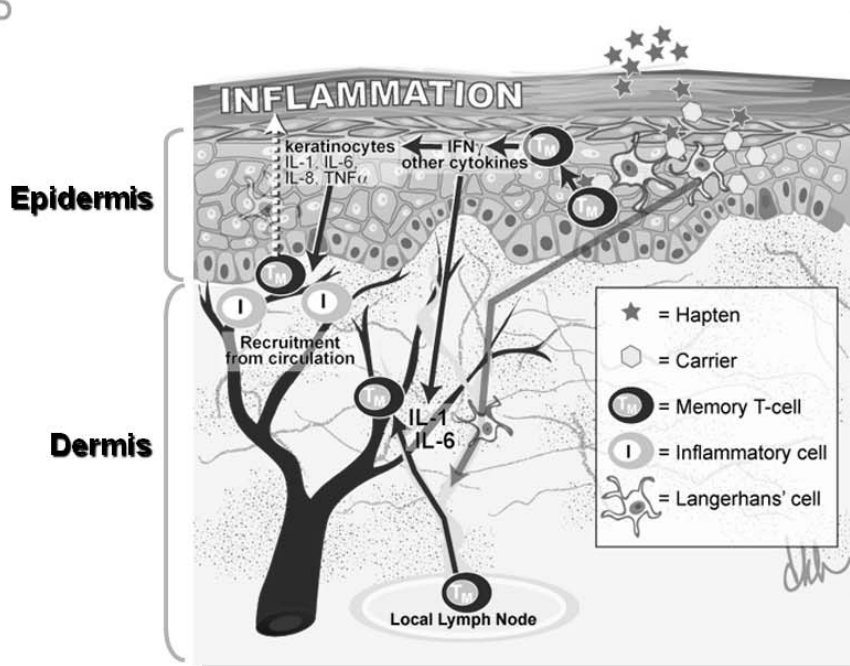


Adverse Outcome Pathways (AOPs)

- *Molecular Initiating Event: Covalent Protein Binding*
 - *In Vivo Effect: Induce immunomodulation through type IV skin sensitization.*
- Adverse Outcome Pathway: Nine steps including:*
- *Induction Phase*
 - *Challenge Phase*

OECD 10 OCDE





Complexity in AOPs

- The amount of detail and linear character of the pathway between a molecular initiating event and adverse outcome can vary significantly.
- This is especially true for health endpoints where effects are the result of:
 - multiple events (e.g., repeat dose toxicity)
 - accumulate over time (e.g., neural toxicity)
 - are particular to a life stage of the organism (e.g., developmental toxicity).



Organizing the Knowledge Bases and Models

- To delineate AOPs will require a flexible framework for:
 - Housing the library of qualitative descriptions of the causal pathways between molecular initiating events and adverse outcomes, and
 - Storing the quantitative response linkages between biological effects in the pathway.

OECD  14 OCDE



Mechanistic Information in the form of AOPs

- will enable the Toolbox to improve the forming chemical categories based on mechanistic understanding of toxicological behavior.
- will improve the capabilities and transparency of predicting no-effect levels and other long term effects with plausible mechanisms of toxic action for observed effects.

OECD  15 OCDE

Information

- Developed into three libraries:
 - The first library is the boundaries of effects used in hazard assessment (i.e., the *in vivo* data).
 - The second library is an expanded listing of profilers to include when possible structural boundaries and QSAR models for each of the hundreds of molecular initiating events.
 - The third library is the array of AOPs which link the profiling events with the hazard outcomes.

OECD  16 OCDE

How to Use Information

- *In vitro* data, such as from molecular screening that define the potential for a specific interaction between a target chemical and a particular “receptor” can be experimentally determined and then modeled as a profiler.
- The chemical category is defined by such profilers and the data gap is filled with the corresponding *in vivo* data.
- The AOPs provide the description of plausible progressions of adverse effects at the different levels of biological organization as well as the response linkages between effects which are needed to quantitatively link the profiled outcome (*in vitro* endpoint) to the hazard outcome (*in vivo* endpoint).

OECD  17 OCDE

Current Use in the Toolbox

- Initial profiling based organic chemistry (i.e., can the chemical react with a protein).
- Secondary profiling based on *in chemico* data, (i.e., experimental potency data on conjugate of the chemical (hapten) in the context of particular protein fragments (thiol or amine).
- Data gap filling by quantitative read-across from *in vivo* data (e.g., LLNA EC3) for the appropriate reaction and appropriate subgroup.

OECD 18 OCDE

Future Use in the Toolbox

- Tertiary profiling based on *in vitro* data on maturation of dendritic cells (i.e., intracellular signaling pathways, gene and receptor expression changes) that based on AOPs are linked to skin sensitization. For example;
- Mitogen-activated kinase pathways are chains of proteins in the cell that communicate a signal from a receptor on the surface of the cell to the DNA in the nucleus. Different sensitizers activate different kinase pathways.
- CD86 is a protein expressed on antigen-presenting dendritic cells that provides signals necessary for T-cell activation and survival. Sensitizers activate CD86 expression.

OECD 19 OCDE



Future Use in the Toolbox

- **CD54 is a transmembrane protein possessing an amino-terminus extracellular domain, a single transmembrane domain, and a carboxy-terminus cytoplasmic domain. The protein's extracellular domain is composed of multiple loops created by disulfide bridges. Disulfide bridges are targets for disulfide exchange electrophiles which act as sensitizers.**
- **Cytokines are small cell-signaling proteins secreted by cells of the immune system which are signaling molecules used in intercellular communication and serve as immunomodulating agents. IL-8 and TNF α secretions are induced by sensitizers.**

ANNEX 3

***Working Draft
10 August 2010
Revised 20 October 2010***

Pathways of Toxicity: Working Definitions and Examples

**Crofton, K.M., Ankley, G., Shafer, T.J., Nichols, J.,
Edwards, S., Villeneuve, D. and Wolf, D.C.**

**National Health and Environmental Effects Research Laboratory
Office of Research and Development
US Environmental Protection Agency
Research Triangle Park, NC and Duluth, MN.**

Regulatory toxicology, at its essence, involves linking exposures to adverse outcomes. Over the past two decades a variety of groups have advocated systems and pathway-based approaches to define the processes by which toxicants elicit adverse outcomes. Early applications of the pathway approach were often referred to as exposure-dose-response models or biologically based dose-response models (eg., Clewell et al., 1995; Shuey et al 1995). In 2001, a framework for using mode-of-action (MOA) information to determine human relevance of animal data was published by the International Programme on Chemical Safety (IPCS) (Sonich-Mullens et al, 2001). In 2007 the US National Academy of Science published a vision for toxicity testing that was based on the concept of a ‘toxicity pathway’, which was viewed as a molecular initiating event and associated cellular responses (NAS, 2007). The pathway concept was extended to include effects at the population level which is an important level of regulatory concern in ecotoxicology (Bradbury et al., 2004). Termed an adverse outcome pathway (AOP), it describes both pathways initiated via non-specific interactions (e.g., narcosis) as well as more specific ligand-receptor interactions and ligand-receptor interactions (Ankley et al. 2010). An overarching description of the continuum of effects from source to outcome, referred to herein as the Source to Outcome Pathway (SOP) has been proposed to cover all the events from initial environmental release to effects at the community level (Figure 1). All of these pathway approaches are based on the concept that toxicity results from chemical first reaching and then interacting with an initial key target in the organism. Further, this primary interaction or “molecular initiating event” begins a series of cellular and organ level events that result in adverse outcomes, in the organism, the population, and the community.

There are currently a number of different publications that provide overlapping definitions and descriptions of portions of the source to outcome continuum. Figure 1 illustrates the relationships between toxicity pathways, mode-of-action pathways, adverse outcome pathways, and source-to-outcome pathways. The intent of this document is to define these terms in order to provide a ‘standard’ basis for discussion in the workshop. The use of a common language will serve to focus discussions around use of the AOP concepts in developing (Q)SARs and chemical categories to advance the use of predictive techniques in

risk assessment. Each term is defined as originally described in a seminal reference. Specific examples are then provided to illustrate distinctions among terms with respect to the extent of mechanistic detail, relevance to progressively higher levels of biological organization, and potential applications.

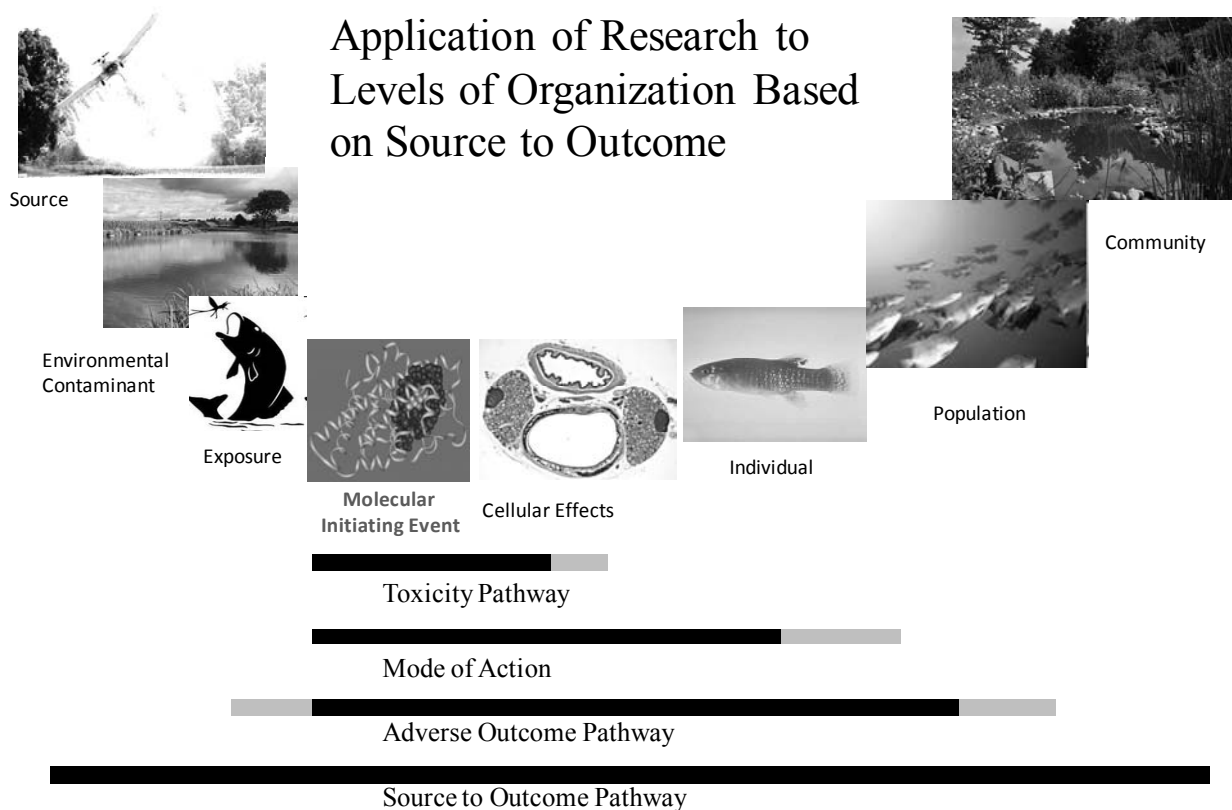


Figure 1. Representation of the relationships between Toxicity Pathways, Mode of Action Pathways, Adverse Outcome Pathways, and Source to Outcome Pathways. The black bars represent the breadth of research common to these concepts. The gray bars represent the theoretical extent of the concepts.

1. Molecular Initiating Event

Definition: The initial point of chemical-biological interaction within the organism that starts the pathway is referred to herein as molecular initiating event. Additional key events further along the pathway that lead to, and are experimentally or toxicologically

associated with, the adverse outcome can be referred to as “key events”. All of these are empirically observable precursor steps that are a necessary element of the mode-of-action or are a biological marker for such an element (USEPA 2005; Boobis, Doe et al. 2008).

Examples: Binding of a xenobiotic to a hepatic nuclear receptor is the molecular initiating events for a variety of pathways, including liver cancer (e.g., Hester et al., 2006). The identification of a molecular initiating event allows the development of methods to screen for chemical interactions with the biological target (e.g., receptor). This is critical for development of QSAR models and use of toxicity pathways in risk assessment (Kavlock and Dix, 2010).

2. Toxicity pathway

Definition: *Cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects are termed toxicity pathways (NRC 2007).*

Example: *Binding of estradiol to estrogen receptors in cells is a normal process that initiates transcriptional pathways important for normal sexual development. However, binding of an environmental xenobiotic (e.g., ER agonist) to the estrogen receptor during development will perturb these normal transcriptional pathways. These perturbations, when large enough or not compensated for, lead to toxicity and disease. In this example, alterations in ER regulated transcription can result in adverse reproductive outcomes.*

3. Mode of action

Definition: The sequence of key events and cellular and biochemical events (measurable parameters) , starting with the interaction of an agent with the target cell, through functional and anatomical changes, resulting in cancer or other adverse health effects (USEPA 2005; Boobis, Doe et al. 2008). Mode of action differs from mechanism, in that the latter implies a more detailed understanding of the molecular basis of the toxic effect (Seed et al., 2005).

Example: Upregulation of hepatic Phase II catabolic enzymes during critical windows of cochlea development leads to hearing loss (Crofton and Zoeller 2005). A number of polyhalogenated aromatic hydrocarbons (e.g., PCBs, dioxins, PBDEs) activate hepatic nuclear receptors (i.e., CAR, PXR). This initiating molecular event triggers upregulation of a number of Phase II enzymes including uridine diphosphoglucuronosyltransferase (UGTs). UGTs will glucuronidate thyroid hormones, which are eliminated via biliary clearance, ultimately resulting in hypothyroxinemia. If this occurs during the development of the cochlea, it leads to malformation of cochlear hair cells, and ultimately, hearing loss.

4. Adverse outcome pathway

Definition: An adverse outcome pathway (AOP) represents existing knowledge concerning the linkage between a the molecular initiating event and an adverse outcome at the individual or population levels (Ankley, Bennett et al. 2009). As such, AOPs by definition, span multiple levels of biological organization. AOPs often start out being depicted as linear processes, however, the amount of detail and linearity characterizing the pathway between a molecular

initiating event and an adverse outcome within an AOP can vary substantially, both as a function of existing knowledge and risk assessment needs.

Example: A wide variety of chemicals cause toxicity via narcosis which, in fish, can be accurately predicted based on propensity of the chemical to interact with cellular membranes/membrane components (Ankley et al. 2009). The octanol-water partition coefficient (K_{ow}) of a chemical is an excellent predictor of the potential of a chemical to cause acute lethality via narcosis, and models relating acute lethality in fish to K_{ow} are widely used in regulatory programs throughout the world, despite the fact that discrete events between the molecular initiating event (interaction with the membrane) and lethality are not well-defined. At the other end of the spectrum are AOPs where the chain of events or cascade of biological responses between a molecular initiating event and an adverse biological outcome are relatively well defined. An example in fish involves the production of vitellogenin (VTG; egg yolk protein) by females. Vitellogenesis is an estrogen-mediated process that occurs in the liver of fish. Chemicals that inhibit the production of sex steroids through different mechanisms, or antagonize the interaction of estrogen with the estrogen receptor, depress hepatic production of VTG, thereby decreasing plasma concentrations of the protein, its deposition to developing oocytes in the ovary, and ultimately decreased egg production and repercussions relative to population-level effects (Ankley et al. 2010). In this instance, having a well-defined AOP has supported the development and application of biomarkers, measurements made at intermediate biological levels of organization (e.g., plasma VTG levels, gonad histopathology), that are predictive of adverse outcomes such as decreased production of viable offspring.

5. Source to Outcome Pathway

Definition: *The continuum or cascade of measurable events starting from release into the environment and ending at an adverse outcome (USEPA 2003).*

Examples: *Source to outcome pathways extend the AOP concept to include a full characterization of exposure. As such, they provide critical information about the sources of compounds in the environment, their relative levels, and the routes of exposure. In addition, source to outcome pathways consider the potential for adverse outcomes in individuals to impact populations and communities. For example, exposure to pyrethroid insecticides may occur via several different sources, such as residues in food and residues from indoor crack and crevice treatments. Market basket surveys provide information about which compounds and the relative level of an individual's exposure based on diet, while swipes from treated areas provide information regarding potential dermal exposure. This information can be incorporated into models that predict exposure levels based on the age, diet and activity levels of different individuals (e.g. adults vs. toddlers). By linking exposure models to toxicokinetic models, environmentally relevant levels of pyrethroid at the target tissue (brain) can be predicted. Pyrethroids disrupt the function of voltage-gated sodium channels that control neuronal firing; at sufficient concentrations, this will lead to alterations in the rate and firing patterns of neurons in the nervous system that could result in pyrethroid toxicity at the individual level.*

Another example is perchlorate, a contaminant of ground and surface water. To characterize sources and exposure, it is necessary to understand the various uses of perchlorate (rocket fuel, explosive mining, automobile airbags, etc) and how its disposal (ponds/lagoons, open-pit burning, hazardous waste landfills) contributes to ground and surface water contamination. Contamination of these sources may lead to

exposure directly via drinking and personal uses (e.g. washing/showering), or indirectly via irrigation of crops which are contaminated and later consumed. Perchlorate disrupts thyroid hormone pathways by inhibiting iodide uptake at the sodium-iodine symporter in the intestines, mammary gland and thyroid gland. Because of the critical role of the thyroid hormone system during development the fetus represents a sensitive subpopulation. Disruption of thyroid hormone homeostasis during development results in permanent neurodevelopmental deficits. Adverse neurodevelopment in human individuals will impact human communities (e.g., increased health care costs and decreased productivity). Perchlorate also has the potential to impact ecological receptors that depend on normal functioning of the thyroid axis to regulate development (e.g., metamorphosis in amphibians), growth, and control of metabolism. These effects would be expected to propagate to the community level. For example, decreased size or increased limb deformities in amphibians may result in greater predation of the abnormal individuals that, over time, would result in decreased amphibian populations.

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EPA
United States
Environmental Protection
Agency

Adverse Outcome Pathways

Working Definitions for the Workshop

Kevin M. Crofton
National Health and Environmental Research Laboratory

Source to Outcome Pathways

Source
Environment Contaminant
Exposure
Molecular Initiating Event
Cellular Effects
Individual
Population
Community

Office of Research and Development
National Health and Environmental Effects Research Laboratory

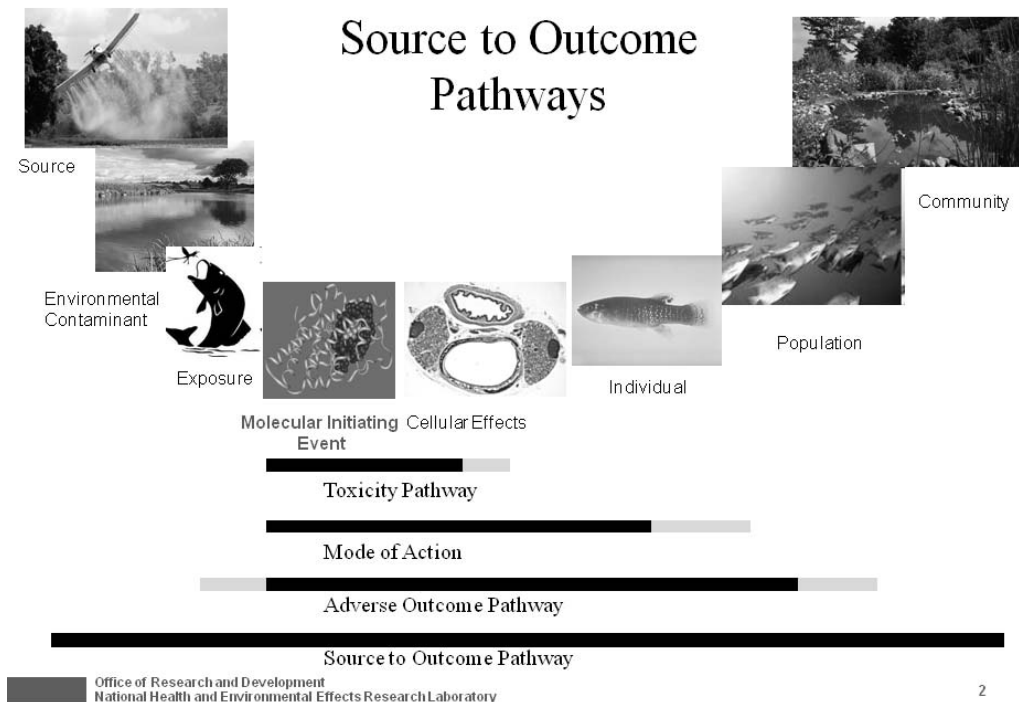
WORKSHOP ON USING
MECHANISTIC INFORMATION IN
FORMING CHEMICAL CATEGORIES
TO FILL DATA GAPS FOR
REGULATORY PURPOSES
8-10 December 2010, Washington DC

Introduction

Goal: to provide a common set of definitions in order to provide a 'standard' basis for discussion in the workshop

Definitions:

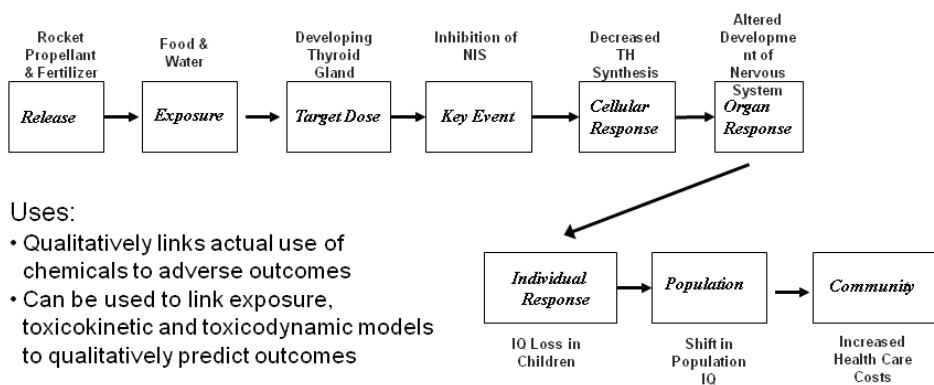
- Source-to-Outcome Pathway
- Adverse Outcome Pathway
- Mode of Action Pathway
- Toxicity Pathway
- Molecular Initiating Event



Source to Outcome Pathway

- **Definition:** The continuum or cascade of measurable events starting from release into the environment and ending at an adverse outcome (USEPA 2003).

- **Example:** Perchlorate

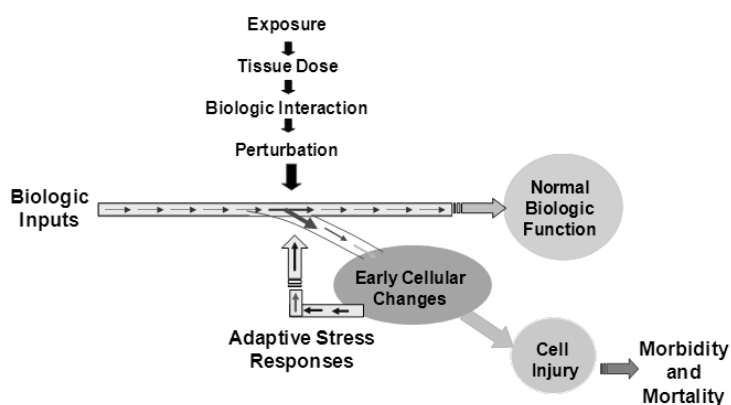


Adverse Outcome Pathway

- **Definition:** An adverse outcome pathway (AOP) represents existing knowledge concerning the linkage between a the molecular initiating event and an adverse outcome at the individual or population levels (Ankley et al. 2009).
- **Examples:** Chemical that antagonize the interaction of estrogen with the estrogen receptor, depress hepatic production of VTG, thereby decreasing plasma concentrations of the protein, its deposition to developing oocytes in the ovary, and ultimately decreased egg production and repercussions relative to population-level effects
- **Use:** Identified key events can be qualitatively and quantitatively linked to an adverse outcome and these events can be used in developing chemical categories.

Toxicity Pathway

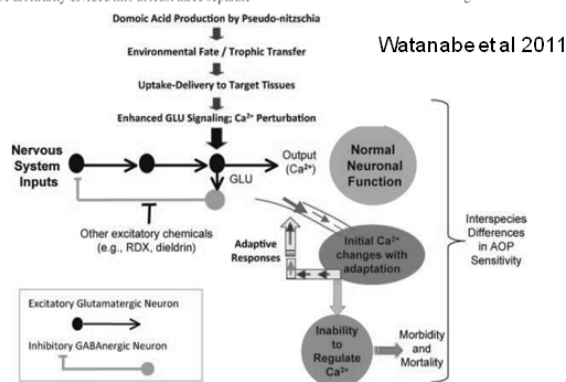
- **Definition:** Cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects are termed *toxicity pathways* (NRC 2007).
- **Example – Normal:** Kainate receptors activated by glutamate open ion channels in neurons regulates ion flux important for neuronal firing.



NRC, 2007

Toxicity Pathway

- **Example - Abnormal:** Domoic acid causes glutamate-induced hyperstimulation of neurons, neurons accumulate excess Ca, and at high enough levels this leads to cell death.



Use: Allows increased understanding of the significance of the perturbation. i.e., how hard does the system need to be hit to result in cell injury.

Molecular Initiating Event

- **Definition:** The initial point of chemical-biological interaction within the organism that results in a cascade of events leading to an adverse outcome.
- **Examples:** Binding of a xenobiotic to a hepatic nuclear receptor is the molecular initiating events for a variety of pathways, including liver cancer (e.g., Hester et al., 2006).
- **Usefulness:** The identification of a molecular initiating event allows the development of methods to screen for chemical interactions with the biological target (e.g., receptor). This is critical for development of QSAR models and use of toxicity pathways in risk assessment (Kavlock and Dix, 2010).

Molecular Initiating Event - Example

PESTICIDES AS PXR LIGANDS

505

Pregnane X Receptor (96-well plate assay)

Usefulness:

- 1) Generate data for chemical categories
- 2) Test chemical categories for relevant AOP

Lemaire et al (2006)

TABLE 2
Effect of Pesticides on hPXR Transactivation in HGPR Cells

Type ^a	Pesticide	Relative activity ^b	SD	Class ^c	Chemical Abstract Service registry number
f	Bupirimate	93.5	5.4	P	41483-43-6
f	Fenarimol	89.6	5.7	P	60168-88-9
f	Propiconazole	85.1	2.7	C	60207-90-1
f	Fenbuconazole	56.1	6.8	C	114369-43-6
f	Prochloraz	50.5	0.1	C	67747-09-5
f	Imazalil	46.5	5.9	C	35554-44-0
f	Mancozeb	29.3	4.9	Dithiocarbamate	7011/18
f	Vinclozolin	19.4	1.5	Oxazole	50471-44-8
h	Pretlachlor	129.5	5.5	CA	51218-49-6
h	Metolachlor	107.2	10.3	CA	51218-45-2
h	Oxadiazon	94.2	7.0	Oxiconazole	19666-30-9
h	Alachlor	71.3	4.3	CA	15972-60-8
h	Isoproturon	50.1	5.0	U	34123-59-6
h	2,4-D	26.1	2.1	PH	94-75-7
h	Diuron	24.5	0.7	U	330-54-1
h	Azinulfuron	23.8	1.9	U	120162-55-2
h	2,4,5-T	23.5	3.5	PH	93-76-5
h	Aminotriazole	21.6	0.2	Triazole	61-82-5
h	Mecoprop	18.0	0.1	PH	93-65-2
h	Atrazine	16.9	2.9	Triazine	1912-24-9
i	Terbufos	114.2	3.8	OC	8001-35-2
i	Permethrin	88.4	6.5	Pyrethroid	52645-53-1
i	Fipronil	58.7	6.9	Pyrazole	120068-37-3
i	Diflubenzuron	33.0	1.0	U	35367-38-5
if	Thiabendazole	31.5	2.1	Thiazole	148-79-8
i	Carbaryl	25.0	3.5	Carbamate	63-25-2
i	Methyl parathion	23.1	1.0	Organophosphorus	298-00-0
i	Nicotine	18.8	0.8	Botanical	54-11-5
i	Heptachlor	18.7	0.6	OC	76-44-8

Note. The dashed lines separate the agonists from the chemicals that failed to activate PXR within each class.

^aType: f, fungicide; h, herbicide; i, insecticide.

^bInduction of HGPR luciferase activity by 10 μ M pesticides expressed as a percent of 3 μ M rifampicin-induced activity. Background value without pesticide was 21.3 \pm 3.1%.

^cClass from Compendium of Pesticide Common Names (www.bcrs.demon.co.uk): P, pyrimidine; C, oxazole; CA, chloracetanilide; U, urea; PH, phenyl; OC, organochlorine.

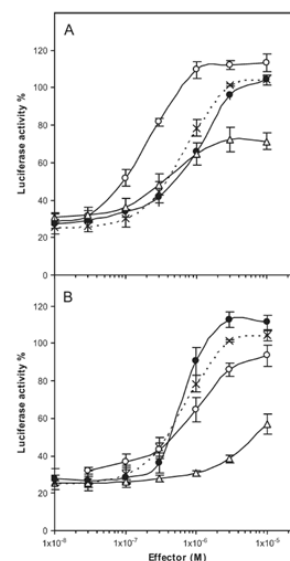


FIG. 3. Induction of luciferase activity by new ligands of hPXR. The activity of HGPR cells was measured as a function of the concentration of (A) herbicides: pretlachlor (○), metolachlor (●), and alachlor (△) as well as rifampicin (dotted line); and (B) various pesticides: oxadiazon (●), bupirimate (○), and fipronil (△) as well as rifampicin (dotted line). The activity was expressed as a percentage of 3 μ M rifampicin-induced activity. Means were calculated from three independent experiments performed in triplicate and expressed \pm SDs.

Thanks For Listening

ANNEX 4

Case study

Name of AOP: Weak Acid Respiratory Uncoupling Outcome: Acute Aquatic Toxicity

Part 1. Building an Adverse Outcome Pathway

Brief summary of the AOP:

For fish acute toxicity the weak acid uncoupling adverse outcome pathway can be described as:

- there is no metabolization of the chemical and distribution of the chemical in the organism is based on partitioning, but also influenced by ionization;
- molecular site(s) of action is the inner mitochondrial membrane;
- molecular initiating event is non-covalent perturbation;
- biochemical effect is the loss of the H-ion gradient that leads to a reduction in ATP formation, which disruptions all ATP-dependent pathways and leads to non-specific inhibition of cellular functions;
- organelle-, cellular-, and organ-level consequences of affecting the ATP-production are reversible;
- target organ(s) or tissue(s) in which the molecular site of action and biochemical effect occur are diverse, but tissue's with high energy demands (e.g., heart muscle) are particularly susceptible;
- key physiological response to the biochemical and cellular effects is increased oxygen consumption (i.e., increased rate of metabolism) and decrease in ATP production;
- there is no key target organ-response to the biochemical, cellular, and physiological effects;
- effect on the organism is reversible, which if left untreated leads to respiratory failure and death.

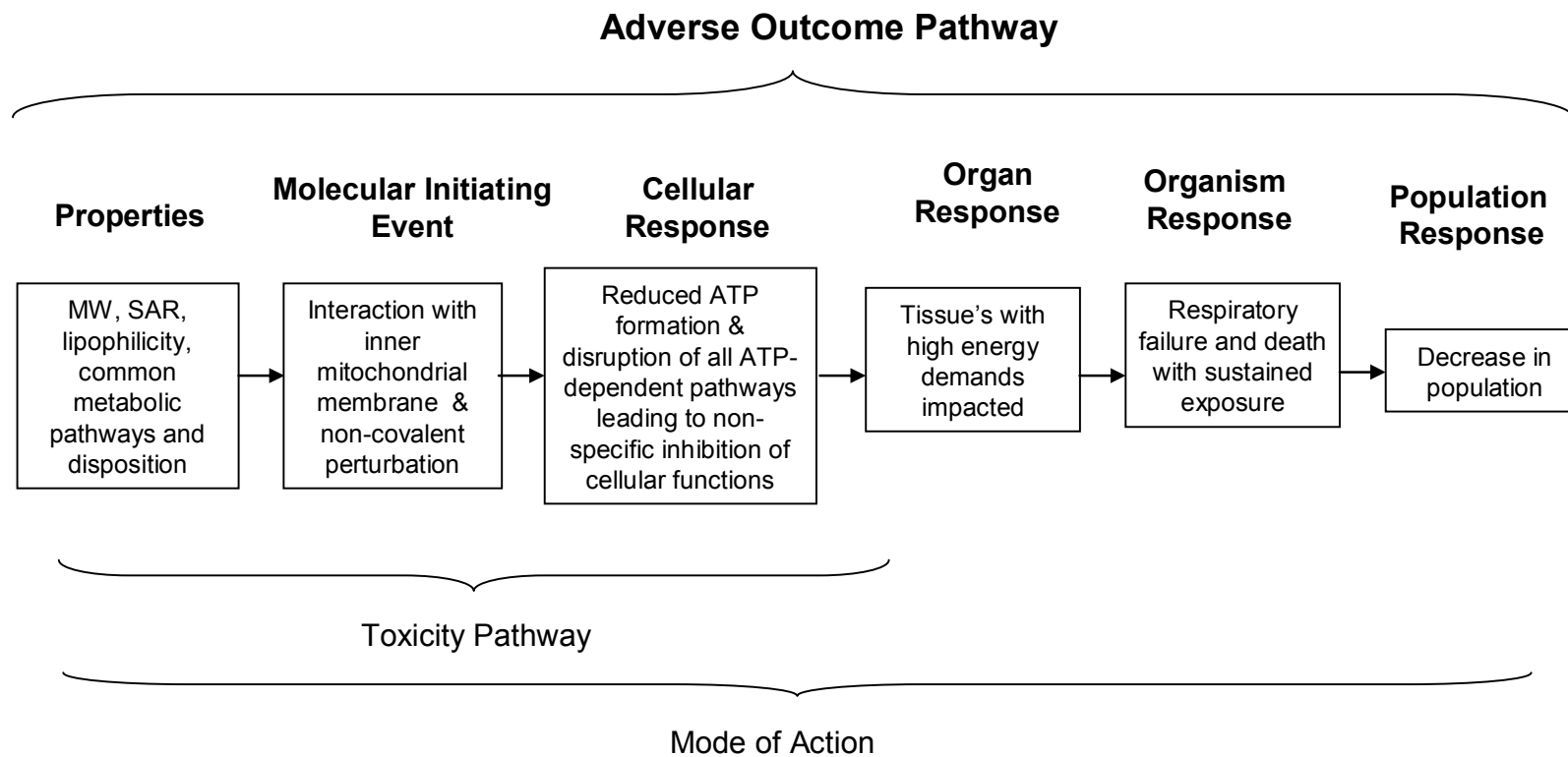


Figure 2. Using a modification of a schematic presented as Figure 1 in Ankley et al. (2010), the proposed adverse outcome pathway (AOP) for weak acid respiratory uncoupling and acute aquatic toxicity is presented for different levels of biological organization.

Discussion of the evidence supporting the AOP:

This section should identify the initiating event (if known), and the sequence of key events (i.e., empirically measurable, precursor steps) across the various levels of biological organization that lead to the adverse outcome. The experimental evidence in support (in vitro, in vivo) of this postulated AOP should briefly be presented drawing on the principles and concepts in the IPCS MOA Framework. In other words, the weight of evidence using criteria based on those described by Bradford Hill.

Background Information:

During normal mitochondria function an H-ion gradient is formed across the inner mitochondrial membrane as a result of metabolically mediated electron transport and the H-ions are used to generate ATP via the ATP-synthetase enzyme complex, which is membrane bound. The movement of electrons down the mitochondrial respiratory chain is coupled to the proton pumping, which builds a large proton motive force (membrane potential) across the mitochondrial inner membrane. The energy transduced by the membrane potential is used for the synthesis of ATP.

An assortment of chemicals is known to interrupt or “uncouple” this process of mitochondrial oxidative phosphorylation; many of these compounds have an ionizable group. Simply put, uncouplers of oxidative phosphorylation (here after referred to as uncouplers) are chemicals, which decrease the efficiency of ATP production or abolish the union between oxygen consumption and ATP production. More specifically uncoupling refers to any energy-dissipating process including the metabolically futile waste of energy, which competes with normal mitochondrial functions. There are a number of types of mitochondrial toxicants or “mitotoxicants” (Wallace and Starkov, 2000). As such there are a number of molecular initiating events leading to the same adverse outcome, each with its own chemical space or applicability domain.

The decoupling of oxidation from phosphorylation by weak acids is explained by mitochondrial proton leaking and the chemiosmotic hypothesis of Mitchell (1966). Briefly, as explained by McLaughlin and Dilger (1980) in the mitochondria, the uncoupler dissolves into the lipid of the inner mitochondrial membrane and crosses it. The mitochondrial matrix is more alkaline than the cytosol so the uncoupler ionization releases a proton into the mitochondrial matrix.

The ionized uncoupler short-circuits the H-ion gradient and provides an alternative process of transporting the H-ions across the membrane (Blaikie et al., 2006). Respiration continues to pump H-ions across the inner membrane into the mitochondrial matrix but no ion gradient is formed because the weak acids dissipate the ions and no ATP is synthesized. The result is that uncoupling chemicals induce a metabolically futile wasting of energy by stimulating resting respiration, while at the same time decreasing ATP yield (Wallace and Starkov, 2000). Molecularly and physiologically, the best studied, weak acid respiratory uncoupler compounds are 2,4-dinitrophenol and pentachlorophenol (Loomis and Lippmann, 1948; McLaughlin and Dilger, 1980; McKin et al., 1987).

Relevant *in Vitro* Data:

The *in vitro* assay Kinspec uses energy transducing membranes extracted from the photosynthetic bacterium *Rhodobacter sphaeroides* (Escher and Schwarzenbach, 1996). In the Kinspec test, a brief “single-turnover” flash of light causes a build-up of membrane potential and the subsequent decay is monitored using time-resolved spectroscopy (Escher and Schwarzenbach, 1996; Escher et al., 1997). It can assess disturbances on energy transduction

such as uncoupling and baseline toxicity but is ineffective in delineating other toxicity categories. The presence of uncouplers acting as protonophores accelerates the decay of the chromatophore membrane potential, because of their ability to transport protons across the membrane. The endpoint for the toxic effect of uncoupling in this assay is the concentration of toxicant needed to induce an observed pseudo-first-order rate constant of uncoupling of 0.5 s^{-1} . Compounds acting as baseline toxicants or narcotics also produce an effect in Kinspec through nonspecific, noncovalent membrane disturbance (Escher et al., 2002). Although the molecular initiating events are very different the same endpoint in Kinspec, (i.e., the concentration of toxicant required to elicit a decoupling rate of 0.5 s^{-1} is used for quantifying uncouplers and baseline narcotics. The main criterion distinguishing the two types of toxicants is the lower effect concentration of uncouplers compared to baseline toxicity (Escher et al., 2002).

Relevant *Tetrahymena* and Microtox Data:

Studies of population growth kinetics and membrane lipid profiles in *Tetrahymena pyriformis* were compared for the classic nonpolar narcotic 1-octanol and the classic weak acid respiratory uncoupler pentachlorophenol (Schultz et al., 2002). The growth kinetics of naive populations of ciliates exposed to these two toxicants was markedly different. Populations exposed to 1-octanol acclimate rapidly to the presence of the toxicant. Ciliates exposed to pentachlorophenol exhibit no indication of acclimation. At low concentrations of pentachlorophenol ciliates accommodate the presence of the uncoupler without impeding population growth. This accommodation probably reflects the fact that cellular ATP production capacity is much larger than is normally required. At high concentrations of pentachlorophenol, ciliate generation time is increased in a concentration-dependent fashion that probably reflects the impairment of cellular energetics.

Analyses of the relative percent of fatty acid methyl esters (FAMES) reveals a pattern of changes linked to toxicant exposure, which are different for uncouplers and narcotics (Schultz et al., 2002). Exposure to the weak acid uncoupler pentachlorophenol results in a decrease in saturated fatty acids and a concomitant increase in unsaturated fatty acids. Exposure to the baseline narcotic 1-octanol results in an increase in saturated fatty acids and a concomitant decrease in unsaturated fatty acids. Exposure to pentachlorophenol results in fatty acid alteration, which are concentration-independent, while exposure to 1-octanol results in fatty acid alteration, which are concentration-dependent. Exposure to pentachlorophenol results in fatty acid alteration in mitochondrial membranes, while exposure to 1-octanol results in no fatty acid alteration in mitochondrial membranes. Taken *in toto* these FAMES alterations are consistent with weak acid uncouplers selectively targeting the mitochondrial membranes in a concentration independent fashion.

Nonpolar narcosis and weak acid respiratory uncouplers are two categories of toxicants where there is good inter-species and inter-endpoint potency agreement (Cajina-Quezada and Schultz, 1990; Schultz and Cronin, 1997; Schultz et al., 1998). Twelve suspected weak acid and 7 weak base respiratory uncouplers have been experimentally evaluated with the *Tetrahymena* aquatic toxicity assay (Schultz et al., 1998). Moreover, 8 suspected weak acid and 10 weak base respiratory uncouplers have been experimentally evaluated with the Microtox aquatic toxicity assay (Schultz et al., 1998).

Relevant Fish Data:

McKim et al. (1997) used fish acute toxicity syndromes based on biochemical and/or physiological effects of exposure, selected as key responses measured *in vivo* from exposure to model chemicals. One syndrome they developed was for weak acid uncouplers based on 2,4-dinitrophenol and pentachlorophenol (McKim et al., 1997) that is different than either the

syndrome for nonpolar narcosis or polar narcosis. The physiological-biochemical responses in spinally transected rainbow trout upon exposure to these two model weak acid uncoupler is a rapid and continuous increase in ventilation volume and O₂ consumption, which corresponds to an increase in the rate of metabolism. These whole fish observations are consistent with the hypothesis of futile metabolism where ventilation increases in an effort to provide more oxygen, which is utilized in respiration, but without the production of ATP. Ten phenolic uncouplers have been tested in *Pimephales promelas* (fathead minnow) 96-hr flow-through acute toxicity assay (Russom et al., 1997).

Relevant Mammalian Data:

Two,4-Dinitrophenol was heralded in the 1930's as a weight loss drug. It was sold as a diet pill under the brand name "Kleenup". However, its narrow therapeutic range resulted in human toxicity, which is characterized by in addition to weight loss, malaise, headaches, increased perspiring, thirst, breathing difficulties leading to respiratory failure and death (Leftwich et al., 1982). In mice the LD₅₀ for 2,4-dinitrophenol is 141 µmol/kg; concentrations inducing 50% uncoupling vary from 30 -100 µmol for ineffective uncouplers like 2,4-dinitrophenol to 5 – 10 µmol for highly effective uncouplers like 2,6-di-tert-butyl-4-(2',2'-dicyanovinyl)phenol.

Data Summation:

- **Concordance of dose-response relationships;** There is agreement among the dose-response relationships (both within and between assays) for 8 to 12 weak acid respiratory uncouplers tested in fish, ciliates, and bacteria.
- **Temporal concordance among the key events and adverse outcome;** There is good agreement between the sequences of physiological events leading to death in fish.
- **Strength, consistency, and specificity of association of adverse outcome and initiating event;** There is good strength and excellent consistence and specificity of association between fish mortality and uncoupling of oxidative phosphorylation, especially for 2,4-dinitrophenol and pentachlorophenol.
- **Biological plausibility, coherence and consistency of the experimental evidence;** The experimental evidence is logical, and consistent with the mechanistic plausibility proposed by the ionic proton shuttle theory.
- **Alternative MOAs (AOPs) that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative MOAs, if supported, require a separate AOP;** Weak base uncouplers, while consistently modeled with weak acid uncouples (see Schultz, 1998), are not supported by the proposed AOP.
- **Uncertainties, inconsistencies and data gaps;** Uncertainty include the structural and physico-chemical cutoffs between phenolic polar narcotics and weak acid uncouplers, the differences between weak acid and other types of uncouplers; there are no inconsistencies within the reported data; the major data gap is the heavy reliance on data for 2,4-dinitrophenol and pentachlorophenol.
- **A clear statement regarding the supporting evidence for the AOP including the level of confidence;** As seen in Table 1 there is excellent experimental support for the seminal events along the pathway.

Table 1. Summary of the experimental support for the AOP.

Key Events	Experimental Support	Strength of Evidence
Key Event 1 (initial event)	Reversible uncoupling of oxidative phosphorylation based on proton shuttle mechanism of action: (Loomis and Lippmann, 1948; Mitchell, 1966; McLaughlin and Dilger, 1980).	Adequate; well accepted mechanism of toxic action.
Key Event 2	Site of action mitochondrial membrane as supported by Kinspec test, (Escher and Schwarzenbach, 1996; Escher et al., 1997) and Tetrahymena FAMES analyses (Schultz et al., 2002).	Limited in number of compounds evaluated, but consistent with mechanistic plausibility.
Key Event 3	Increase in the rate of metabolism as supported by rapid and continuous increase in ventilation volume and O ₂ consumption (McKim et al., 1997).	Limited in number of compounds evaluated, but consistent with mechanistic plausibility.
Adverse Outcome	Fish mortality EC50 values for several chemical (Russom et al., 1997).	Robust <i>in vivo</i> data set supported by similar results with alternative species.

In summarizing the strength of the evidence in support of the AOP, the following should be discussed:

- ***How well characterized is the AOP?***
- ***How well the initiating and key events are causally linked to the outcome?***
- ***What are the limitations in the evidence in support of the AOP?***
- ***Is the AOP specific to certain life stages / age classes? i.e., are there critical life stages where exposure must occur to result in the adverse effect? Or, is the AOP known to be initiated regardless of life stage but key events along the pathway are different dependent on life stage?***
- ***Are the initiating and key events expected to be conserved across taxa?***

Because the molecular initiating event is based on a general cell function- oxidative energetic, the down-stream biological events are well characterized and causally linked. For the same reason the AOP is conserved across taxa and is not life stage- sex- or age-dependent. While data is often limited to two compounds- 2,4-dinitrophenol and pentachlorophenol, this limitation is also a strength as it allows for the direct comparisons between *in vivo*, *in vitro* and alternative species-based testing.

Part II: Building a Category for the AOP Pathway (Identify the chemical space, defined as describing the group of compounds where the

similarity assumption is valid for building the AOP-based chemical category)

This section should discuss considerations in support of building the chemical space (*i.e., those chemical structures that trigger a specific AOP*):

- ***What chemicals are known to trigger the molecular initiating event in the AOP?***
- ***What is “similar” (i.e., pChem, sub-structural fragments), about chemicals associated with this AOP?***
- ***Within a single initiating event, are there unique responses at the cellular / tissue level? If so, describe these linkages, providing supporting evidence, and level of confidence for each response as it relates to the AOP.***
- ***Are there similar key events among the chemicals that could tie them to a common AOP?***
- ***Are there differences among the chemicals that would result in sub-categorization, e.g., different types of chemical interactions that trigger the same initiating event?***

Structural alerts for identifying uncouplers of oxidative phosphorylation were defined in the OECD Guidance Document of Aquatic Effects Assessment (OECD, 1995). These alerts are similar to those implemented in ASTER (Russom et al., 1997), which includes some additional fragments and rules (Table 2).

Table 2. 2D structural features associated with monophenols acting as weak acid uncouplers.

2D Features

- a) Polyhalo-substituted (three or more)
- b) Dinitro-substituted
- c) Polyhalo- and mononitro-substituted (two or more)

Terada (1990) building on earlier work of Stockdale and Selwyn (1971), Miyoshi et al. (1987) and subsequent investigations including the most recent studies of Spycher et al. (2005, 2008a, b) describe in addition to structural alerts physico-chemical properties for phenolic weak acid uncouplers. Physico-chemical properties are restricted to ionization constants (pK_a) and partitioning ($\log K_{ow}$), which according to Spycher et al. (2008a) is related to a low enough energy barrier to allow the membrane permeability of the anion. The question of the range of pK_a values associated with weak acid uncouplers is one of long discussion in the literature. In its most robust form the range of pK_a values associated with weak acid uncouplers varies from 3 to 9; yet there is little if any evidence that all phenols in that pK_a range primarily act as uncouplers. Schultz et al. (1996) with analyses of *Tetrahymena pyriformis* 48-hr IGC₅₀ data developed a series of $\log K_{ow}$ -dependent regression equations for iso- pK_a groups. Through a comparisons of slopes and intercepts they observed that phenols with pK_a values of 6.3 were the most toxic and least influenced by $\log K_{ow}$. A plot of toxicity versus pK_a revealed a bilinear distribution (Schultz et al., 1996). The toxicity of phenols with pK_a values below 6.3 are directly related to pK_a , while the toxicity for phenols with pK_a values above 6.3 are inversely related to pK_a values.

Phenols act by way of several mechanism among these are polar narcosis and weak acid uncoupling (Bradbury, 1994; Russom, 1997; Aptula et al., 2002). In acute aquatic toxicity

narcosis is the basis for observed toxicity unless superseded by a more specific means. The question that therefore arises is what if any is the pK_a value for phenols at which weak acid uncoupling supersedes narcosis? Current work with the largest available data set (i.e., *Tetrahymena pyriformis* 48-hr IGC₅₀ data) shows that the pK_a value between 3 and 6 are related to phenols where uncoupling is predominant. On the other hand pK_a value between 7 and 10 are related to phenols where polar narcosis is predominant. It is uncertain which mechanism predominates for phenols with pK_a value between 6 and 7. This uncertainty is related to the inability to accurately predict pK_a values from structure and the fact that acid phenols impose a pH stress in the *Tetrahymena* assay (Schultz and Burgan, 2003), which impact the experimental IGC₅₀ values.

Part III: Conclusions about the AOP Pathway and Conclusions

Provide a statement regarding the level of confidence of the data completeness and quality in support of the AOP and the identified chemical category.

- ***How strong is the relationship that links the chemical stressor, in a causative manner, to the adverse outcome?***
- ***What is the overall level of Confidence with the chemical category?***
- ***To add a new substance/chemical to this category – what are the necessary data?***
- ***Are there data deficiencies at any step along the AOP that would limit the utility of the AOP concept being applied for a specific purpose (e.g., a greater level of uncertainty may be acceptable for an AOP used for prioritization for further testing than for an AOP used to support a numerical risk determination)?***

Structural alerts (see Table 2) and physico-chemical properties for identifying and modeling phenolic weak acid uncouplers are well documented. Data for adding a new chemical to the category include 2D structure meeting the structural alerts in Table 2 and an ionization constants (pK_a) value. (Q)SAR modeling for potency estimation for acute fish toxicity required a pK_a value and a log Kow value. Current data deficiencies included *in vivo* or *in vitro* data that provides a means of separating weak acid uncouplers from phenolic polar narcotics for chemicals in the a pK_a range of 7.0 to 6.0. An increase in the level of uncertainty is brought on by the lack of an accurate means of estimating pK_a values.

Both the OECD Guidance Document and ASTER also consider anilines and pyridines (i.e., weak bases) as uncouplers of oxidative phosphorylation (OECD, 1995; Russom et al., 1997). However, there is no agreement if weak organic bases act via a proton shuttle mechanism across energy transducing membranes. Specifically, no experimental evidence has been found for chloro- and/or nitro-substituted anilines with submitochondrial particles (Argese, 2002). Moreover, the strong positive dipole potential of the membrane interior of 220–280mV (Clarke, 2001) suggests that the permeability of cations is very limited (Smejtek, 1995). Therefore, it is most likely that the observed effects of weak bases (Cajina-Quezada and Schultz, 1990; Bradbury, 1994; Russom et al. 1997; Schultz et al., 1997; Schultz and Cronin, 1997; Schultz et al., 1998) are caused by another target in mitochondrial membranes.

The discrepancy between the structural alerts for weak acids acting as uncouplers and the diversity of the known active uncouplers can be resolved in one of two ways; either by 1) defining additional structural fragments for the nonphenolic chemicals, or 2) by finding descriptors that are related to the global molecular processes of the uncoupling mechanism, which allow the classification of nonphenolic and phenolic uncoupler. The use of an additional descriptor, while

transparent would require examination of the substituent effects for each class of uncouplers. The former would require more testing to prevent the classification of too many false positive. There is also the possibility that some uncoupler classes will remain undiscovered. A third way addresses this discrepancy, and the one favored by the pathway approach would be to develop separate pathways for each type of respiratory uncoupling.

Part IV; Lessons Illustrated

Discuss the lessons learned from this case study regarding the concept of the Adverse Outcome Pathway and its utility in forming toxicologically meaningful chemical categories for filling data gaps by non-test methods (e.g., read-across and structure-activity relationships) for regulatory purposes.

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The Use of an Adverse Outcome Pathway (AOP) in Developing a Chemical Category for Weak Acid Respiratory Uncouplers (WARUs)

Workshop on using mechanistic information in forming chemical categories
8-10 December 2010, Washington DC

OECD  1 OCDE



AOP for WARU

- **Documentation for this AOP is provided in a background document prepared for this workshop.**

OECD  2 OCDE



Background

- Many types of mitochondrial toxicants.
- Uncouplers induce abolishment of the union between O₂ consumption and ATP production.
- Loss of H-ion gradient across inner mitochondrial membrane.
- Several types of uncouplers, weak acid best studied.

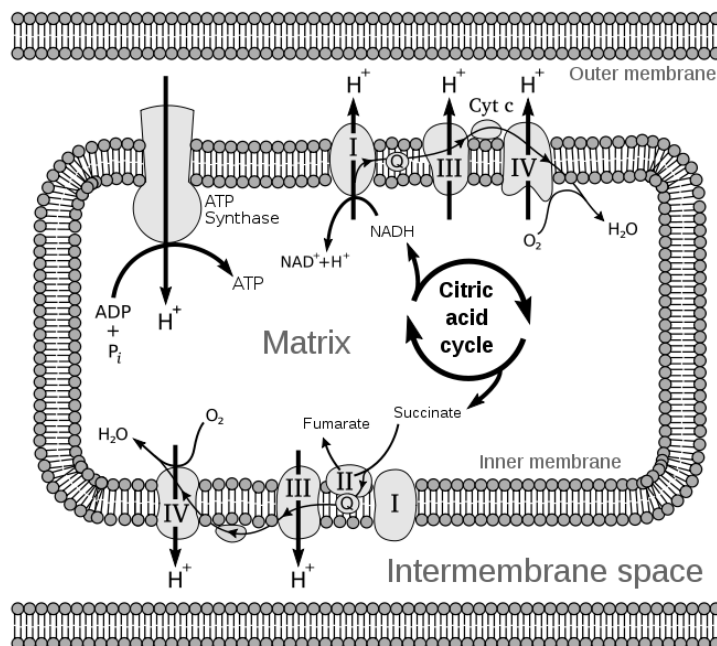
OECD  3 OCDE



Mechanistic-Basis for Toxicity

- During normal mitochondria function an H-ion gradient is formed across the inner mitochondrial membrane as a result of metabolically mediated electron transport and the H-ions are used to generate ATP via the ATP-synthetase enzyme complex, which is membrane bound.
- The WARUs short-circuits the H-ion gradient and provides an alternative process of transporting the H-ions across the membrane.
- Respiration continues to pump H-ions across the inner membrane into the mitochondrial matrix but no ion gradient is formed because the WAs dissipate the ions and no ATP is synthesized.

OECD  4 OCDE



Key Literature Data for Weak Acid Uncoupling AOP

- Biochemical-proton shuttle theory
- In Vitro- Kinspec assay
- Tetrahymena- population growth kinetics; membrane fatty acid alterations; pH-stress
- Fish- Fish Acute Toxicity Syndromes
- Mice- LD50 data
- Human- “Kleenup”



Key Features of Weak Acid Uncoupling AOP

- **Site of Action:** Inner membrane of mitochondria
- **Molecular Initiating Event:** Non-covalent perturbation leading to loss of H⁺ gradient
- **Biochemical Pathway:** Disruption of all ATP-dependent pathways leading to inhibition of general cellular functions
- **Physiological Response:** Increase in ventilation & O₂ consumption; decreased ATP production
- **Target Tissues/Organs:** High energy requirement (e.g., heart muscle).
- **Organism Response:** Respiratory failure & death

OECD  7 OCDE



Key *in vitro* Evidence

- Kinspec assay uses energy transducing membranes extracted from the photosynthetic bacterium (Escher and Schwarzenbach, 1996).
- In the Kinspec test, a flash of light causes a build-up of membrane potential and the subsequent decay is monitored using spectroscopy (Escher et al., 1997).
- The presence of uncouplers acting as protonophores accelerates the decay of the membrane potential, because of their ability to transport protons across the membrane and they induce a pseudo-first-order rate constant of uncoupling of 0.5 s⁻¹.
- Compounds acting as narcotics also produce an effect in Kinspec but it is nonspecific, noncovalent membrane disturbance (Escher et al., 2002).

OECD  8 OCDE

Key Cellular Evidence

- Analyses of the relative % percent of membrane fatty acid methyl esters (FAMES) reveals a pattern of changes linked to toxicant exposure, which are different for uncouplers and narcotics (Schultz et al., 2002).
- Exposure to pentachlorophenol results in a decrease in saturated fatty acids and a concomitant increase in unsaturated fatty acids, which are concentration-independent.
- Exposure to the 1-octanol results in an increase in saturated fatty acids and a concomitant decrease in unsaturated fatty acids, which are concentration-dependent.
- Exposure to pentachlorophenol results in fatty acid alteration in mitochondrial membranes, while exposure to 1-octanol results in no fatty acid alteration in mitochondrial membranes.

OECD  9 OCDE

Key *in vivo* Evidence

- The fish acute toxicity syndrome based on biochemical and/or physiological effects of exposure developed for 2,4-dinitrophenol and pentachlorophenol (McKim et al., 1997) is different than either the syndrome for nonpolar narcosis or polar narcosis.
- The physiological-biochemical responses to the WARUs include a rapid and continuous increase in ventilation volume and O₂ consumption, which corresponds to an increase in the rate of metabolism.
- These whole fish observations are consistent with the hypothesis of futile metabolism where ventilation increases in an effort to provide more oxygen, which is utilized in respiration, but without the production of ATP.

OECD  10 OCDE

Data Summation-1

- ***Concordance of dose-response relationships;*** There is agreement among the dose-response relationships (both within and between assays) for 8 to 12 WARUs tested in fish, ciliates, and bacteria.
- ***Temporal concordance among the key events and adverse outcome;*** There is good agreement between the sequences of physiological events leading to death in fish.
- ***Strength, consistency, and specificity of association of adverse outcome and initiating event;*** There is good strength and excellent consistence and specificity of association between fish mortality and uncoupling, especially for 2,4-dinitrophenol and pentachlorophenol.

OECD  11 OCDE

Data Summation-2

- ***Biological plausibility, coherence and consistency of the experimental evidence;*** The experimental evidence is logical, and consistent with the mechanistic plausibility proposed by the proton shuttle theory.
- ***Alternative MOAs (AOPs) that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative MOAs, if supported, require a separate AOP;*** Weak base uncouplers, while consistently modeled with WARUs (see Schultz, 1998), are not supported by the proposed AOP.

OECD  12 OCDE



Data Summation-3

- ***Uncertainties, inconsistencies and data gaps;*** Uncertainty include the structural and physico-chemical cutoffs between phenolic polar narcotics and WARUs, the differences between WAs and other types of uncouplers; there are no inconsistencies within the reported data; the major data gap is the heavy reliance on data for 2,4-dinitrophenol and pentachlorophenol.
- ***A clear statement regarding the supporting evidence for the AOP including the level of confidence;*** There is excellent experimental support for the seminal events along the pathway.

OECD  13 OCDE



Summary of the experimental support for the AOP

Key Events	Experimental Support	Strength of Evidence
Key Event 1 (initial event)	Reversible uncoupling of oxidative phosphorylation based on proton shuttle mechanism of action (Loomis and Lippmann, 1948; Mitchell, 1966; McLaughlin and Dilger, 1980).	Adequate; well accepted mechanism of toxic action.
Key Event 2	Site of action mitochondrial membrane as supported by Kinspec test (Escher and Schwarzenbach, 1996; Escher et al., 1997) and Tetrahymena FAMES analyses (Schultz et al., 2002).	Limited in number of compounds evaluated, but consistent with mechanistic plausibility.
Key Event 3	Increase in the rate of metabolism as supported by rapid and continuous increase in ventilation volume and O ₂ consumption (McKim et al., 1997).	Limited in number of compounds evaluated, but consistent with mechanistic plausibility.
Adverse Outcome	Fish mortality EC ₅₀ values for several chemical (Russom et al., 1997).	Robust <i>in vivo</i> data set supported by similar results with alternative species.



Building a Category for the AOP

- 2,4-dinitrophenol and pentachlorophenol trigger the molecular initiating event.
- “Similar” chemicals include
 - a) Polyhalo-substituted (three or more)
 - b) Dinitro-substituted
 - c) Polyhalo- (two or more and mononitro-substituted)

OECD  15 OCDE



Mechanistic-Basis for Key Physico-Chemical Properties

- The WARU partitions into the lipid of the inner mitochondrial membrane. Thus, the requirement of lipophilicity for such uncouplers.
- The mitochondrial matrix is more alkaline than the cytosol so the uncoupler ionizes releases a proton into the mitochondrial matrix. Thus, the requirement of a weak-acid moiety for such uncouplers.

OECD  16 OCDE



WARUs Based on *Tetrahymena* Data

- Phenolic (weak acids)
- Log K_{ow} between 1.5 and 5.5 (partitions into mitochondrial membranes)
- pK_a between 6.5 and 3 (ionized in mitochondrial matrix)

OECD  17 OCDE

Data Matrix for Selected Phenols

Phenol	log Kow	pKa	log (1/Tox)
3-NO ₂	2.00	8.36	0.51
2,4-Cl	3.17	7.85	1.04
4-CH ₃ -2-NO ₂	2.15	7.11	0.57
4-Cl-3-NO ₂	2.46	7.73	1.27
2,4,6-NO ₂	1.33	0.42	-0.16
2,4-NO ₂	1.54	4.08	1.08
2-CH ₃ -4,6-NO ₂	2.12	4.31	1.72
2-t-butyl-4,6-NO ₂	3.64	4.80	2.25
2,3,4,6-Cl	3.88	5.40	2.18
2,3,4,5,6-Cl	5.18	4.75	2.42

OECD  18 OCDE



Data Deficiencies and Uncertainty

- Current data deficiencies included *in vivo* or *in vitro* data that provides a means of separating weak acid uncouplers from phenolic polar narcotics for chemicals in the pK_a range of 7.0 to 6.0.
- An increase in the level of uncertainty is brought about by the lack of an accurate means of estimating pK_a values.

OECD  19 OCDE



AOP-Based Category Membership

- Toxicologically meaningful category can be formed based on 2D structure and physico-chemical properties.
- Verification of membership can be based on an *in vitro* assay or battery of assays monitoring O₂ consumption, ATP production, or decay of membrane potential.

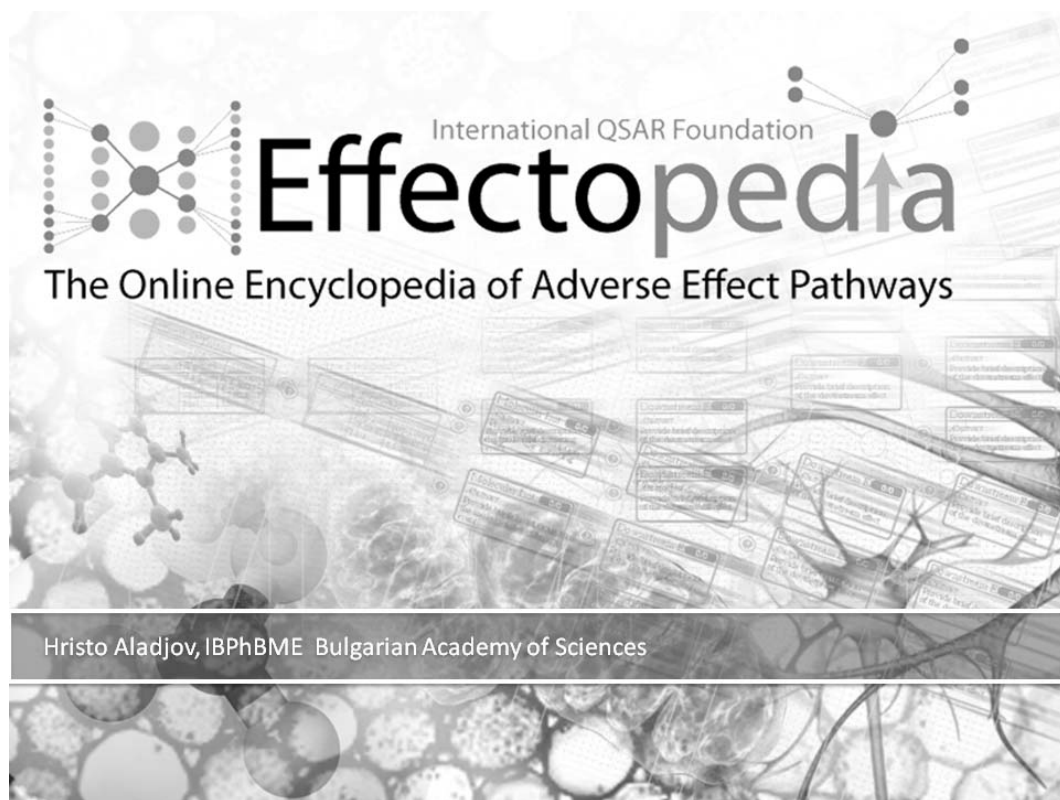
OECD  20 OCDE



Data Gap Filling

- Done by read-across or (Q)SAR modelling based on *in vivo* fish toxicity data (96h LC50 for *Pimephales promelas*).

ANNEX 5

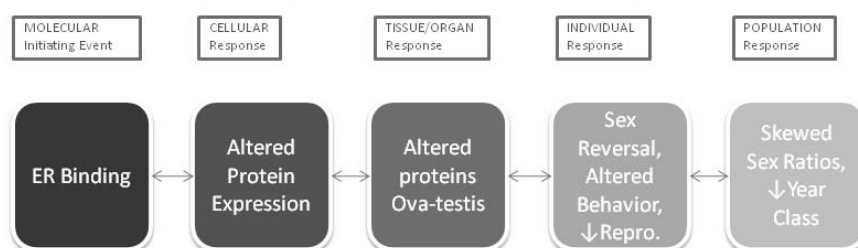


What is the available information and existing solutions to organize it.

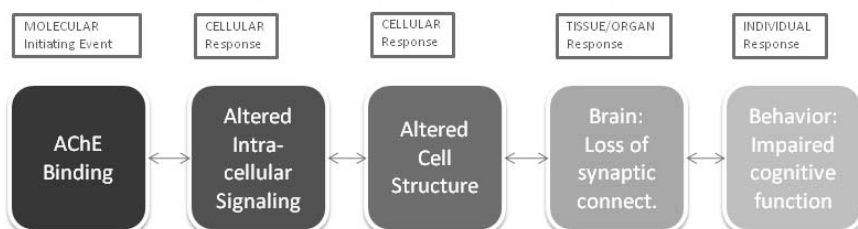
BACKGROUND

ADVERSE OUTCOME PATHWAYS

Generalized Estrogen Receptor (ER) Adverse Effect Pathway

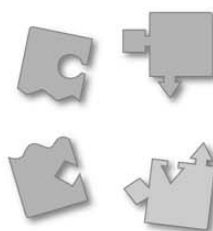


Adverse Effect Pathway of Developmental Neurotoxicity in Humans



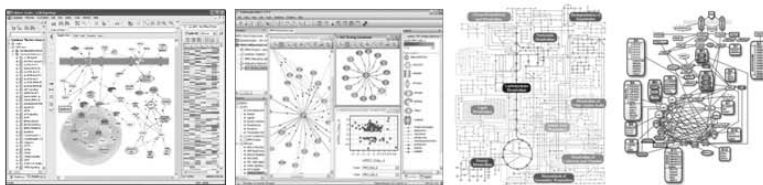
AVAILABLE INFORMATION

- Expert knowledge
- Partial Pathways
- Metabolic trees
- Different test data
 - chronic, acute
 - *in-vivo*, *in-vitro*, *in-chemico*
 - *Public*, *proprietary*



CHALLENGES

- Complexity

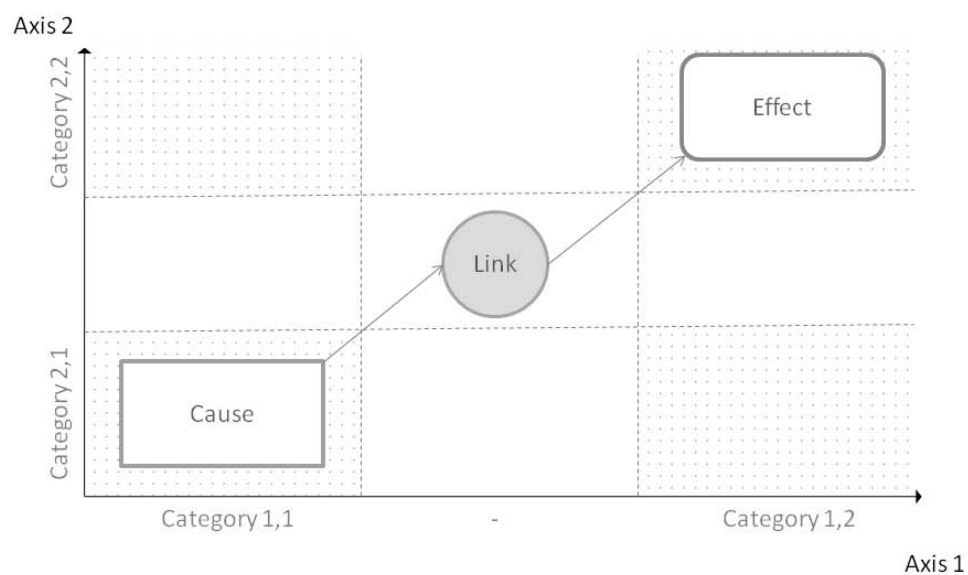


- Multidisciplinary
- Lack of unified pathway representation language
- Accessibility to the scientific community and the public

Organizing the existing information in adverse outcome pathways

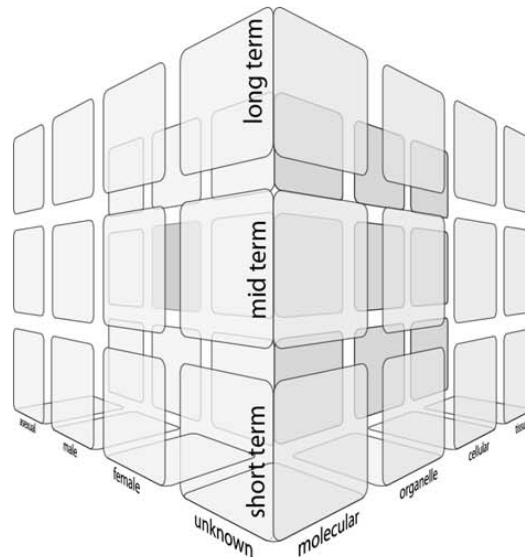
EFFECTOPEDIA

AOP AS DIAGRAM AND CHART

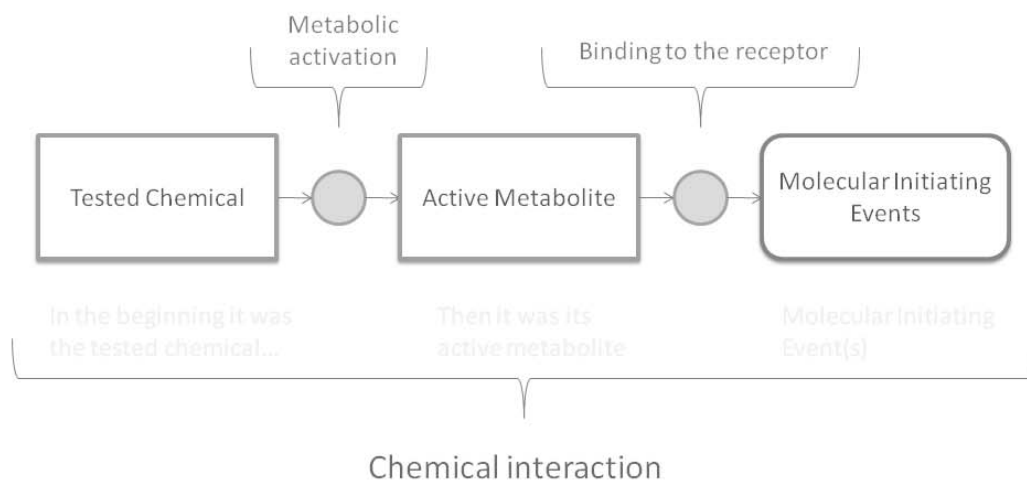


DEFINE PATHWAY SPACE

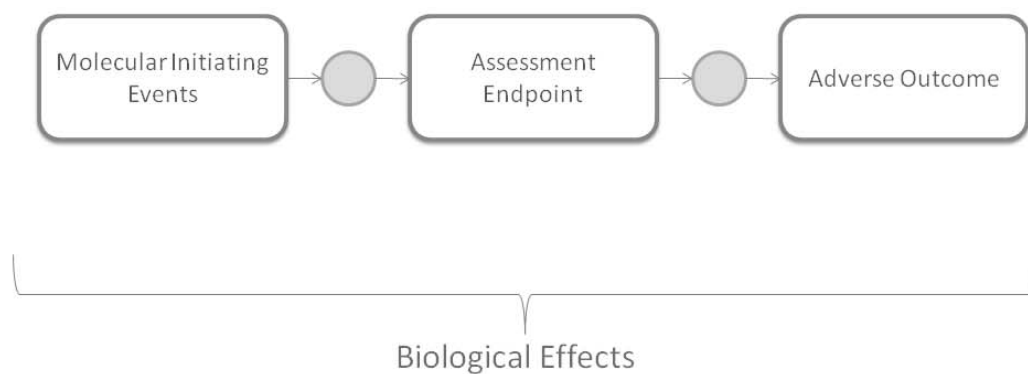
- Life stage
- Taxonomy
- Gender
- Time to effect
- Level of biological organization
- ...



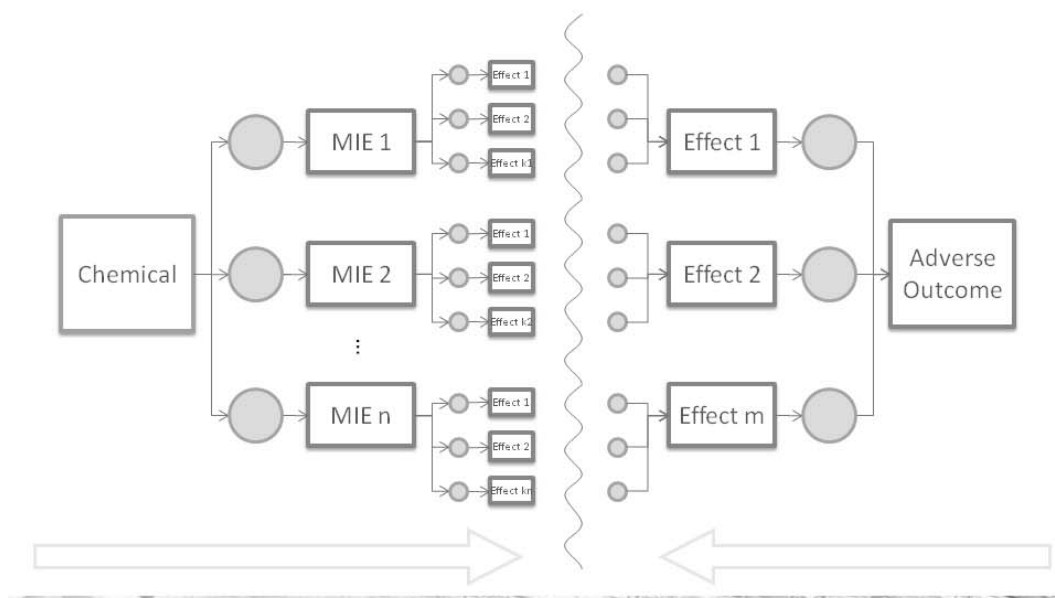
DEFINING AN ADVERSE OUTCOME PATHWAY



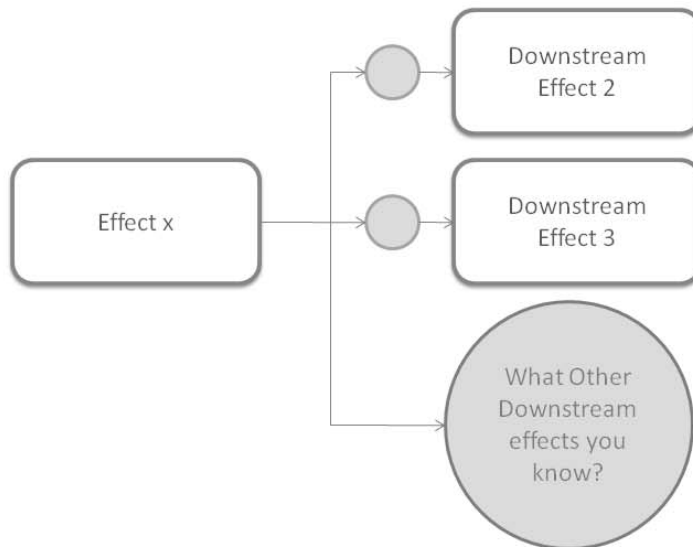
DEFINING AN ADVERSE OUTCOME PATHWAY



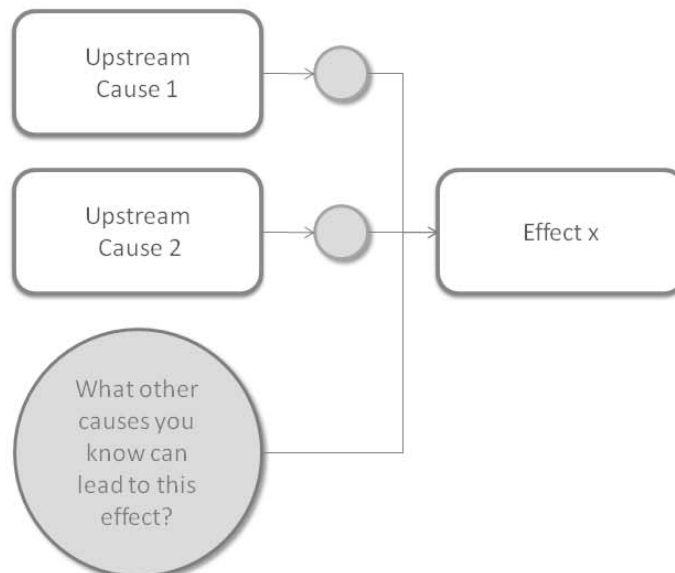
BREAKING COMPLEXITY



BREAKING COMPLEXITY



BREAKING COMPLEXITY



TECHNOLOGY

- Based entirely on open source platform independent solutions



- Accessible with standard web browser
 - For viewing: search engine optimized static HTML pages.
 - For editing : no installation needed web start or applet based editors.
- Standard XML based language for direct data import / export
- Modular, Plug-in architecture
- Collaborative and distributed by design

IMPLEMENTATION

- More than 30 000 lines of pure Java own code
- Core functionalities
 - Visual editor
 - Search Engine
 - History tracking module
 - Suggestion engine and filters
 - XML support
 - ...
- Planned features
 - Chemical database connectivity
 - Reference repository
 - Static HTML pages generation
 - User roles
 - ...

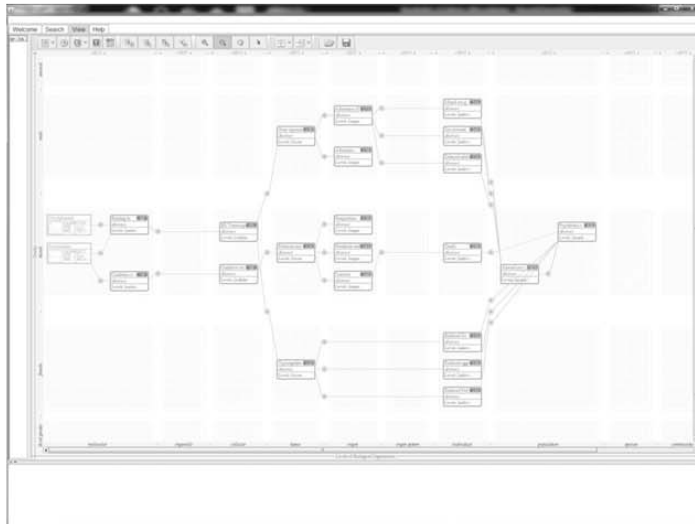
AVAILABILITY

- GNU General Public License version 3.0



- Source repository **SOURCEFORGE.NET®**
- Demo page www.effectopedia.org/go

DEMO






POWER FEATURES

- Instant notification
 - When user contribution is discussed
 - When user contribution is linked
- Create and fund data gap challenges
- Advanced pathway visualization / analysis tools:
 - Show pathways in different relevant pathway projections
 - Sorting and filtering data.
 - Vulnerability slider.



APPLICATIONS

- Toxicology uses case studies of individual test chemicals to understand the adverse effects of chemicals and possible mechanisms (effect pathways)
 - QSAR methods can extrapolate those adverse effects of one chemical to similar chemicals if the chemicals have the same mechanisms and effect pathways (AOP)
 - The OECD category approach is based on grouping chemicals based on similar metabolism and hazardous effect profiles
 - Effectopedia will organize expert data and knowledge of important AOPs and facilitate the grouping chemicals into categories
- 



APPLICATIONS

- Effectopedia will aid hazard assessment with a catalog of AOPS that explain linkages of biological effects at different levels of observation
- Effectopedia will aid hazard assessment with a library of molecular initiating events (MIEs) for important adverse outcome pathways.
- Effectopedia will extend QSAR methods by integrating existing models of hundreds of *in vitro* endpoints with relevant assessment endpoints and outcomes
- Effectopedia will extend the OECD Toolbox with chemical profilers for identifying chemicals with similar receptor binding profiles (MIEs)—“what other chemicals in the inventory might bind to this receptor)



APPLICATIONS

- Effectopedia will provide new order to the complexity of chemicals which are capable of multiple molecular initiating events and causing observed effects arising from multiple pathways.
- Effectopedia will simplify the assessment of which MIEs are most important for each chemical category member under specific test guidelines and species
- The general idea that the relative position of an AOP is dependent on biological vulnerability of the species to the AOP as well as the chemical binding affinity of a specific chemical.
- Complexity increases when the MIEs are the result of activated metabolites in addition to the parent chemicals. Effectopedia will simplify this complexity by extending the AOPs to all metabolites derived from QSAR-driven simulators for specific organ/tissue metabolisms

ESTABLISHING EFFECTOPEDIA

- Sufficient funding for:
 - Building critical mass of knowledge
 - Facilitating user interaction with the system
- Find the balance
 - Simple enough to make it manageable, complex enough to be useful
- Accessibility and advertisement
- Scientific community support

ACKNOWLEDGMENTS

- International QSAR Foundation
- IBPhBME, Bulgarian Academy of Sciences
- OECD, Paris
- National Science Foundation



ANNEX 6

Case Study November 9, 2010

Name of AOP: ER-mediated Reproductive Impairment Outcome: Reproductive Impairment

ER Case Study Background & Context:

The ER-mediated AOP presented and the chemical categories established were used to address the following regulatory need. The USEPA is faced with large numbers (hundreds to thousands) of chemicals that need to be assessed for their potential to cause endocrine disruption. Time and resources dictate that all chemicals cannot be evaluated at once. The challenge is to determine which chemicals should be tested first. Ideally a hypothesis-based approach would be used which focuses on the potential for adverse effects, and prioritizes for testing those chemicals most likely to cause an effect. It is known that interference with hormone receptors (e.g., the estrogen receptor (ER)) can adversely affect reproduction, and further, that diverse chemicals structures can bind ER. The case study presented here relates how the ER-mediated AOP was used in establishing which chemicals (grouped in categories) are higher priority for testing for potential reproductive effects, thus providing an hypothesis-based approach to chemical prioritization instead of choosing chemicals randomly for testing from among the hundreds on specific Agency lists.

Use of the ER-mediated reproductive impairment AOP helped organize what was known and what information was needed to prioritize the Agency chemical lists in question. Understanding of events along the AOP allowed researchers to focus on the molecular and cellular level for making in-lab (*in vitro*) measurements on strategically chosen chemicals within categories found on the chemical inventory. The new knowledge gained was used to build an Expert System using a chemical groups/sub-groups (chemical category) approach to predict an untested chemicals potential to bind ER based on structure. Again, the likelihood of chemical-ER binding is an effects-based rationale used to select chemicals from a large inventory to be tested first for reproductive effects, thus minimizing use of resources, time, and animals on chemicals less likely to produce an effect and instead focusing on those of greater concern.

The QSAR Expert System, built on *in vitro* test data collected at successive points along the ER-mediated AOP, and the AOP itself, could be used for other purposes besides chemical prioritization. However, in applying the Expert System to answer additional questions there should first be an examination of the AOP and an appreciation for where there is test data linking effects at various levels of biological organization along the AOP, where the greatest uncertainties lie, and whether the information is sufficient to answer any new question being asked.

Part 1. Building An Adverse Outcome Pathway

Brief summary of the AOP:

An estrogen receptor (ER)-mediated reproductive impairment adverse outcome pathway (AOP) can be described for fish as shown in Figure 1. The AOP connects the event that initiates the pathway (*i.e.*, chemical binding to the ER) with a series of measures that can be made at successively higher and more complex levels of biological organization that are plausibly linked to an adverse outcome of concern in risk assessments. The key events in this pathway include:

- Initiation of events by a chemical binding the ER as a result of sufficient chemical uptake into the organism and partitioning to a target tissue with ER-containing cells;
- Cell and tissue level gene transcription and translation, *e.g.*, activation of ER responsive genes indicated by vitellogenin (Vtg; an egg-yolk pre-cursor) protein production in fish liver;
- Organ effects (*e.g.*, appearance of ova in male fish testicular tissue); and
- Adverse reproductive and developmental outcome(s) observed in the individual (*e.g.*, change in secondary sex characteristics (feminization of males); cessation of spawning in females; complete sex reversal (genetic males with fully developed and functioning ovaries).

The boxes in the Figure 1 describe responses that may be observed at the various levels of biological organization. This pathway can also be extended to potential consequence to a fish population as represented in the Population Responses box in Figure 1 where, for instance, sex reversal in individuals can result in skewed sex ratios in the population, or where cessation or decrease in spawning in individuals may potentially result in reduction in a year class in the population.

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Figure 1. The Adverse Outcome Pathway for ER-mediated reproductive impairment in fish.

Discussion of the evidence supporting the AOP - Data Summation:

- **Concordance of dose-response relationships;** Studies have been conducted that match chemical concentrations inducing Vtg in fish liver tissue slices with fish liver concentration *in vivo* where Vtg induction is observed.
- **Temporal concordance among the key events and adverse outcome;** There is good agreement between the sequences of biochemical events leading to reproductive impairment in fish. Studies show ER binding and transcriptional/translational changes can occur rapidly while effects at the organ or individual level occur later.
- **Strength, consistency, and specificity of association of adverse outcome and initiating event;** There have been studies across different laboratories and fish species demonstrating the specificity of association between ER binding/gene activation and adverse histopathological changes (*i.e.*, fish ova/testis and sex reversal), especially for potent chemicals (*e.g.*, EE2) as well as for weaker ER agonists (*e.g.*, alkylphenols, etc).
- **Biological plausibility, coherence and consistency of the experimental evidence;** This AOP is well established for multiple chemicals across different fish species. This pathway is also established in other taxa (*e.g.* *Xenopus*) and mammals, albeit with differences in the measured effects along the pathway.
- **Alternative MOAs (AOPs) that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative MOAs, if supported, require a separate AOP;** Adverse reproductive consequences do

occur by other mechanisms. To be grouped in this proposed AOP, demonstration of ER binding and/or Vtg are essential.

- **Uncertainties, inconsistencies and data gaps;** There is uncertainty around the degree of chemical affinity for the ER (e.g., measured *in vitro* relative to E2 affinity) needed to lead to *in vivo* adverse consequences.
- **A clear statement regarding the supporting evidence for the AOP including the level of confidence;** As shown in Table 1 there is good experimental support for the key events along the pathway.

Table 1. Summary of the experimental support for the AOP.

Key Events	Experimental Support	Strength of Evidence
Key Event 1 (initial event)	Chemical binding to the Estrogen Receptor (ER agonism*): (e.g., SAP 2009 and references there-in).	Extensive; Hundreds of chemicals shown to bind ER from humans, rat, mouse, multiple fish species, etc)
Key Event 2	Change in gene expression and protein production (vitellogenin (Vtg)), (e.g., SAP and references there-in).	Considerable; Dozens of chemicals shown to induce Vtg <i>in vitro</i> (isolated fish hepatocytes, tissue slices, reporter cell lines).
Key Event 3	Histopathological changes including reduced testicular growth, appearance of ova in testes (e.g., Jobling et al., 1996; Gray et al., 1999).	Adequate; Many chemicals have been shown to produce ova/testis <i>in vivo</i> in lab and field studies.
Adverse Outcome	Reproductive Impairments in fish e.g., sex reversal, reduced fecundity leading to population collapse (e.g., Seki et al., 2003; Kidd et al., 2007).	Adequate; Relatively fewer studies but sufficient number of compounds evaluated in lab and field studies

*Note that this example focuses on ER agonism. Chemical ER antagonism has also been demonstrated in fish and mammals using pharmaceuticals specifically designed to block ER, however the majority of concern with environmental chemicals is ER-agonism.

ER Binding Affinity: An Indicator of Potential Reproductive Effects in Fish

There are multiple protein targets and pathways through which chemicals may interfere with normal processes resulting in impaired reproduction. Predicting which chemicals may be capable of interfering with any given pathway remains a challenge. Many of the research efforts over the past two decades in the area of endocrine disruption have focused on increasing understanding of how chemicals perturb these endogenous hormone systems. Despite the complexity of events that can lead to reproductive impairment, it has long been appreciated that chemical binding to the ER is one important conserved mechanism of interfering with processes involved in reproduction. The ER-mediated AOP (Figure 1) is a conceptual model that is useful to illustrate how ER binding can be linked through a series of measurable events to adverse outcomes of regulatory concern. As chemicals likely to initiate or block the ER-mediated pathway are identified, the conceptual model is useful for generating testable hypotheses at various levels of biological organization along the pathway. The AOP provides risk assessors and managers a readily articulated and testable rationale for determining, in the context of the current case study, the order (priority) in which chemicals should undergo required screening.

It is important to recognize that while the determination of potential adverse reproductive effects in whole organisms or populations is an important risk assessment issue, it was not a goal of the prioritization effort described in this case study. The potency of a chemical for producing a specific adverse outcome *in vivo* cannot be assumed to be the same as its potency for ER binding in a cell-free assay. This is due to many considerations, for example, chemical kinetics, interactions and feedback between cell and organ systems, etc. While a broad generalization of a single quantitative relationship between ER binding affinity and *in vivo* response across all chemicals cannot be drawn at this time, there is ample evidence that potent ER binders, such as the endogenous estrogen 17- β -estradiol (E2) and the pharmaceutical ethinylestradiol (EE2), when found in the environment cause adverse effects ranging from cessation of egg production in exposed females demonstrated in the lab to complete population collapse in a whole lake study (e.g., Kidd et al., 2007). These potent ER binding chemicals have been shown to bind ER, produce Vtg (*in vitro* and *in vivo*) in male fish where it is normally absent, induce formation of ova in fish testicular tissue, cause cessation in spawning in females, alter secondary sex characteristics resulting in feminized males, and cause the development of fully functional ovaries in genetically male fish exposed during gonad development (SAP 2009 and references there-in).

A somewhat more challenging question is whether chemicals with relatively low ER binding affinity that were not designed to interact with the ER can elicit significant reproductive effects in fish. There are a numerous studies that demonstrate adverse consequences of *in vivo* exposure to chemicals having low *in vitro* ER binding affinities relative to that of E2. Alkylphenols are a well known example where relative binding affinities (RBA) of chemicals in this class can be four to six orders of magnitude less than that of E2. Effects noted upon exposure to lower potency chemicals include reduced testicular growth and ova present in male testicular tissue, changes in secondary sex character and behavior in males, reduced fecundity in females, as well as complete phenotypic sex reversal (e.g., Jobling *et al.*, 1996; Gray *et al.*, 1999; Seki *et al.*, 2003a,b; Yokota *et al.*, 2005). These are just a few citations from a large body of literature on endocrine disruption in fish in lab and field studies.

It should be emphasized that the use of the ER-mediated AOP in this case study is not to predict *in vivo* potency of ER binding chemicals, which is a more complicated and different problem than the specific application of this AOP in developing a hypothesis-driven approach to predict chemicals with ER binding potential. The ER-mediate AOP in fish is used as the scientific basis for focusing on chemical binding to the ER as the parameter to prioritize chemicals for *in vivo* testing. The results of subsequent *in vivo* tests conducted on the priority list of chemicals will then be used to answer the higher tier risk assessment question of whether or not reproductive effects are likely to occur upon environmental exposure to these chemicals. It is especially low affinity chemicals for which prioritization approaches are needed in the current case study example, to identify the likely few chemicals of concern that are unlikely to initiate this pathway from among a large number of chemicals on EPA lists..

The amount of evidence required to support an AOP that will be useful for risk assessments depends upon the question being asked. For instance, use in prioritizing chemicals to identify when further testing is needed would likely require less detail in the AOP than if the pathway was to be used to support a refined risk assessment decision. For the current case study, the fish ER-mediated reproductive impairment AOP is used as the mechanistic basis underlying development of a QSAR-based Expert System for chemical prioritization. The purpose of the system is to prioritize which chemicals, within a specified chemical inventory, should be selected for further testing in *in vitro* or *in vivo* endocrine disruptor assays. Chemicals that bind

the ER, one important pathway leading to endocrine disruption, are more likely to impair reproduction through this pathway than chemicals that are non-binders.

The QSAR-based Expert System is developed by gaining an understanding of which chemical structures bind ER *in vitro*. The system is then used to extrapolate from a smaller number of chemicals shown to bind ER to the larger chemical space of a defined regulatory chemical inventory. In developing the Expert System, chemicals are chosen for testing to bound the chemical space of the inventory based on chemical parameters found to be associated with ER binding within chemicals groups. These chemicals groups that have like-parameters associated with like-activity (ER binding) form the basis for establishing AOP-relevant chemical categories. The work to develop the ER prioritization Expert System is concentrated at the left side of the AOP (Figure 1) to gain better toxicological understanding of how and what chemicals can initiate the pathway. The subsequent testing of chemicals that were prioritized using the Expert System increases the knowledge on the right side of the AOP (Figure 1) where there is more relevance to a risk determination. Thus, as more *in vitro* and *in vivo* testing is done the understanding of, and confidence in use of the pathway will increase.

As mentioned previously, the boxes in Figure 1 list examples of responses (effects) that occur *in vivo* at various levels of biological organization which link the initiation of the pathway to the adverse outcome. In Figure 2 the ER-mediated AOP is shown as it is used to develop a QSAR-based Expert System to predict chemical ER binding to prioritize chemicals for endocrine screening. Figure 2 includes ellipses that: a) encircle the portion of the pathway where *in vitro* assays are applied to determine the range of chemical structures that can initiate the pathway by ER binding, and that can elicit ER-mediated gene activation in cells at the tissue level; and, b) the *in vitro* assay and level of biological organization that serves as the focus area for *in silico* (quantitative structure-activity relationship (QSAR)) model development. As is described in the current example, chemical binding to the rainbow trout ER (the point of AOP initiation), is used to develop a QSAR (*in silico* model).

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Figure 2. The *in vitro* assay and QSAR focus areas within the fish ER-mediated reproductive impairment AOP are used to develop an Expert System for prioritizing chemicals for further testing in *in vivo* and *in vitro* endocrine disruptor assays. Chemical interactions measured in a cell-free rainbow trout ER binding assay were confirmed in a second *in vitro* trout liver slice assay to ensure that binding translated to a higher order effect. Chemical groups or sub-groups were formed based on like-acting chemicals, *i.e.*, those that yielded similar responses in the assays.

A training set of *in vitro* ER binding data was used to build an expert system to predict rainbow trout ER binding affinity, and is relevant to the discussion of building categories in Part II. Additionally, an *in vitro* rainbow trout tissue slice assay (representing a higher level of biological organization; Figure 2) was employed to confirm the chemicals binding ER also resulted in ER-mediated gene activation within a metabolically-competent tissue. Rainbow trout liver slices have been shown to be ER responsive to parent chemicals and their metabolites (Shilling and Williams, 2000; Schmieder et al., 2000; Schmieder et al., 2004). The liver slice assay provides confirmation that the ER binding translates to gene activation or antagonism at the next, higher level of biological organization along the ER-mediated AOP (*e.g.*, tissue/organ level; Figure 1) and increases confidence in the linkage between low affinity ER binding and gene activation. Additional work is on-going to test chemicals giving positive responses in *in vitro* assays in fish *in vivo* assays, to gain further confidence in the AOP for the types of chemicals in the specific inventory to which the Expert System is applied.

The case study presented focuses on rainbow trout *in vitro* data. It should be noted that a good concordance exists between ER binding to the trout ER and binding to the fathead minnow ER (Denny et al., 2005) and other fish receptors (e.g., Nimrod and Benson, 1996; Olsen et al., 2005).

Relevant Mammalian Data

There is also good concordance with chemical binding to ER of mammalian species (SAP, 2009 and references therein). In general the same types of chemicals bind the ER across vertebrate species although the potency may differ.

Part II: Building a Category for the AOP Pathway (Identify the chemical space, defined as describing the group of compounds where the similarity assumption is valid for building the AOP-based chemical category)

Use of the AOP to Investigate Structure Space associated with ER binding.

An understanding of the energetic and steric characteristics of the ER binding domain, therefore, provides the means to establish a mechanistic basis for defining a chemical structure space associated with ER ligands. The receptor is sufficiently promiscuous to permit binding with a diverse array of chemical structures. However, only a small percentage of pesticide and industrial chemicals appear capable of binding the ER.

Earlier models had been developed for ER binding based on higher affinity chemicals (e.g., Weise and Brooks, 1994; Tong et al., 1997; Shi et al., 2002; reviewed in Schmieder et al., (2003a) demonstrating that a diverse array of chemical structure seem to be capable of interacting with the receptor. These previous models, however, did not include sufficient overlap of chemical structures available in the EPA chemical inventories of interest and were thus not adequate for predicting interaction of these low ER affinity compounds. In the current approach, systematic study of chemicals with low binding affinity was undertaken to establish a (Q)SAR training set that reflected current understanding of the ER binding domain, and also was representative of the chemical groups in the specific chemical inventories to which the expert system was to be applied. The Expert System was developed to cover two specific inventories, food use pesticide inert ingredients (FI), and antimicrobial active ingredients (AM), to prioritize chemicals within these inventories for subsequent EDSP Tier I screening.

As mentioned in Part I, *in vitro* assays along the ER-mediated AOP (Figure 2) were used to determine which chemical structures bind ER and induce gene activation. The information is then used to extrapolate from a smaller number of ER binding chemicals to the larger chemical space of the defined inventory. Chemicals selected for ER binding assays were chosen to bound the FI and AM chemical space based on chemical structural group or sub-groups established with consideration also of the chemical parameters (properties) associated with ER binding group. Thus, chemical similarity is determined for grouping “like-acting” chemicals by considering structure and properties associated with ER binding.

Chemicals triggering the AOP initiating event.

The low affinity ER binders within the FI and AM inventories that were found to trigger the ER-mediated AOP, as evidenced by trout ER binding and Vtg induction in trout liver slices, were found to be of two types. Chemicals interacting at Site A (Figure 3) contain a phenolic group and are believed to interact in the ER binding pocket similar to the steroidal A-ring of 17 β -estradiol. Chemicals may instead interact at the B-site (Figure 3) similar to hydrogen bonding with the D-ring of 17 β -estradiol. A series of p-alkylphenols served as a training set for chemicals

interacting at Site A, while a series of *p*-alkylanilines served as the training set for Site B. Chemicals for each series were tested across a Log K_{ow} range starting with the most basic chemical structure, phenol and aniline, respectively, and going up to the 12 carbon alkyl substituted *p*-dodecylphenol and the 8 carbon *p*-octylaniline. For both the lead A-site and B-site series a relationship was found between ER binding affinity and Log K_{ow} for the chemical series as shown in Figure 4.

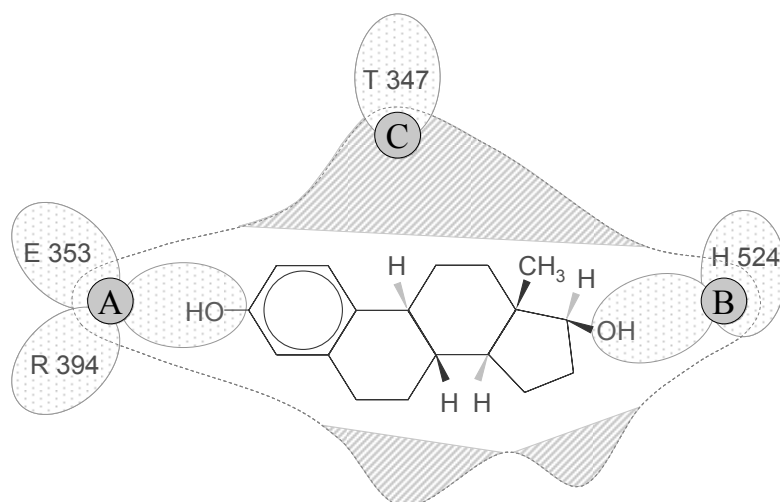


Figure 3. The estrogen receptor ligand binding domain showing sub-pockets A, B, C where chemical ligand hydrogen bonding interactions occur. Receptor protein amino acids involved in interactions with chemical ligands are indicated by letter and sequence number. 17 β -Estradiol is shown in the binding pocket (SAP, 2009).

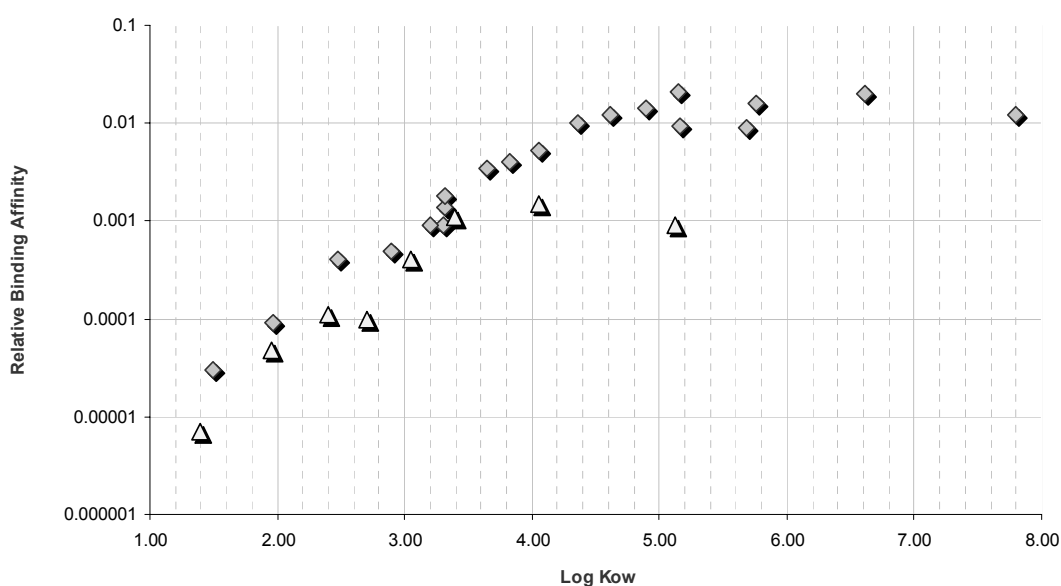


Figure 4. Relationship between rainbow trout estrogen receptor binding (RBA) and Log K_{ow} for chemicals interacting at Site A (e.g., *p*-alkylphenols; blue diamond) or Site B (e.g., *p,n*-alkylanilines; green triangle).

Representative chemicals in > 30 chemical classes were tested to cover the >600 structures in the FI and AM inventories as well as gain a basic understanding of chemical structural attributes associated with rainbow trout ER binding. The information gained from the testing and examination of relationships between Log Kow and binding affinity (e.g., Figure 4) was used to develop an Expert System to prioritize the FI and AM chemicals [SAP, 2009]. The system's decision tree is shown in Figure 5.

Overall there were many types of chemicals found to initiate the ER-mediated AOP including: alkylphenols, alkoxyphenols, parabens, salicylates, alkylanilines, alkoxyanilines, phthalates, branched phenones, etc. The chemicals all initiated at the same key event in the pathway, that is, they all bind to the ER. However, there were differences among the chemicals that we used to sub-categorize them. A major division was whether a chemical was believed to hydrogen bond at Site A or Site B. There is an additional parameter (calculated local atomic charge on O or N) that distinguishes Site A and Site B chemicals. Within Site A and B general grouping the chemicals were further categorized into a chemical class grouping (e.g., p-alkylphenols; p-alkylcyclohexanols, etc). It was also further determined that within some chemical groups there were optimal Log Kow ranges where binding occurred, and below and above which binding did not occur. Thus Log Kow ranges resulting in ER binding > 0.00001% are specified with the chemical group in Figure 5 as appropriate.

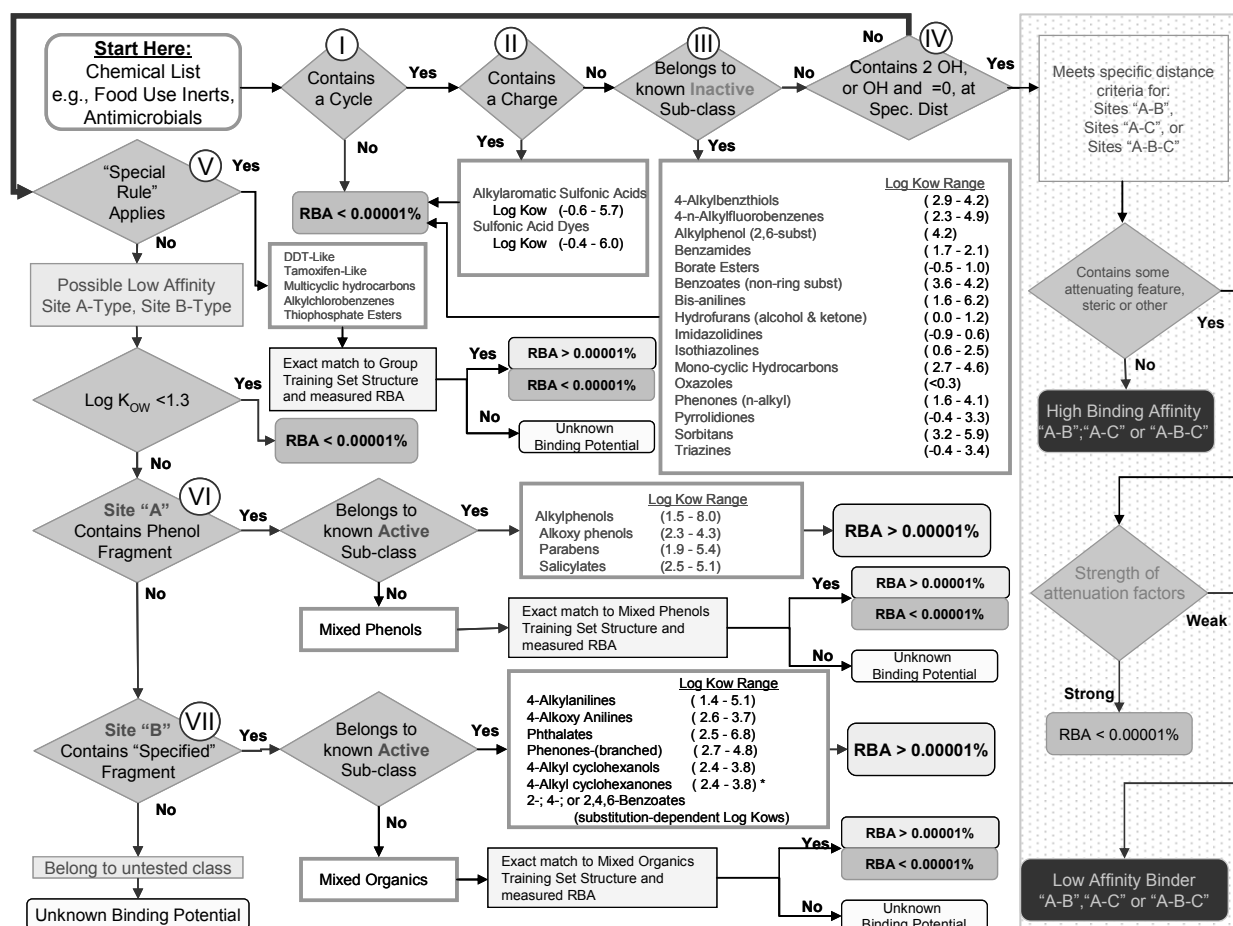


Figure 5. The expert system decision tree based on rainbow trout estrogen receptor binding affinity and trout liver slice transactivation knowledgebase.

There were also chemicals with mixed functionalities found in the FI and AM inventories. The Site A interacting chemicals are listed on Figure 5 as “mixed phenols” because all contain at least one phenolic group in addition to other substituents. For Site B they are identified as “mixed organics”. In the current iteration of the decision tree any Site A or Site B chemicals in the two inventories with mixed functionalities were all included in the training sets. Thus, their ER binding potential is determined in the system by looking for an exact match to the tested training set structure.

It should be noted that the majority of chemicals in both inventories were found not to bind ER. Sections I, II, and III in the decision tree contain: I - all the acyclic chemicals; II - charged compounds; and III – 15 chemical groups with no activity found in the Log Kow ranges tested. Chemicals falling into any of the classes in these sections of the tree are all assigned an ER RBA less than 0.00001%. The assay cutoff reflects the chemical with the lowest apparent binding that resulted in significant Vtg induction in the trout liver slice assay.

The ER expert system decision tree represents an approach to defining the groups (or categories) of chemicals that interact with the receptor and initiate the AOP, as well as the chemical groups that have been shown not to initiate the pathway.

Part III. Strength of association between chemical stressor and the adverse outcome.

In the case of the ER-mediated AOP in fish there is reasonably strong evidence that chemicals that bind the ER produce adverse reproductive effects in the whole organism (e.g., Kidd et al., 2007; Seki et al., 2003 and references there-in). Strong evidence exists that exposure to the endogenous hormone 17 β -estradiol at the wrong time in the reproductive cycle, or with inappropriate timing and concentration, or exposure of males will result in adverse outcomes. Additionally, there are many studies that show ethinylestradiol (the main constituent of birth control pills) found in sufficient concentrations in sewage treatment effluents to cause the same adverse effects that have been demonstrated in the lab cross multiple fish species as well as amphibians (Kidd et al., 2007 and references there-in). There is also ample evidence that weaker ER binders, including those of interest in this case study, produce the same type of adverse effects (e.g., Seki et al., 2003). Numerous studies with octylphenol and nonylphenols have been done that measure effects both in the lab and in field studies, with both short and long-term studies that demonstrate the effects. The relationships found in developing the Expert System presented here raise the hypothesis that similar shorter chain chemicals may also produce adverse effects through this pathway. Work is on-going to systematically test the hypothesis to build a correlation between ER binding potency and *in vivo* effects.

The level of confidence in the multiple chemical categories (the multiple branches) in the decision tree is good for the prediction of ER interaction. The training sets have been tailored to cover all the chemical groups in the FI and AM inventories and to bound all the Log Kow ranges within those groups. Therefore, the confidence is good for this stated application of the ER-mediated AOP, that is, for chemical prioritization. It would be inappropriate to use this same information to predict *in vivo* potencies at this time. As more *in vivo* assay results become available for these low affinity ER binders, it may be possible in the future to determine a trout ER RBA cutoff below which an ER-mediated adverse reproductive effect in fish is unlikely.

To add new chemicals to existing groups (sub-categories) in the current expert system the Log Kow of the chemical is needed. If it is within the tested range then no further testing would be needed (see Figure 5). If it is outside the current Log Kow bounds of the training set for that group then *in vitro* trout ER binding information would be needed. To add a new chemical

group to the system requires *in vitro* testing within that group, ER binding and confirmation of ER gene activation or lack thereof for a few representative chemicals in the group which should be chosen to bound the Log Kow range of the entire group.

Part IV; Lessons Illustrated—Break out group discussion

Discuss the lessons learned from this case study regarding the concept of the Adverse Outcome Pathway and its utility in forming toxicologically meaningful chemical categories for filling data gaps by non-test methods (e.g., read-across and structure-activity relationships) for regulatory purposes.

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The Use of an Adverse Outcome Pathway (AOP) in Developing Chemical Categories for ER-mediated Reproductive Impairment

ER-mediated AOP

Significant evidence exists linking ER binding chemicals to adverse outcomes (ER AOP case study write-up):

- hh - drug design of anti-estrogens for breast cancer research and treatment

- env - multiple studies linking chemical ER binders to adverse effects

 - potent pharmaceuticals - e.g., EE2

 - weak affinity environmental chemicals - e.g., APs

This presentation will focus on:

- using an ER-mediated AOP to form chemical categories for addressing a specific risk assessment application

ER-mediated AOP

The risk context to which AOP is applied influences the approach:

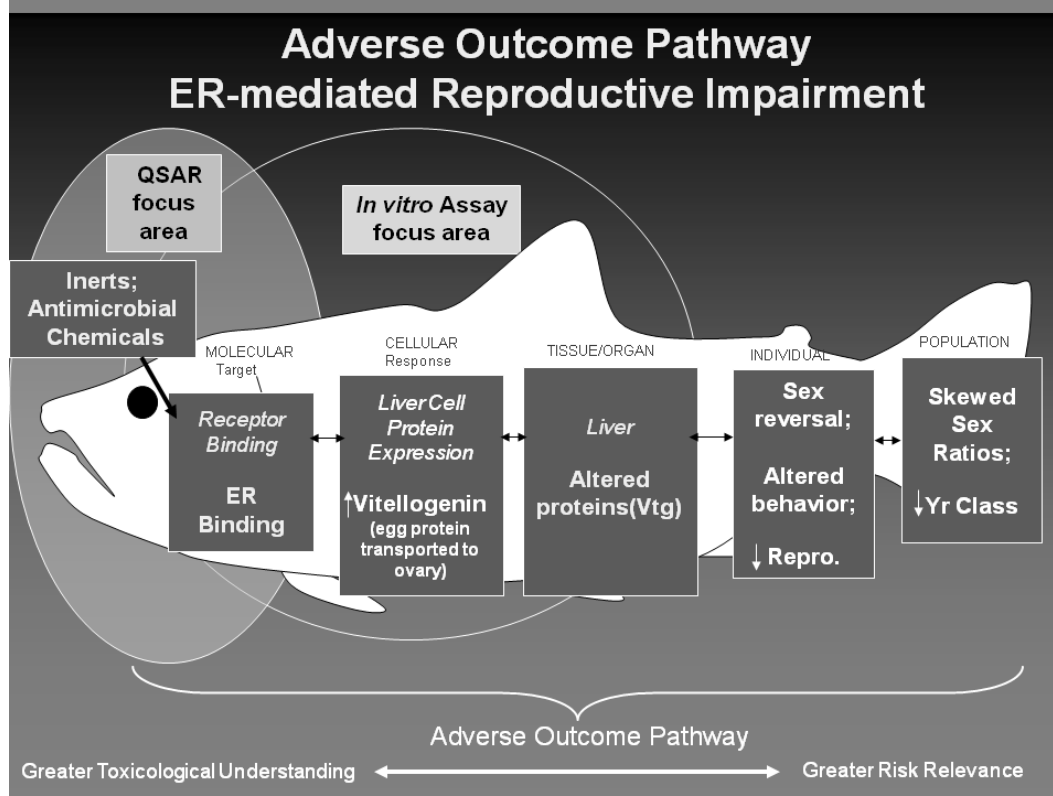
This example:

Risk context – USEPA needs to evaluate large lists of data-limited chemicals for ED potential; how can predictive tools be used to identify which of these chemicals have the greatest potential to cause an adverse effect because of their estrogenic potential;

Goal - Given limited testing resources, prioritize chemicals on targeted inventories, so that those with the highest likelihood of producing an adverse outcome are tested first

Targeted chemical inventories:

- inert ingredients in pesticides used on food
- antimicrobial active ingredients



OECD Principles for QSAR Validation

- Well-Defined Endpoint
 - Well-defined biological endpoint –
 - Informing important risk endpoint – ER AOP - plausible linkage of assay measurement (initiating event) to higher level adversity
 - Well-defined chemistry -
 - Assay(s) used to gather information for category classification allow testing of the types of chemicals (range of properties) found on the regulatory inventories to be prioritized
 - Is the chemical form and concentration in the assay understood?
- Mechanistic interpretation
 - Can estimates be explained mechanistically - chemistry & biology ?
 - Is there a linkage between what's measured and used to build chemical categories and an adverse effect of concern?
 - ER-mediated AOP – MIE related to adverse outcome
 - Relationship of underlying chemical parameter to measured activity (important basis for forming chemical category)

OECD Principles for QSAR Validation (cont.)

- Defined Model Applicability Domain
 - Well-defined application
 - Is the regulatory question well-defined – priority setting is different than risk assessment?
 - Is the QSAR model domain coverage well-defined?
 - Does the QSAR chemical domain adequately cover the regulatory chemical domain i.e., the regulatory question?
- Appropriate measures of goodness of fit, robustness, ability to predict
 - Measures appropriate for a regression model are not necessarily appropriate to evaluate an expert system
- Unambiguous algorithm
 - Expert Systems – logic tree, rules/queries, supporting information
 - Multiple chemical categories coded in logic tree based on mechanistic hypotheses (chemistry & biology)

Application of OECD Principles to Forming Chemical Categories: Example:

Transparency

- How reasonable is the estimate compared with data for similar chemicals ?
- Can the QSAR estimate be explained mechanistically?

Usefulness

- Are the predictions applicable to all the chemicals of regulatory concern?
- Does the model/expert system answer the regulatory question?

Mechanistic Basis of the Expert System to Predict Relative Estrogen Receptor Binding Affinity

- ER Binding Affinity: An Indicator of Potential Reproductive Effects
 - ER AOP
- ER Binding Domain
 - Knowledge/theories of chemical-receptor interactions
 - ER sub-pockets
- The Regulatory Chemical Domain
 - Characterizing the FI and AM inventory chemicals
 - Building from existing information
- The Receptor Binding Assay Domain
 - Optimizing assays considering properties of inventory chemicals

ER Binding Affinity: An Indicator of Potential Reproductive Effects

Mechanistic linkage exists between the risk assessment endpoint (ER-mediated reproductive impairment) and the hazard identification endpoint (ER binding)

Foundation of the assays used to build a category-based ER binding Expert System is the ER-mediated AOP

- The molecular initiating event of the pathway is identified (ER binding)
- The expert system identifies which chemical structures can initiate the pathway
 - Pathway context provides conceptual model useful for generating testable hypotheses
 - Pathway context provides decision-making rationale for the regulatory community

ER-mediated Adverse Outcome Pathway:

- Area of focus consistent with legislative directive
- Chemical binding to the ER is known to have potential to cause adverse effects
- Evidence existed that diverse chemical structures bind ER

ER Binding Affinity: An Indicator of Potential Reproductive Effects

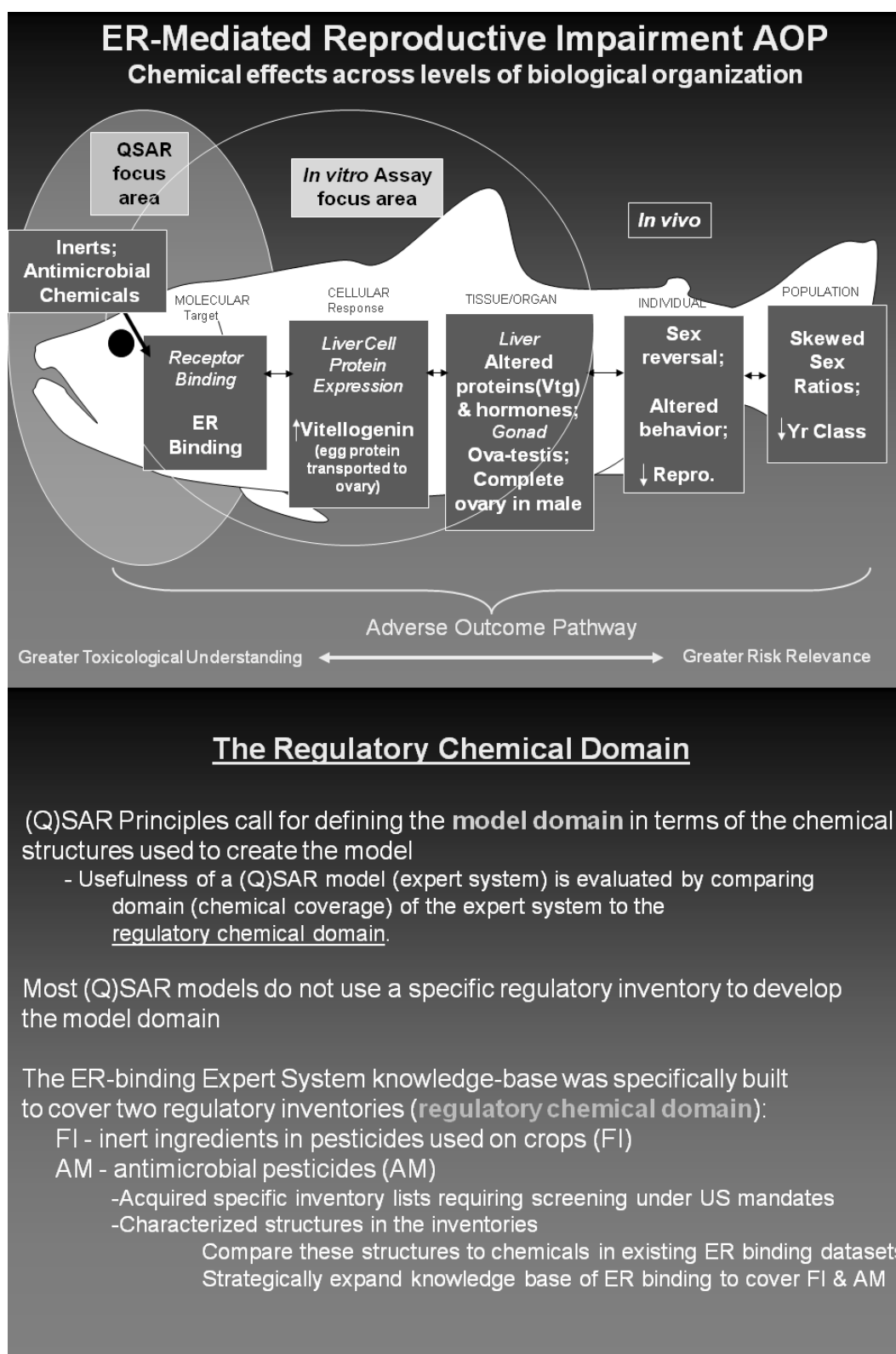
Mechanistic linkage exists between the risk assessment endpoint (ER-mediated reproductive impairment) and the hazard identification endpoint (ER binding)

Foundation of the assays used to build a category-based ER binding Expert System is the ER-mediated AOP

- The molecular initiating event of the pathway is identified (ER binding)
- The expert system identifies which chemical structures can initiate the pathway
 - Pathway context provides conceptual model useful for generating testable hypotheses
 - Pathway context provides decision-making rationale for the regulatory community

ER-mediated Adverse Outcome Pathway:

- Area of focus consistent with legislative directive
- Chemical binding to the ER is known to have potential to cause adverse effects
- Evidence existed that diverse chemical structures bind ER
- Bioassays for ER binding were available from drug-design, although extant methods were focused on potent anti-estrogens
 - assays were optimized to cover the chemical domain in question
- Drug-design research provided mechanistic insights on multiple types of interaction within the ER binding site
 - led to hypotheses, testing, and ultimately underlying rationale for grouping chemicals (CATEGORIES/SUB-CATEGORIES)



**Apply knowledge/theory of_
ER Binding Domain (MIE)
to_
The Regulatory Chemical Domain**
(continuing to seek MECHANISTIC understanding)

Hypothesize ER interactions of Inventory Chemicals

Inert ingredients and antimicrobial pesticides are non-steroidal and do not contain multiple H-bonding groups at distance needed for steroid-like interactions

Hypotheses:

- Any pesticide inert or antimicrobial that does bind ER will do so through an interaction mechanism that results in low affinity binding
- Only a small % of these chemicals are likely to bind ER
- A chemical group approach will facilitate regulatory application
- Chemicals can be grouped based on how they interact with the ER (within specific ER sub-pockets)

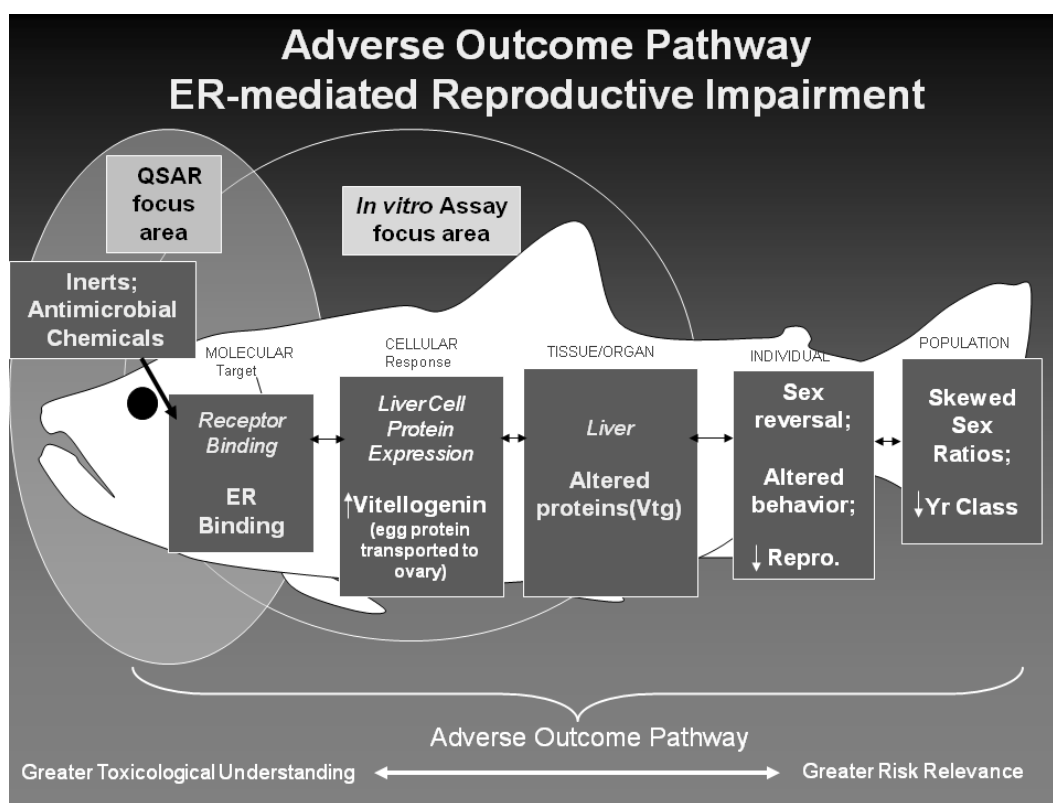
The Receptor Binding Assay Domain

Well-Defined Endpoint: the Biology

Focus on the MIE (ER binding) within ER-mediated AOP

Optimize bioassay methods for FI and AM chemicals (chemical properties) to detect low affinity ER interactions:

- 1) rtER binding is assessed using a standard competitive binding assay optimized for FI and AM chemicals;
 - chemicals are tested until binding displacement is observed or solubility in assay media, whichever comes first, to determine any potential to bind ER
- 2) binding curves are interpreted by assaying for gene expression (ER-mediated vitellogenin mRNA production in metabolically competent trout liver slices);
- 3) additional experiments to verify competitive binding (e.g., K_i , dosimetry) are done as needed (e.g., charged alkylaromatic sulfonic acids)



The ER Binding Assay Domain Well-Defined Endpoint: Chemical Dosimetry

Additional information is gathered to better understand chemical behavior in the assays.

- 1) Chemical purity
- 2) Metabolism
 - Is the test system used for collection of empirical data capable of xenobiotic metabolism?
 - If so, is activity (or lack of activity) due to parent chemical or a metabolite?
- 3) Bioavailability of the test chemical in the assay
 - Rate of chemical 'disappearance' within the system (e.g. hydrolysis; partitioning to surfaces in assay system)
 - Chemical solubility
 - Freely dissolved vs. bound and unavailable

Expanding the Knowledge-base to Cover Regulatory Chemical Inventories

Process:

- subdivide chemical on lists into groups
- group by chemical attributes **mechanistically** related to the hazard endpoint - "testable rules" (e.g., binding Site A or Site B)
- during development iteratively evaluate **model domain** against inventory chemicals to ensure sufficient data and rules to cover all types of chemicals on FI and AM lists
- if: an inventory chemical is not defined by a subgroup (e.g., outside the domain)
then: expand data (**well-defined endpoint**) and rules to cover inventory chemical
- continue until entire Regulatory Chemical Domain is covered

Thus, chemicals were selected for testing (in **well-defined assays**) to achieve two goals:

- to investigate mechanisms of binding the ER
- to adequately cover all inventory chemicals within a mechanistic group

Application of OECD Principles to Forming Chemical Categories

Example:

Transparency

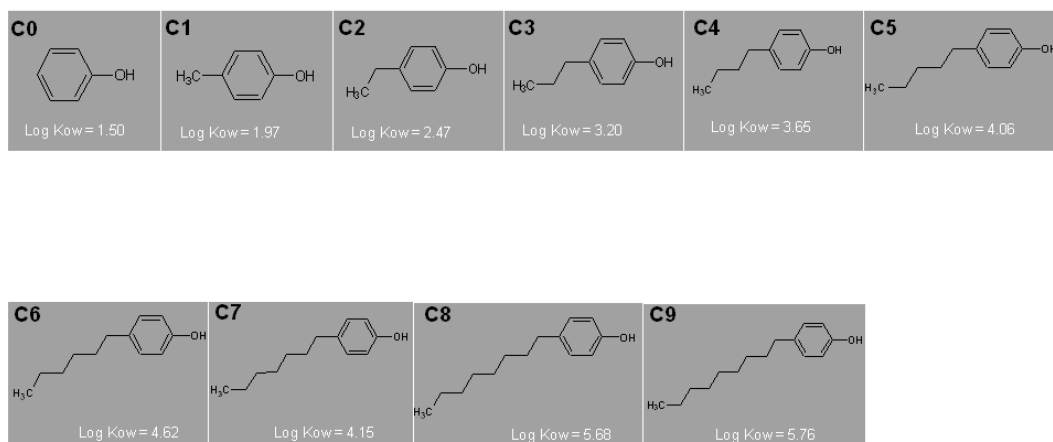
- How reasonable is the estimate compared with data for similar chemicals ?
- Can the QSAR estimate be explained mechanistically?

Usefulness

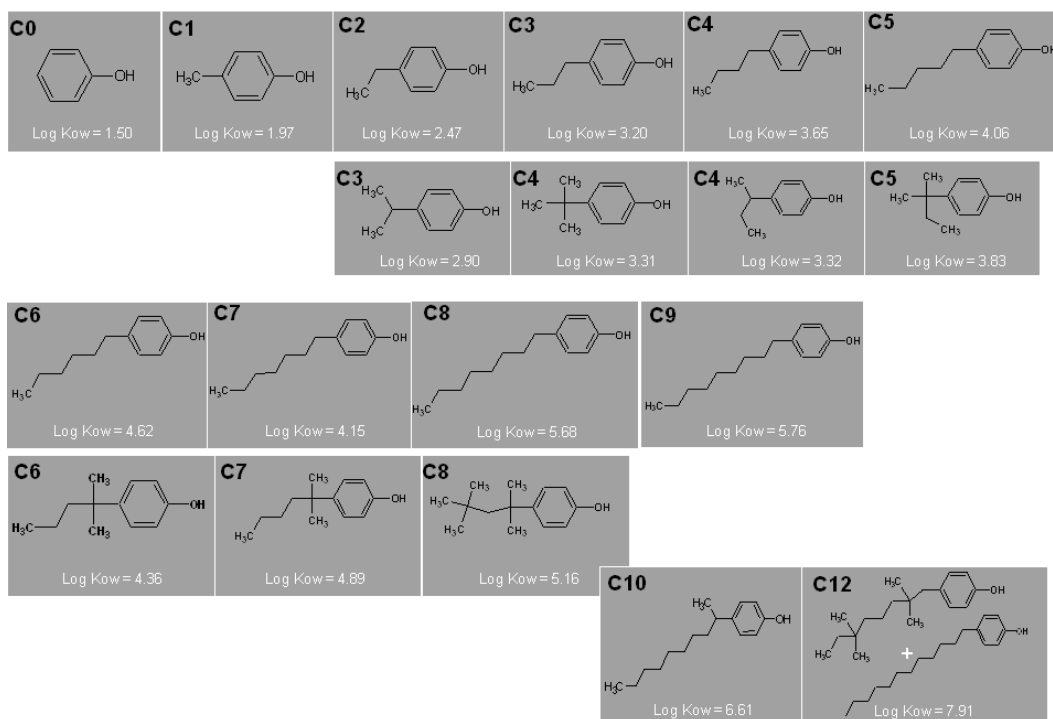
- Are the predictions applicable to all the chemicals of regulatory concern?
- Does the model/expert system answer the regulatory question?

ER Binding Site A Homologous Series

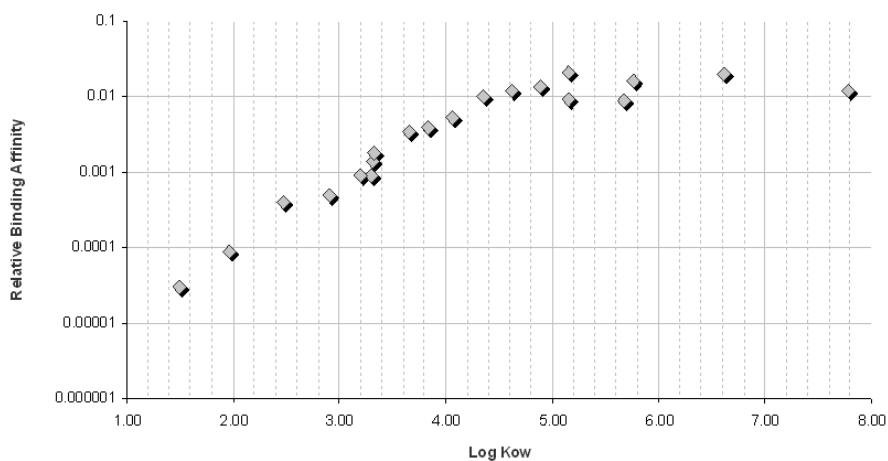
4-n-Alkylphenols



4-t-Alkylphenols were also assayed



Relationship between Chemical Parameter (Log K_{ow}) and Activity (RBA)
4-alkylphenols



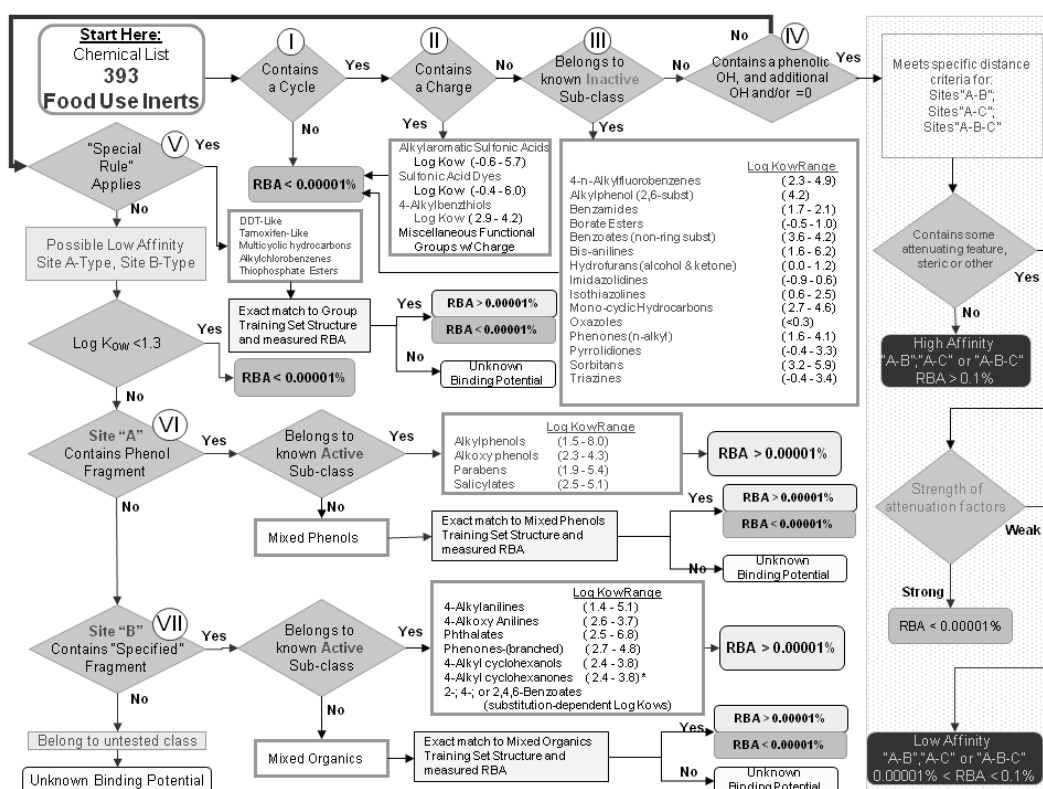
Application of OECD Principles to Forming Chemical Categories Example:

Transparency

- How reasonable is the estimate compared with data for similar chemicals ?
- Can the QSAR estimate be explained mechanistically?

Usefulness

- Are the predictions applicable to all the chemicals of regulatory concern?
- Does the model/expert system answer the regulatory question?



Expert System Predictions for Food use Inerts and Antimicrobials

	<i>Food use Inerts</i>	<i>Antimicrobials</i>
	<u>Total Chemicals (%)</u>	<u>Total Chemicals (%)</u>
	<u>393 (100%)</u>	<u>211 (100%)</u>
Predicted RBA < 0.00001	378 (96%)	196 (93%)
Predicted RBA > 0.00001	15 (4%)	15 (7%)

Conclusions

- An Expert System based upon the ER-mediated AOP
 - Knowledge of common initiating event across chemical classes facilitates development of QSARs and read-across methods to predict toxicity potential of untested chemicals
- OECD QSAR Validation Principles
- ICPS MOA

ANNEX 7

Case Study
November 9, 2010

Name of AOP: Voltage Gated Sodium Channels (VGSC)
 Outcome: Neurotoxicity

Part 1. Building An Adverse Outcome Pathway

Brief summary of the AOP:

The development and regulation of pesticides has provided a rich history of data and information important to both toxicology and risk assessment. Pyrethrins are natural insecticides that come from the chrysanthemum plant. In order to improve the insecticidal action of these natural chemicals, numerous synthetic pyrethroids have been developed that are structurally related to the pyrethrins (Figure 1). In developing and researching these pyrethroids much has been learned and published about the toxicity, the mode of action, and the adverse (neurotoxicological) outcome in insects and mammals following exposure to these compounds.

Figure 2 shows the proposed adverse outcome pathway² for neurotoxicity in mammals following exposure to an appropriate dose of a pyrethroid. This AOP is based on a previous model (Shafer et al, 2005) as well as information taken from a variety of recent reviews (US EPA (2009); Soderlund et al, 2002; Shafer and Mayer 2004; Wolansky and Harrill, 2008)).

Briefly, a weight-of-evidence (WOE) analysis indicates the following key events that have been demonstrated in a variety of mammalian and non-mammalian species, both *in vitro* and *in vivo*:

1. Pyrethroids interact with voltage-gated sodium channels (VGSCs) to alter the normal flux of sodium ions across the neuronal cell membrane by prolonging (slowing) the kinetics of VGSC activation (opening) and inactivation (closing);
2. The prolongation of the inactivation rate can be short (e.g. 10 msec) or long (e.g. >100 msec) lasting, and is dependent on the structure of the pyrethroid; and
3. The alterations in VGSC function lead to increased sodium influx and depolarization of the neuronal membrane, resulting in increased excitability of neurons, and
4. The short lasting changes in VGSC inactivation produce small increases in membrane excitability leading to generation of repetitive firing of neuronal action potentials, while the long-lasting changes in VGSC inactivation inhibit neuronal firing because they depolarize the membrane above the threshold for action-potential generation; and

² The use of this term (adverse outcome pathway) and all associated language follows the definition in the paper "*Pathways of Toxicity: Working Definitions and Examples*" developed for this workshop.

5. Alterations in firing rates *in vivo* result in measurable clinical signs of neurotoxicity as shown in *in vivo* studies (both mammalian and non-mammalian species). The characteristic clinical signs of pyrethroids that cause short changes in inactivation rate include fine tremors, hyper-excitability, and myoclonus and are designated as Type I or T. Those pyrethroids that cause long-lasting changes in VGSC inactivation rate cause choreoathetosis, salivation, hyperactivity, and clonic/tonic convulsions and are designated as Type II or CS.

The adverse outcomes described above are based on characterization in rodents and depend on the pyrethroid structure. Type I pyrethroids include chemicals that contain the descyano-3-phenoxybenzyl or other alcohols. Type II pyrethroids contain an α -cyano-3-phenoxybenzyl alcohol which increases the insecticidal activity greatly compared to the Type I insecticides (Figure 1a). Figure 1b shows the structure of a natural pyrethrin from the chrysanthemum plant

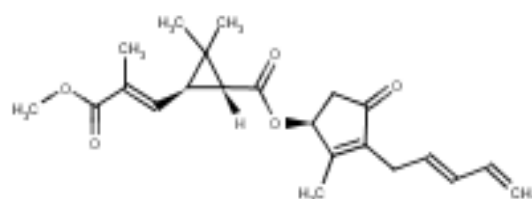
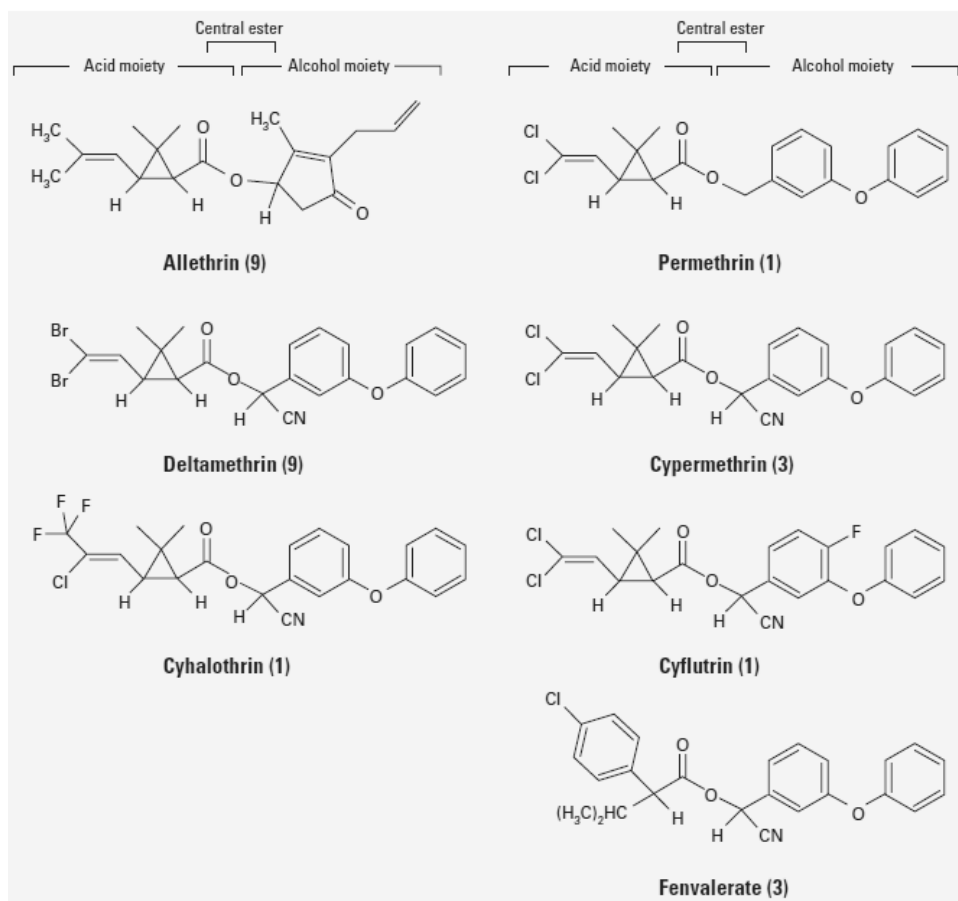


Figure 2A,B. Structures of common pyrethroids. Each structure contains an Acid moiety and an alcohol moiety joined by an ester linkage. Synthetic pyrethroids are on the left (A) and an example of a natural pyrethrin (Pyrethrin I) is on the right (B).

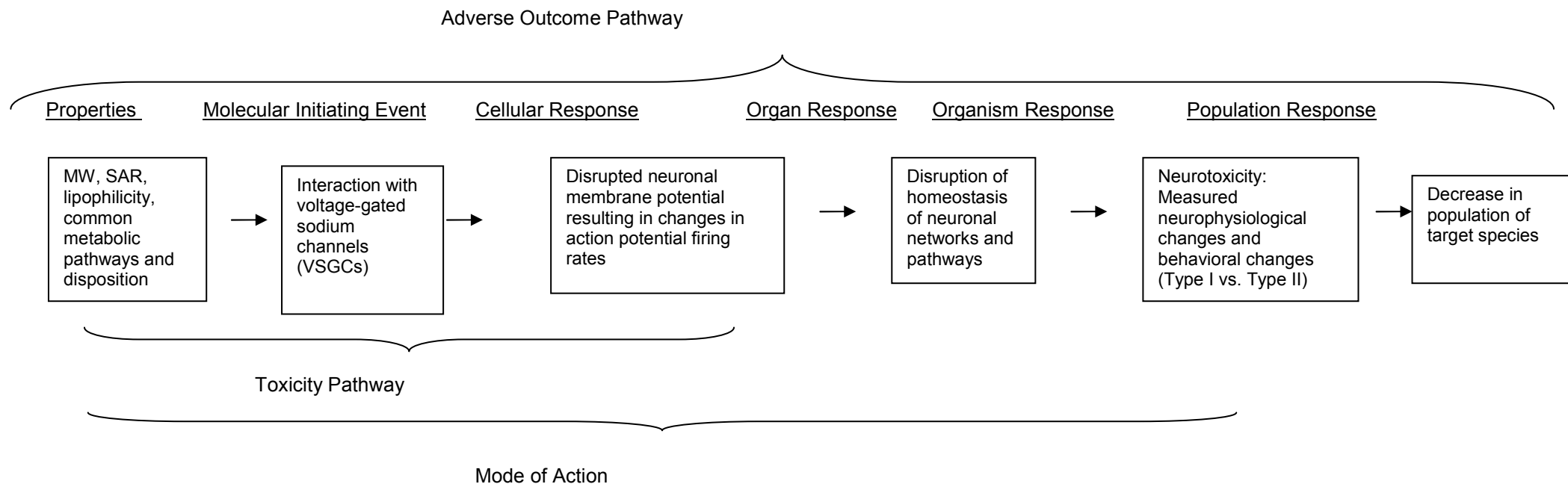


Figure 2. Using a modification of a schematic presented as Figure 1 in Ankley et al. (2010), the proposed adverse outcome pathway (AOP) for the neurotoxicity of synthetic pyrethroids is presented for different levels of biological organization.

Discussion of the Evidence Supporting the AOP:

The Experimental Evidence

The following “state of knowledge” is presented by level of biological organization:

1. Initiating Molecular Event. Voltage-gated sodium channels (VGSCs) are protein structures common to vertebrate and invertebrate species. They are composed of multiple subunits (alpha and beta subunits in mammals). The alpha subunit forms a pore through neuronal membranes and controls sodium flux across the neuronal membrane. The beta subunits influence the function and expression of the alpha subunits. Opening of the VGSC increases sodium entry into the neuron and is responsible for generating the action potential (movement of an electrical signal along the neuron that represents a change in membrane excitability). There are a number of VGSC subtypes, and there is evidence that channel subtypes differ in sensitivity to different pyrethroids.

A variety of techniques have been developed to measure alterations in sodium flux through these channels. *In vitro* data using neurons from different species and different tissues have shown that pyrethrins and synthetic pyrethroids alter the sodium flux through VGSC by changing the rates at which they open (activate) and close (inactivate). These alterations are consistent, reproducible, and indicative of the initiation of the eventual neurotoxicity observed *in vivo* in insects and mammals.

2. Cellular (Organ) Response. It is well established in a number of species, using both *in vivo*, *ex vivo* and *in vitro* preparations that pyrethroid modification of VGSC leads to changes in neuronal membrane excitability with subsequent changes in firing rates of action potentials. For example, using crayfish giant axon, it has been shown that actions of fenvalerate on VGSC function as well as membrane excitability (Salgado et al 1989) are both temperature-dependent. Lund and Narahashi (1981) demonstrated that tetramethrin disrupts VGSC function, depolarized the membrane, and upon stimulation of the nerve, induces repetitive action potential firing. These same authors demonstrated that tetramethrin altered VGSC function and induced repetitive firing in squid axon (Lund and Narahashi 1982). Similar findings have also been reported in mammalian neurons *in vitro*, wherein pyrethroids modify VGSC function and produce changes in membrane excitability in dorsal root ganglion neurons (Tabarean and Narahashi 1998) and cerebellar Purkinje neurons (Song and Narahashi 1996). *In vivo*, the changes in VGSC function are manifested as increased rat tail nerve excitability (as measured by compound action potentials; Parkin and Le Quesne 1982; Nozaki et al., 1995); and epileptic activity (as measured by EEG) followed by clinical signs (Condes-Lara et al., 1999).
3. Organism Response. Changes in VGSC inactivation rates and membrane excitability correlate with neurotoxicity symptoms observed in *in vivo* studies from both mammalian and non-mammalian species. Many studies in rodents have documented a variety of neurotoxic effects following exposure to Type I and Type II synthetic pyrethroids (Table 1). The rate constants (τ) for inactivation of VGSC tail currents modified by different pyrethroids are provided in Table 2. The des-cyano pyrethroids cause short-lasting modifications of VGSC inactivation (small τ values), and Type I signs, while the cyano-containing compounds cause long-lasting modifications of VGSC inactivation (large τ values) and type II signs. However, the range of τ values is more continuous than bimodal, and consistent with this, some pyrethroids with intermediate τ values produce syndromes containing both Type I and Type II signs.

Table 1. Signs of Pyrethroid Poisoning Across Rodent Species¹

Table 1. Signs of Pyrethroid Poisoning Across Rodent Species ¹						
	Mice		Rat			
	Intracerebral Exposure		Intravenous Exposure		Oral Gavage Exposure	
	Type I	Type II	Type I	Type II	Type I	Type II
Mild Poisoning Signs	Hyperactivity Circling (mostly ipsilateral direction)		Hyperexcitability, Aggressive sparring	Restlessness; Chewing; Nosing; Pawing; Burrowing	Episodes of restlessness	
					Chewing; Nosing; Pawing; Burrowing; Head bobbing; whole body shakes	
	↑ Sensitivity	Salivation	↑ Responsivity	Salivation	↑ Responsivity	Salivation; ↑↓ Responsivity
Moderate to Severe Poisoning Signs	Whole body tremors	Tremors followed by CA ²	Fine tremors progressing to whole body	Impaired movements followed by CA (tail, limbs)	Aggressive sparring	
					Fine tremors progressing to whole body	Tremor followed by CA
	Prostration	Sinuous writhing	Uncoordinated twitches	Hind limb extensor rigidity; Rolling gait	Spontaneous muscular contractions	Motor incoordination; ↑ Hindlimb extensor tone
			Hyperthermia	Normothermia or hypothermia	Hyperthermia	Normothermia or hypothermia
Nearly Lethal Syndrome	Clonic seizures	Tonic seizures	Rigor occasionally just before death	Loss of consciousness; Tonic seizures	Clonic seizures	Loss of consciousness; Severe writhing spasms; Tonic seizures
	Death @ 10-60 minutes		Death @ 0.5 – 2 hours		Death @ 2-8 hours	

¹ Taken from Table 3 in US EPA, 2009.
² CA = choreoathetosis.

¹ Taken from Table 3 in US EPA, 2009.² CA = choreoathetosis.

Table 2. Time Constants of Sodium Tail Currents Induced by Various Pyrethroids In Frog Nerve Fibers and in Cultured Mouse Neuroblastoma Cells Compared to poisoning Syndromes Reported in Mammals and Insects*

		<u>T_{tail} (ms)</u>		<u>Syndrome</u>	
Compound	Configuration	Frog ^a	Mouse ^b	Rat ^c	Cockroach ^d
Non-Cyano pyrethroids					
Phenothrin	(1 <i>R</i>), <i>trans</i>	6		N	I
Permethrin	(1 <i>R</i>), <i>trans</i>	7		N	I
Allethrin	Mixture	10			I
Phenothrin	(1 <i>R</i>), <i>cis</i>	13	4	N	I
Cismethrin	(1 <i>R</i>), <i>cis</i>	21		T	I
Permethrin	(1 <i>R</i>), <i>cis</i>	28		T	I
Fenfluthrin	(1 <i>R</i>), <i>cis</i>	105	14-30		I
NAK1963	Mixture	150			I
S-5655	Mixture	150		T(S)	I
Cyano pyrethroids					
Cyphenothrin	(1 <i>R</i>), <i>trans</i>	290		T	II
Cyphenothrin	(1 <i>R</i>), <i>cis</i>	385	140	CS	II
Fenvalerate	(2 <i>RS</i>),	545		CS	II
Fenvalerate	(2 <i>S</i>),	600		CS	II
Cypermethrin	(1 <i>R</i>), <i>cis</i>	1115		CS	II
Deltamethrin	(1 <i>R</i>), <i>cis</i>	1770	440-800	CS	II

*From Wolansky and Harrill, 2008

^aMeasured in frog myelinated nerve fibres at 15°C.^bMeasured neuroblastoma cells at 18°C.^cN = no symptoms; T = tremor; S = salivation; C = choreoathetosis.^dtopical application; I = incoordination; II = convulsions

4. **Population Response.** Pyrethroid insecticides are designed to kill insects, and the proposed AOP is the pathway that mediates insecticidal activity of these compounds. Thus, decreases in insect (both target and non-target species) population levels would be expected following pyrethroid applications. Resistant strains of insect pests following pyrethroid use are well documented, and a number of mutations in the insect VGSC have been documented in these resistant populations, all of which decrease the sensitivity of the channel to pyrethroid modification. However, pyrethroid applications have been demonstrated to elicit population-level responses in non-target species as well. Work by Woin (1998) demonstrated that direct effects of fenvalerate on insects and arthropods was followed by a >10 fold increase in oligochaetes in simulated freshwater mesocosms. Similar changes in ecosystem balance have been reported following pyrethroid use (for review, see Fleeger et al., 2003).

For human populations, no data is available regarding population level effects of pyrethroids. This includes assessments or reports of the sensitivity of different populations based on genetic differences. It is possible that such populations may exist, as there are a number of known polymorphisms in VGSCs in humans that alter the function of the channel and are associated with neurological diseases such as epilepsy. However, whether these polymorphisms change the interaction of pyrethroids with the channel have not been determined.

Weight-of-Evidence (WOE) Analysis

- *Concordance of dose-response relationships*
 - Studies have been conducted that demonstrate a relationship between channel gating and neuronal firing (Lund and Narahashi, 1981). A quantitative relationship has been established that estimates only ~1% or less of VGSCs need to be modified to produce changes in membrane excitability and firing rate (Lund and Narahashi 1982 and Narahashi et al, 1998)
- *Temporal concordance among the key events and adverse outcome*
 - Following exposure to pyrethroids in both mammalian and non-mammalian species, the onset of effects is rapid following distribution to the nervous system. This is consistent with the role of VGSCs as rapid modulators of membrane excitability and neuronal firing.
- *Strength, consistency, and specificity of association of adverse outcome and initiating event*
 - Mutations in insect VGSCs that make them less susceptible to modification by pyrethroids also make the insect less susceptible to pyrethroid toxicity
 - *In vitro* work with invertebrates [crayfish giant axon (Salgado et al., 1989) and squid axon (Lund and Narahashi 1981, 1982), and node of Ranvier and peripheral nerves (Vijverberg and van den Berken, 1979 and 1982)]: and vertebrates (dorsal root ganglion neurons (Tabarean and Narahashi, 1998) and cerebellar Purkinje neurons (Song and Narahashi, 1996) demonstrate consistency of pyrethroid effects on VGSC function and neuronal excitability across species.
 - Compounds that decrease VGSC activity have also been demonstrated to antagonize pyrethroid effects at higher levels of organization, including motor effects (Joy et al. 1990; Bradbury et al., 1983 (Review: Ray 2001))
 - *In vivo*: increased rat tail nerve excitability (Parkin and Le Quesne 1982; Nozaki et al., 1995); and epileptic activity (as measured by EEG) followed by clinical signs (Condes-Lara et al., 1999) are consistent with altered VGSC function.
- *Biological plausibility, coherence and consistency of the experimental evidence,*
 - VGSCs underlie neuronal excitability and action potential generation across all vertebrate and invertebrate species
 - Pyrethroids cause similar effects on VGSC function, membrane excitability and neurotoxicity across many different invertebrate and vertebrate species, with differences in sensitivity related to differences in VGSC structure.
 - Stereospecificity- isomers that are more active in alteration of VGSC function are also more toxic (Eells et al. 1993; Brown et al 1988; Lund and Narahashi et al., 1982,.
- *Alternative MOAs (AOPs) that logically present themselves and the extent to which they may distract from the postulated AOP:*
 - Other molecular targets besides VGSCs have been proposed: chloride-permeable ion channels, voltage-gated calcium channels and ligand-gated chloride channels. However, it is unclear whether pyrethroid interactions with these targets constitute key initiating events. Alterations in the function of these targets also contribute to changes in membrane excitability and firing and moreover may be secondary to pyrethroid modification of VGSCs. Evidence for these alternative pathways is less robust (Shafer and Meyer 2004). Experiments conducted by Ogata et al. (1988) demonstrated that GABA_A currents are not modified at concentrations that modify VGSC function.
- *Uncertainties, inconsistencies and data gaps:*
 - VGSC function is difficult to measure *in vivo*, and there are no good biomarkers of VGSC modification

- Multiple isoforms of VGSCs exist, with differing sensitivity to pyrethroids. Studies have examined this differential sensitivity of VGSCs to pyrethroids (Smith and Soderlund, 1998; 2001; Choi and Soderlund, 2006; Meacham et al., 2008; Tan and Soderlund, 2009; 2010) but these studies used VGSCs expressed in exogenous systems (e.g. frog oocytes or CHO cells). Results of these studies are not easily extrapolated to mammalian neurons, thus, the particular VGSC isoforms involved are uncertain
- In mammals, there is some evidence of differences in sensitivity to Type II pyrethroids based on lifestage, with young animals being more sensitive than adults (Sheets, 2000; Cantalamessa, 1993). These differences are largely accounted for by differences in metabolic capability. However, assessment of clinical signs in rodents younger than postnatal day (PND) 10 is difficult, thus the susceptibility of younger animals has not been determined. Expression of the multiple VSCC isoforms in mammals is developmentally regulated, and Meacham and co-workers (2008) has demonstrated that mammalian “embryonic” VSCC isoforms expressed in oocytes are more sensitive to modification by Type II compounds than are “adult” isoforms. Excitability and patterns of neuronal firing are important in mammalian CNS development, and VGSCs are integral to this process. Thus, the initiating events could take place in the developing nervous system, but other key events are likely different and the adverse outcome (developmental neurotoxicity) would be different. Compared to the literature available for acute neurotoxicity in adult animals, the published literature regarding developmental neurotoxicity of pyrethroids is very small. A recent review (Shafer et al., 2005) of pyrethroid developmental neurotoxicity studies concluded that there was not a consistent neurotoxic outcome (syndrome?) following developmental exposure to pyrethroids
- Differences in tissue sensitivity are most likely related to differences in the expression of different VGSC isoforms.
- There is some uncertainty as to whether Type I and Type II pyrethroids should be considered as two different AOPs (see Breckenridge et al., 2009; and FIFRA Scientific Advisory Panel [2009] response to US EPA, 2009).

Thus, the evidence for this proposed AOP is overall very strong; a large body of work that has been generated in numerous laboratories using a wide variety of experimental models supports the proposed pathway (Table 3).

Table 3 Summary of the experimental support for the AOP.

Key Events	Experimental Support	Strength of Evidence
<u>Key Event 1 (molecular initiating event)</u> Interaction with voltage-gated sodium channels (VGSCs)	Numerous <i>in vitro</i> studies a variety of mammalian and nonmammalian species	Very strong
<u>Key Event 2</u> Changes in neuronal excitability and firing rates	Primarily <i>in vitro</i> studies in a variety of mammalian and nonmammalian systems.	Very strong
<u>Key Event 3</u> Altered neuronal pathway function	Physiology of a variety of neural pathways is altered by pyrethroids, as measured by EEGs, compound action potentials, sensory and sciatic nerve function	Moderate to Strong
<u>Key Event 4 - Adverse Outcome</u> Neurotoxicity/clinical signs of pyrethroid poisoning	Numerous <i>in vivo</i> studies in mammalian and non-mammalian systems, some incident human data	Very Strong

Part II: Building a Category for the AOP Pathway (Identification of the Applicability Domain)

A wide variety of different drugs, chemicals and natural toxins are capable of interacting with VGSCs and altering the function of this important regulator of nerve excitability. However, subtle differences in how these different compounds alter the function of the channel can influence dramatically the outcome. For example, the potent block produced by tetrodotoxin results in paralysis and rapid death of the organism due to respiratory failure. By contrast, less potent blockers such as phenytoin are used to successfully treat epilepsy and other pathological conditions. Thus, clear definition of the AOP is important to separate potentially useful therapeutic compounds from compounds that may result in deleterious effects.

The AOP discussed above can be distinguished based on several characteristics, including the following:

- Chemical structure. As described above, pyrethroids have a well defined chemical structure that consists of a central ester bond, an acid moiety and an alcohol moiety. Although considerable structural diversity exists within the pyrethroid compounds, all pyrethroids contain these general characteristics. The structures of other compounds acting on VGSC do not conform to this general description and they alter the function of the channel in subtly to dramatically different ways from pyrethroids, resulting in different outcomes at the organism level. However, chemical structure alone may not be sufficient to define the AOP, as DDT, which is not a pyrethroid, also acts on VGSC function like a type I pyrethroid and produces poisoning syndrome similar to a type I pyrethroid.
- Binding site on the channel. VGSCs have a number of known binding sites for toxins. However, pyrethroids bind at a site or sites distinct from these described toxin binding sites. As such, this represents a unique aspect of the AOP.

There are subcategories of the AOP, as the Type I and Type II compounds cause distinctly different types of clinical syndromes. This is linked well through differences in chemical structure, with the presence of a cyano-group on the Type II compounds producing longer delays in the closing of VGSCs, greater depolarization of the neuronal membrane and ultimately depolarization-dependent block of neuronal firing, which is related to the clinical type II syndrome. However, some compounds produce characteristics of both Type I and II syndromes, and have intermediate effects on VGSC function, thus, it is possible that the separation of pyrethroids into two types is not a bimodal distribution of effects (at all levels of the AOP) but rather exists on a continuum wherein not enough of pyrethroids with intermediate effects have been adequately characterized to define clearly this portion of the continuum.

Part III: Conclusions

This AOP has been well characterized with nearly 50 years of studies that have examined it at the level of the molecular initiating event through the level of the Adverse Outcome. Clear structure activity relationships have been demonstrated at the level of the initiating event that corresponds to differences in Adverse Outcome. This includes the above mentioned relationships between Type I and II pyrethroids, presence of the cyanogroup (on Type II compounds), differences in inactivation of VGSC and poisoning syndromes. In addition, individual compounds exhibit stereospecificity that is conserved throughout all events in the AOP. This AOP appears to be conserved across many different vertebrate and invertebrate taxa, with differences in sensitivity related to differences in the sensitivity of the initiating event and differences in pyrethroid PBPK. Thus, the initiating events and adverse outcome are well linked.

Part IV; Lessons Illustrated - Break out group discussion

Discuss the lessons learned from this case study regarding the concept of the Adverse Outcome Pathway and its utility in forming toxicologically meaningful chemical categories for filling data gaps by non-test methods (e.g., read-across and structure-activity relationships) for regulatory purposes.

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Case study 2: AOP: Voltage-gated sodium channel mediated neurotoxicity

Timothy Shafer, PhD
US EPA

Pyrethroid Neurotoxicity



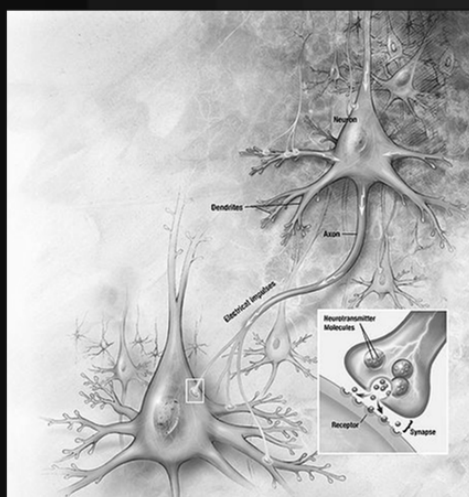
Administration to test animals and insects has identified two distinct poisoning (e.g. HIGH DOSE) syndromes:

- **T or Type I:** Aggressive sparring, increased sensitivity to external stimuli, fine tremor progressing to whole body tremor and prostration
- **CS or Type II:** Pawing and burrowing, profuse salivation, coarse tremor progressing to choreoathetosis and clonic seizures

Endpoint of concern is Acute Neurotoxicity- 40+ yrs of research

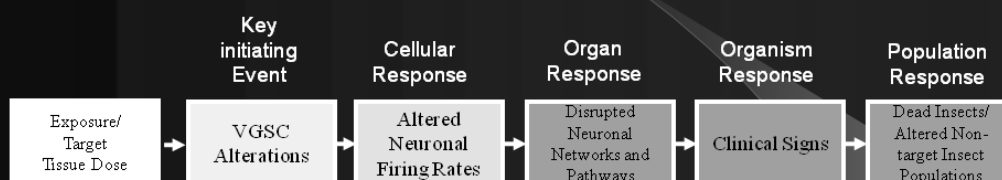
- **Mixed:** A minority of compounds cause signs of both syndromes
- **Not all measures show divergent effects:** e.g. Motor Activity, Network Activity

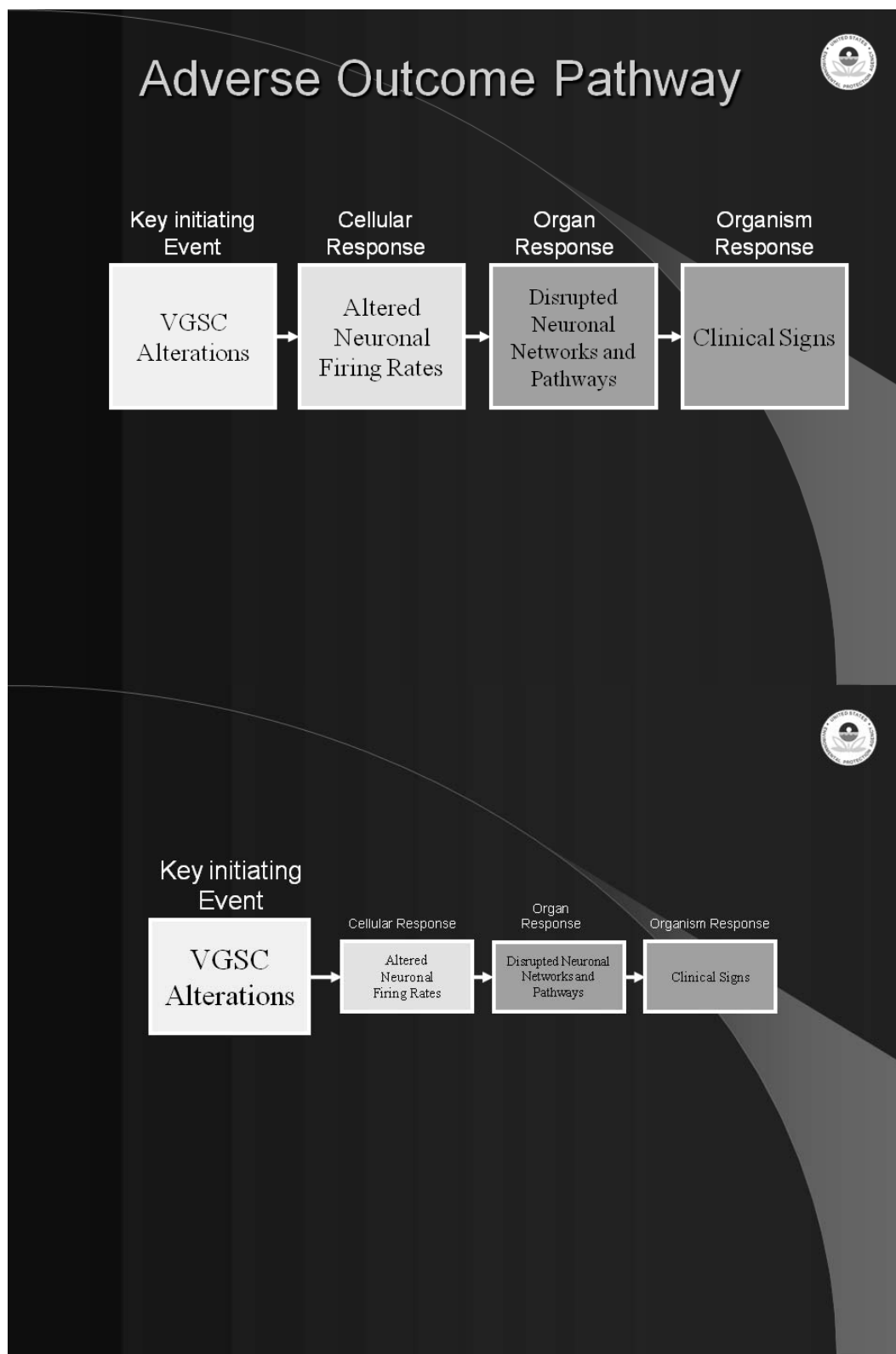
Neurobiology 101



- Electrical activity is critical to nervous system function
- Neurons “talk” to each other by firing brief electrical signals called action potentials
- Voltage-gated sodium channels (VGSCs) are the “gate keepers” of action potentials

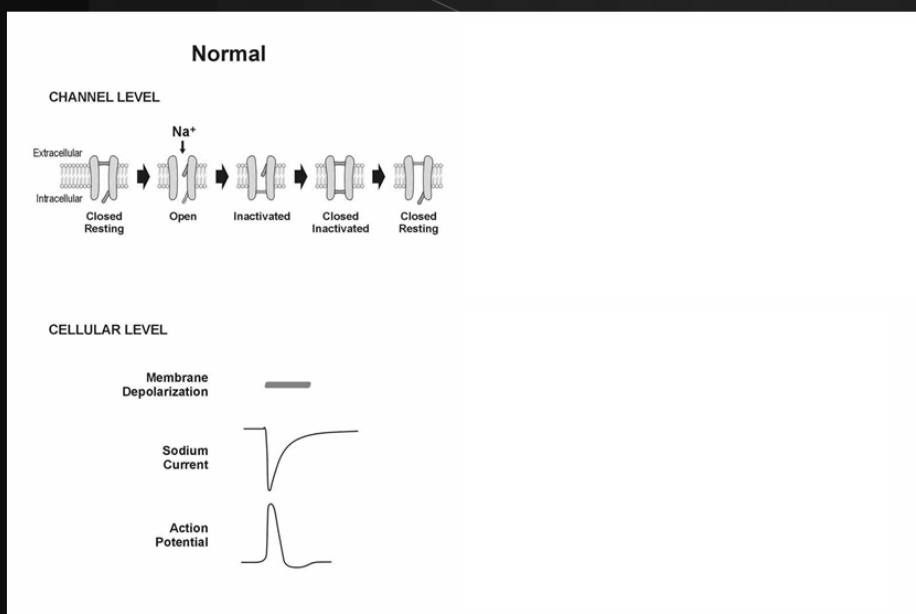
Adverse Outcome Pathway



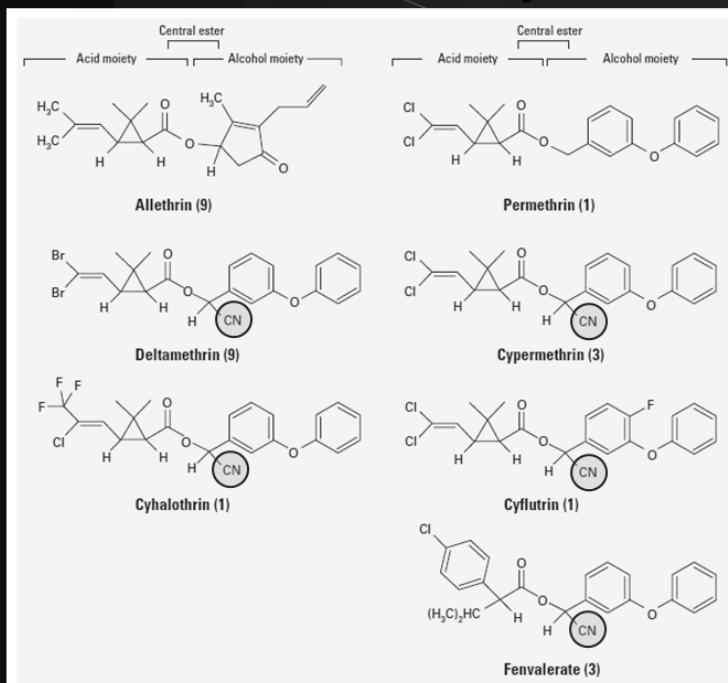




Key Initiating Event In Pyrethroid Neurotoxicity



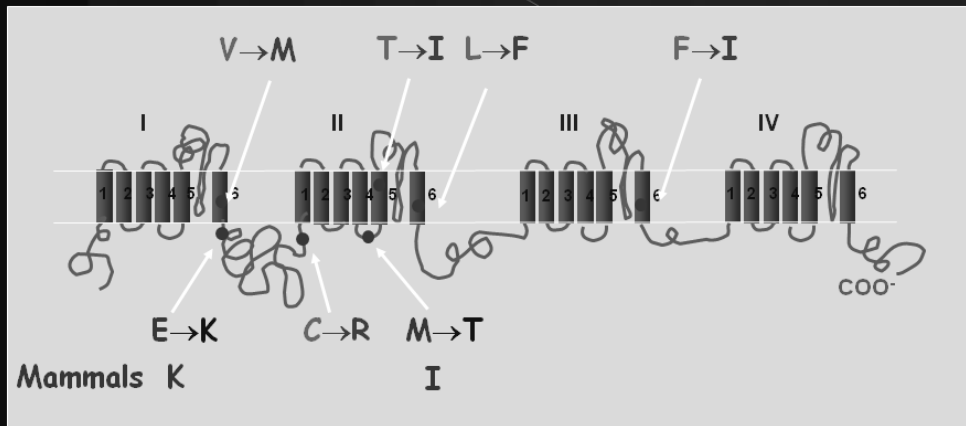
Structure-Activity Relationships



In general, compounds without a CN group cause short-lasting VGSC modification,

while those with a CN group cause long-lasting VGSC modification.

Linking the Key Initiating Event to the Toxicity: Evidence from Insect Resistance



Insects that are resistant to pyrethroids have mutations in the sodium channel that make it less sensitive to pyrethroid modulation
Mammalian channels can be made more 100x more sensitive to pyrethroids by making them more "insect like"

Pyrethroid Effects on VGSC as Key Initiating Event

Strengths



Consistent effects on VGSC across:

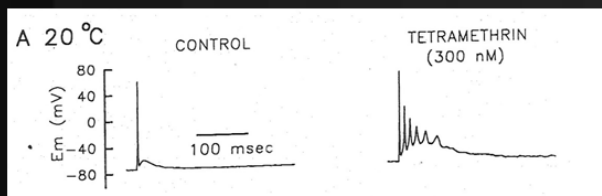
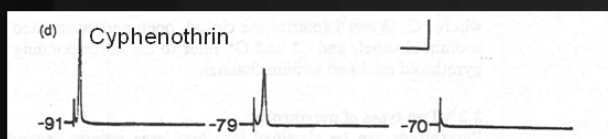
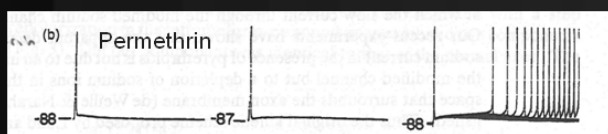
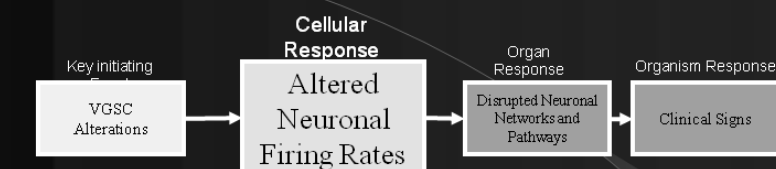
- Multiple Species, Preparations, Laboratories
- Chemical Structure/stereospecificity
- Mutations in channel alter channel sensitivity and toxicity outcome

Pyrethroid Effects on VGSC as Key Initiating Event

Uncertainties

- Multiple (10) VGSC Isoforms in Mammals
- Difficult to measure in vivo
- No easy Biomarker of Effect

Altered Cellular Firing Rates



Crayfish Giant

Axon From: Song and Narahashi, JPET 277, 445-53, 1996.

Rat Purkinje

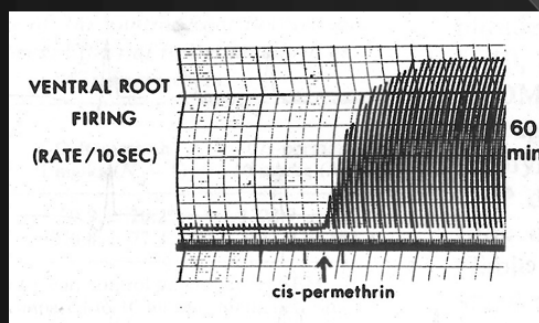
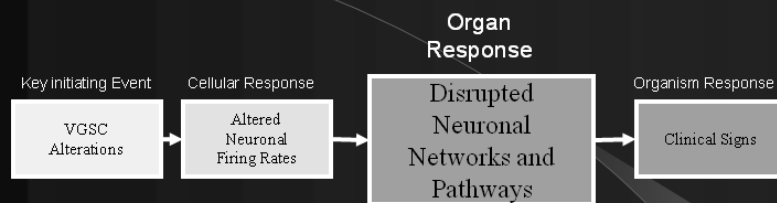
Neuron From Lund and Narahashi, 1983

Pyrethroid AOP

- VGSC channel disruption and subsequent changes in firing rates have been demonstrated in:
 - Insects
 - Crayfish giant axon
 - Squid giant axon
 - Frog
 - Rat DRG and cerebellar Purkinje cells in vitro

Therefore, changes in channel function and membrane firing are well linked and occur in multiple species.

Altered Excitability in Vivo

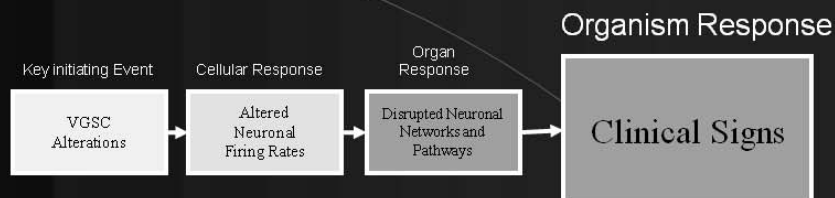


Effects of Permethrin on spontaneous firing in rat spinal ventral roots. From: Staatz-Benson and Hosko, Pest Biochem Physiol 25, 19-30, 1986)



Pyrethroid AOP

- In vivo, pyrethroids alter Compound Action Potentials in tail nerve and dorsal root, and alter EEG recordings
- These data come from multiple preparations and laboratories.
- The data are consistent with modification of VGSC
- The overall number of in vivo studies is lower than in vitro studies.

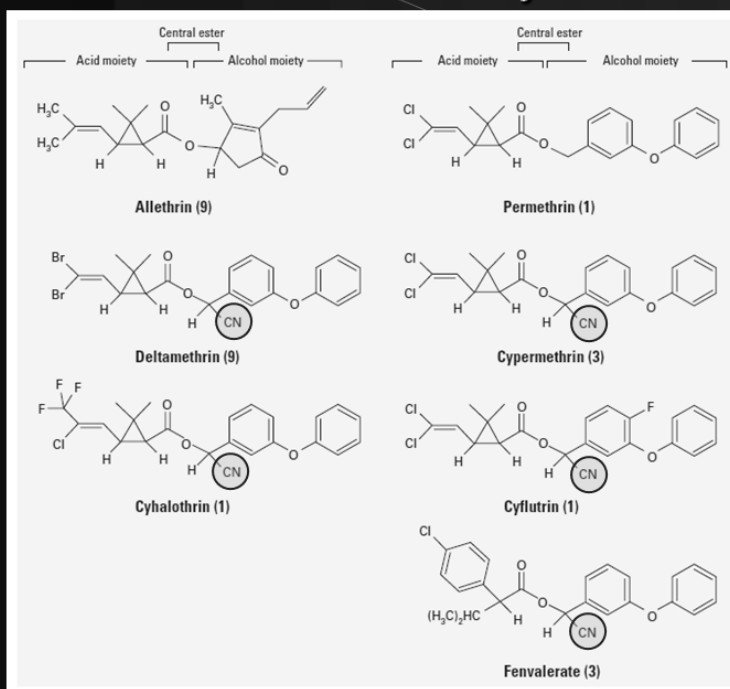


- Difficult to measure directly VGSC activity in vivo
- No easy Biomarker of Effect
- Behavioral measures are apical, and involve many different pathways

Thus, linking organism response to rest of AOP is not easy, but....



Structure-Activity Relationships



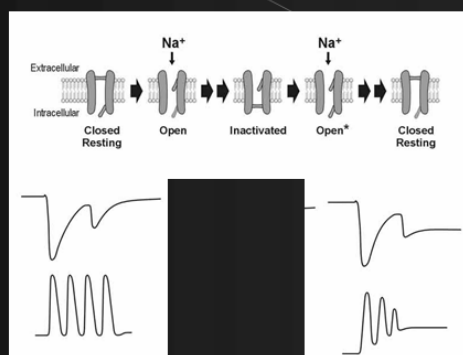
In general, compounds without a CN group cause short-lasting VGSC modification, and T-syndrome

while those with a CN group cause long-lasting VGSC modification and CS syndrome.

Pyrethroid Neurotoxicity AOP...or AOPs?



"Short-lasting"
VGSC modification



"Long-lasting"
VGSC modification

T-Syndrome

"T/CS"

CS-Syndrome

High
Dose



Table 2. Time Constants of Sodium Tail Currents Induced by Various Pyrethroids In Frog Nerve Fibers and in Cultured Mouse Neuroblastoma Cells Compared to poisoning Syndromes Reported in Mammals and Insects*

Compound	Configuration	I _{tail} (ms)		Syndrome	
		Frog ^a	Mouse ^b	Rat ^c	Cockroach ^d
Non-Cyano pyrethroids					
Phenothrin	(1 <i>R</i>), <i>trans</i>	6		N	I
Permethrin	(1 <i>R</i>), <i>trans</i>	7		N	I
Allethrin	Mixture	10			I
Phenothrin	(1 <i>R</i>), <i>cis</i>	13	4	N	I
Cismethrin	(1 <i>R</i>), <i>cis</i>	21		T	I
Permethrin	(1 <i>R</i>), <i>cis</i>	28		T	I
Fenfluthrin	(1 <i>R</i>), <i>cis</i>	105	14-30		I
NAK1963	Mixture	150			I
S-5655	Mixture	150		T(S)	I
Cyano pyrethroids					
Cyphenothrin	(1 <i>R</i>), <i>trans</i>	290		T	II
Cyphenothrin	(1 <i>R</i>), <i>cis</i>	385	140	CS	II
Fenvalerate	(2 <i>RS</i>),	545		CS	II
Fenvalerate	(2 <i>S</i>),	600		CS	II
Cypermethrin	(1 <i>R</i>), <i>cis</i>	1115		CS	II
Deltamethrin	(1 <i>R</i>), <i>cis</i>	1770	440-800	CS	II

Pyrethroid Neurotoxicity AOP...or AOPs?

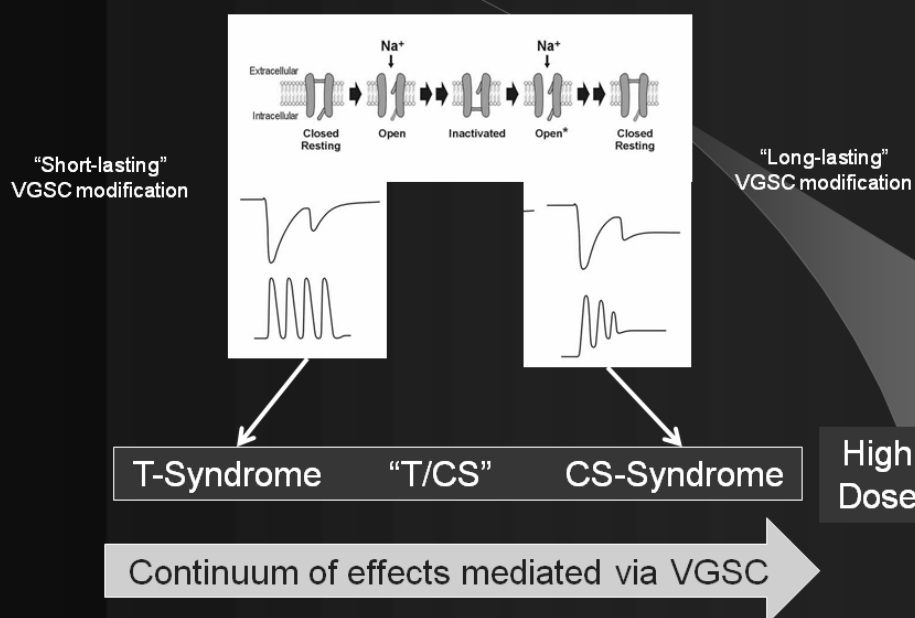




Table 3 Summary of the experimental support for the AOP.

Key Events	Experimental Support	Strength of Evidence
<u>Key Event 1</u> (molecular initiating event) Interaction with voltage-gated sodium channels (VGSCs)	Numerous <i>in vitro</i> studies a variety of mammalian and non-mammalian species	Very strong
<u>Key Event 2</u> Changes in neuronal excitability and firing rates	Primarily <i>in vitro</i> studies in a variety of mammalian and non-mammalian systems.	Very strong
<u>Key Event 3</u> Altered neuronal pathway function	Physiology of a variety of neural pathways is altered by pyrethroids, as measured by EEGs, compound action potentials, sensory and sciatic nerve function	Moderate to Strong
<u>Key Event 4</u> - Adverse Outcome Neurotoxicity/clinical signs of pyrethroid poisoning	Numerous <i>in vivo</i> studies in mammalian and non-mammalian systems, some incident human data	Very Strong



Building a Category for the AOP

A wide variety of compounds are known to interact with VGSCs;

How are those acting via this AOP distinguished from other actions??

Are there other environmental compounds that act via this AOP? How do we best identify these?



Other compound classes acting on VGSCs

- Local Anesthetics (e.g. Lidocaine)
- Anti-Arrhythmics (e.g. Quinidine)
- Anti-Convulsants (e.g. Carbamazepine)

These decrease channel activity and have different clinical outcomes

- Natural Toxins (e.g. Scorpion Toxin)
- p,p'-DDT

Toxin Classes acting on VGSC



Table 1
Neurotoxin receptor sites on voltage-gated sodium channels

Receptor site	Neurotoxin	Functional effect
Site 1	Tetrodotoxin Saxitoxin μ -Conotoxin	Pore block
Site 2	Batrachotoxin Veratridine Grayanotoxin Aconitine	Persistent activation enhanced activation, and block of activation
Site 3	α -Scorpion toxins Sea anemone toxins Atrachotoxins	Slowed inactivation
Site 4	β -Scorpion toxins	Enhanced activation
Site 5	Brevetoxins Ciguatoxin	Enhanced activation and block of inactivation
Site 6	δ -Conotoxin	Slowed inactivation

Various toxins differ from pyrethroids in where they bind to the VGSC as well as how they modify its function.

From: Catterall et al., *Toxicon* 49, 124-41, 2007.



Building a Category for the AOP

- **Structure**: Pyrethroid structure has some commonalities, but is also fairly diverse; DDT, which is not a pyrethroid has actions very similar to type I pyrethroids
- **Binding**: The pyrethroid binding site is not yet well defined.
- **Function**: Modification of VGSC function can be easily and quickly assayed by a variety of approaches. This may be the most efficient manner of identifying other environmental chemicals that interact with this AOP.



Building a Category for the AOP

- Identification of compounds that act via this AOP may need to assess SAR, binding, and function at the channel level to determine whether or not they should be included in this category.



Pyrethroid AOP- Summary

- AOP described is useful for building a category.
- Designing testing approaches for this category is currently a challenge

ANNEX 8

Case Study

**Name of AOP: Hemolytic anemia induced by anilines
and nephrotoxicity induced by 4-aminophenols
Outcome: Repeated dose toxicity**

In this case study, categorization of anilines for the repeated dose toxicity (RDT) is discussed, based on two AOPs related to the hemolytic anemia (AOP1) and the nephrotoxicity (AOP2).

Part I. Building An Adverse Outcome Pathway

Brief summary of the AOP:

AOP1 : Hemolytic anemia induced by anilines

In repeated dose toxicity tests of anilines, the findings on hemolytic anemia are frequently observed as a primary reason for the decision of NOEL value. Aniline is known to induce methemoglobinemia. The toxicant of methemoglobinemia induced by anilines is considered to be N-hydroxyl anilines that are metabolites of anilines in the liver. The hemolytic anemia induced by anilines is considered to be related to the oxidation of erythrocyte by N-hydroxyl anilines. Figure 1 shows the AOP of hemolytic anemia induced by anilines in repeated dose toxicity test.

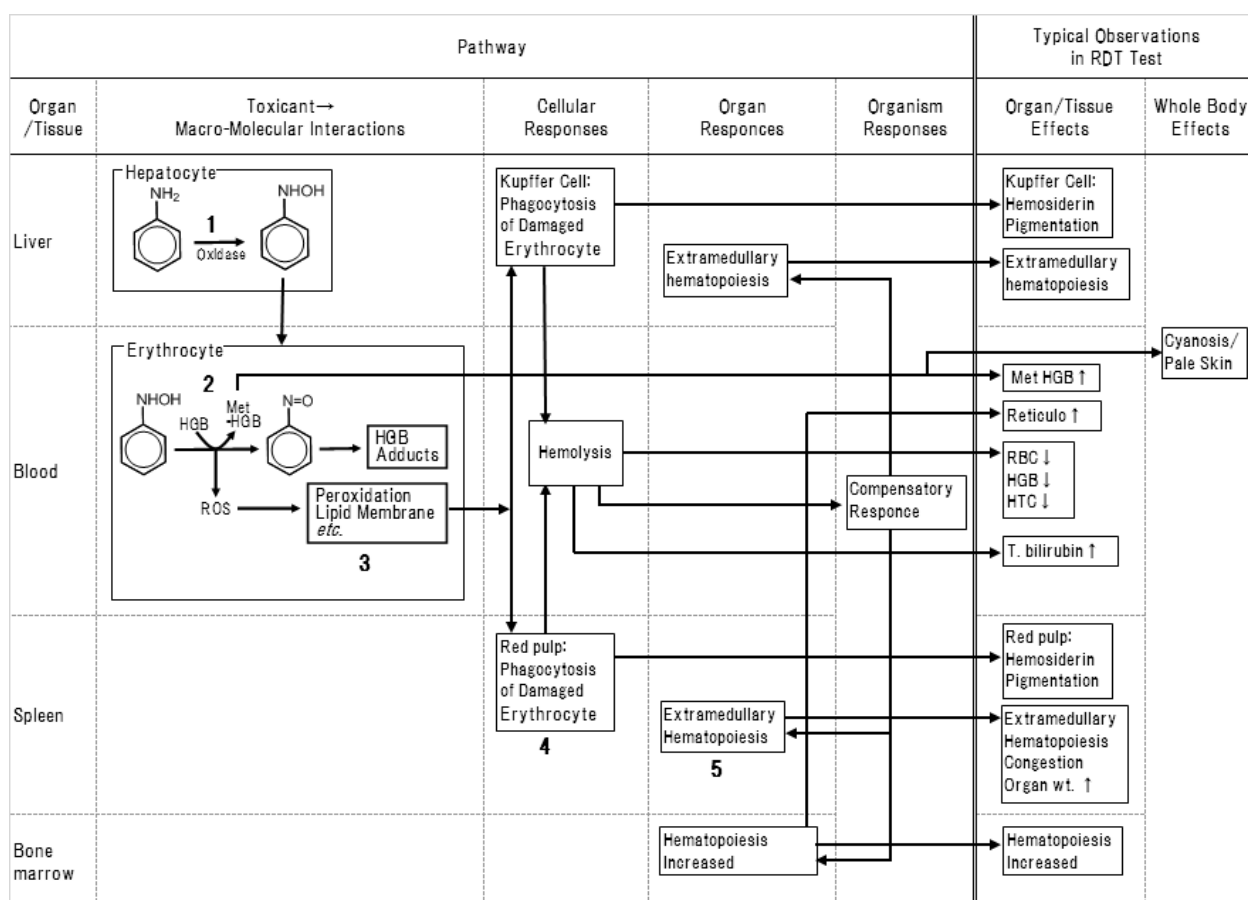


Figure 1. AOP of Hemolytic anemia induced by anilines

The key events of the AOP1 are the followings:

1. Anilines are metabolized in hepatocytes by oxidases such as P450 to N-hydroxyl anilines.
2. N-hydroxyl anilines react with hemoglobin (HGB) in erythrocytes to produce nitrosoaniline and methaemoglobin (Met-HGB).
3. Erythrocytes are degenerated (peroxidation of lipid membrane etc.) by reactive oxygen species (ROS) produced in the above reaction.
4. Phagocytosis of degenerated erythrocytes mainly in the spleen results in hemolysis.
5. Extramedullary hematopoiesis occurs in the spleen and/or liver as a compensatory response.

The following findings in repeated dose toxicity test can be the evidences for the key events (2-5) described in Figure 1.

2. Increase in the concentration of methaemoglobin (Met-HGB) is observed in hematological examination. Cyanosis is observed.
3. Decreases in red blood cell (RBC), hemoglobin (HGB) and hematocrit (HTC) are observed in hematological examination.
4. Pigmentation of hemosiderin and congestion are observed in the spleen in histopathological examination. Spleen weight increases.
5. Extramedullary hematopoiesis in the spleen and/or liver is observed in histopathological examination.

AOP2 : Nephrotoxicity induced by 4-aminophenols

It is known that 4-aminophenols induce nephrotoxicity by the AOP shown in Figure 2. The key events in the AOP are the following:

1. 4-aminophenols are metabolized by oxidase in hepatocyte to benzoquinoneimine, and it follows to form 4-aminophenols glutathione S-conjugates.
2. The glutathione S-conjugates are excreted with bile and reabsorbed in the small intestine. And then, the glutathione S-conjugates are hydrolysed to cysteine S-conjugates to be absorbed and concentrated in the renal proximal tubules.
3. The cysteine S-conjugates are metabolized in renal proximal tubules to benzoquinoneimines. The benzoquinoneimines induce oxidative stress by redox cycling or form covalent binding to cellular macromolecules in renal proximal tubules. These cause cytotoxicity in renal proximal tubules.

As a results, degeneration or necrosis of renal tubule are observed in histopathological examination in repeated dose toxicity test.

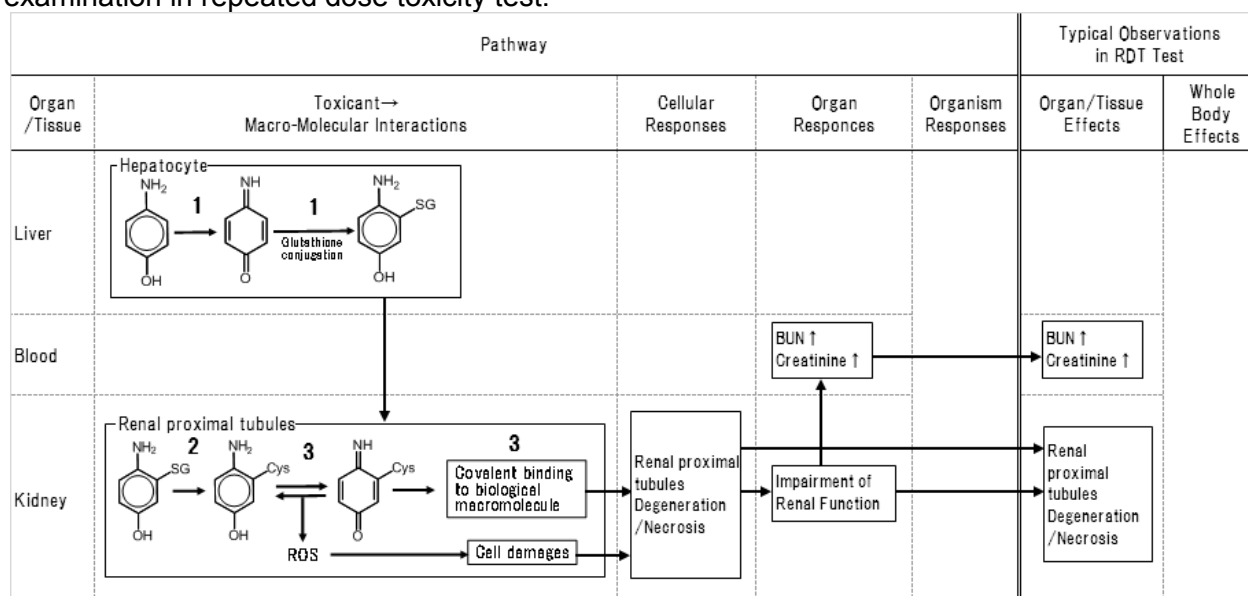


Figure 2. AOP for Nephrotoxicity induced by 4-aminophenols

Discussion of the evidence supporting the AOP:

AOP1 : Hemolytic anemia induced by anilines

1. N-hydroxylation of various anilines was observed *in vitro* tests using liver microsome of mammals such as rats and rabbits (Kiese, 1966). Nitroso benzenes were observed as metabolite of anilines (N-hydroxyanilines is rapidly oxidized to nitrosobenzene) *in vivo* test using mammals such as dogs and cats (Kiese, 1966). [Strength of evidence: Strong].
2. Anilines themselves do not cause hemoglobin oxidation by the incubation with erythrocyte suspension. On the other hand, production of methaemoglobin and nitrosobenzene were observed in case N-hydroxyl anilines were incubated with erythrocyte suspension (Kiese, 1966). [Strength of evidence: Strong].
3. Production of free radicals were identified in rat erythrocyte exposed to hemolytic concentration of N-hydroxylanilines (Bradshaw et al., 1995). However, it is unclear how the internal oxidative damage in the erythrocyte is transmitted across the cell membrane to activate signaling mechanism for macrophage recognition (McMillan et al., 2005). [Strength of evidence: Moderate].
4. Hemosiderin pigmentation were observed in spleen in a dose-dependent manner in a repeated dose toxicity test of anilines in rats (Pauluhn, 2004) . [Strength of evidence: Strong].

- 5 Increased extramedullary hematopoietic cell in spleen were observed in a dose-dependent manner in a repeated dose toxicity test of anilines in rats. (Pauluhn, 2004) [Strength of evidence: Strong].

As mentioned above, there is enough evidence to support the AOP shown in Figure 1. It can be explained via the relationship between the molecular initiating event and observations in repeated dose toxicity test.

The AOP1 can be applied to mammals and birds. It is known that there are large species differences in the susceptibility to methaemoglobinemia after administration of aniline due to their differences in metabolism. (Kiese 1974; Blaauboer et al.; 1979). For example, N-phenylacetamide, which is one of the major metabolite in rats of aniline does not produce methaemoglobin (Brodie and Axelrod, 1948) and dogs do not have N-acetyltransferase (Trepanier 1997). Therefore, dogs show higher susceptibility to methaemoglobinemia by anilines in comparison with other mammals having N-acetyltransferase.

AOP2 : Nephrotoxicity induced by 4-aminophenols

1. It was observed that 4-aminophenols were metabolized by oxidase in hepatocyte to benzoquinoneimine (Calder et al., 1979). Synthesized quinoneimine reacts non-enzymatically with glutathione (Eckert et al., 1990). [Strength of evidence: Strong].
2. The glutathione S-conjugates as a metabolite of aminophenols were detected in bile of rats (Klos et al., 1992). Biliary cannulation partially protected rats from p-aminophenol nephrotoxicity (Gartland et al 1990). Depletion of glutathione by buthionine sulfoximine, which inhibits glutathione synthesis completely protect rats against p-aminophenol induced nephrotoxicity (Gartland et al 1990). [Strength of evidence: Strong].
3. It was observed that 4-aminophenol produced a dose-dependent decrease in renal glutathione and covalently bound to renal proteins, both in vitro and in vivo (Crowe et al., 1979; Calder et al., 1979) [Strength of evidence: Moderate].

As mentioned above, there is enough evidence to support the AOP shown in Figure 2. This AOP was well established in rat.

Part II: Building a Category for the AOP Pathway (Identify the chemical space, defined as describing the group of compounds where the similarity assumption is valid for building the AOP-based chemical category)

AOP1 : Hemolytic anemia induced by anilines

Such anilines as normal aniline, alkyilanilines, haloanilines, alkoxyanilines and N-alkylanilines were observed to produce methaemoglobin in blood *in vitro* tests or *in vivo* tests focusing on methemoglobinemia (Cauchon, D. and Krishnan, K. 1997; Kiese, M. 1966; Sabbioni, G. 1993). The summaries of the results of repeated dose toxicity (RDT) tests of anilines in rats are shown in the Appendix. These tests were conducted by the Japanese government since the institution of the Chemical Substances Control Law (NIHS; NITE). The findings related to hemolytic anemia were observed in a dose-dependent manner in the RDT test of the chemicals with log Kow>1 (Nos. 1-12). Increase of the concentration of methaemoglobin is observed in hematological examination in the RDT test of the chemicals Nos. 2, 6, 7 and 8 (Methaemoglobin concentration were not measured in RDT test of the other chemicals in the appendix). The primary reason of the decision of NOEL values for the most of these chemicals were based on the findings related to hemolytic anemia.

On the other hand, two chemicals (amino phenols: Nos. 13 and 14) with low log Kow of 0.24 showed only a weak potential to induce hemolytic anemia even at high dose-levels. Three chemicals of amino benzene sulfonic acids (Nos. 15-17) having lower log Kow (negative values) lack the potential to induce hemolytic anemia even at high dose levels. One reason is the reduction in their bioavailability due to their relatively high water solubility.

According to the above information, we can define the chemical space of the category for AOP 1 as the log Kow > 1 for the chemicals such as anilines normal aniline, alkylanilines, haloanilines, alkoxyanilines and N-alkylanilines. However, the nitro-substituted anilines (Nos. 11,12) need to be further subcategorized because it is known that a different pathway of hemolytic anemia may occur (Akintonwa, D. A. A. 2000).

AOP2 : Nephrotoxicity induced by 4-aminophenols

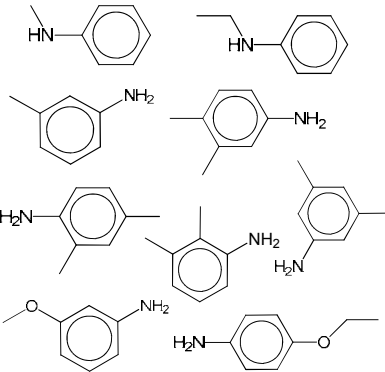
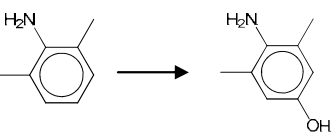
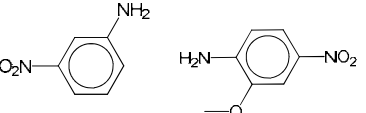
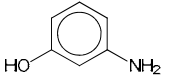
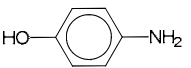
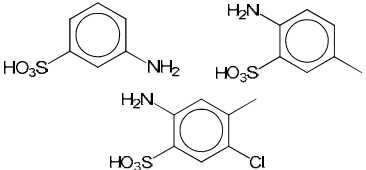
4-Aminophenols and 2-aminophenols have possibility to induce nephrotoxicity by the AOP2, since these compounds can form benzoquinoneimine. In addition, anilines could be metabolized to the compounds having 4-aminophenols or 2-aminophenols substructure, have possibility to induce nephrotoxicity by the AOP2 (Cnubben et al. 1996).

The renal responses of 4-aminophenol (No. 14) in the RDT test were indicated at a dose of 100 mg/kg as shown in the appendix. It is reported that the predominant endpoint exerted by anilines is considered to depend on the metabolism of anilines (Cnubben et al. 1996). Metabolites with 4-aminophenols substructure were observed in vivo test of the compounds Nos. 3 and 8 (Cheever et al, 1980; Short et al. 1989), and, the findings indicating the renal effects were observed in the RDT test of the compound (No. 8).

The chemical space of the AOP2 is not clear, because of the small category members which are validated in RDT test. Whereas AOP2 is not major pathway for most of anilines, AOP2 can still give the indication of different pathway from AOP1 to a part of aniline with a specific substructure.

The summary of the categorization of anilines on RDT test based on AOP1 and AOP2 are shown in Table 1.

Table 1. The summary of the categorization of anilines on RDT test based on AOP1 and AOP2.

	AOP1	AOP2	logKow	Category Member	LOEL in RDT test (mg/kg/days)		
					Total	Anemia	Kidney Effects
1	Relevant		>1		2-250	2-250	≥60
2	Relevant	Possible			50	250	250
3	Possible				15, 100	15, 300	15, -
4	Possible		0-1		240	720	720
5	Possible	Relevant			100	500	100
6			<0		>1000		

Part III: Conclusions about the AOP Pathway and Conclusions

There is enough mechanistic evidence to support the AOP1 and AOP2 (Part I). Both AOPs are validated to be consistent with the results of repeated dose toxicity test of anilines (Part II). Therefore, the confidence of the AOP1 and AOP2 could be considered high.

All the category members of AOP1 defined in Part II showed the hemolytic anemia in the RDT test with the dose rate of less than 250mg/kg and the primary reason of the decision of NOEL values in these RDT test for almost all the category members are based on the hemolytic anemia.

In order to add new category member, the information such as the reactivity to erythrocyte, metabolites and log Kow are important. It is very effective to utilize QSAR prediction for logKow and the metabolites for the compounds with no experimental data.

It would be possible to use the AOP1 category for evaluating NOEL values of anilines for the screening purpose in the risk assessment. Recently, the relationship between the chemical structure of some anilines and the intensity of hemolytic test anemia in RDT test was reported (Sakuratani et al, 2008). It might be possible to fill the data gaps of NOELs by read-across, in case the target chemical has very similar chemicals available in the category with RDT test data.

Part IV; Lessons Illustrated

Discuss the lessons learned from this case study regarding the concept of the Adverse Outcome Pathway and its utility in forming toxicologically meaningful chemical categories for filling data gaps by non-test methods (e.g., read-across and structure-activity relationships) for regulatory purposes.

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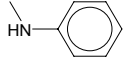
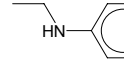
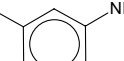

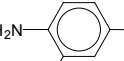
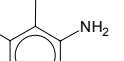
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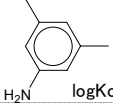
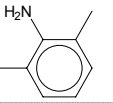
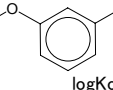
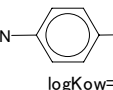
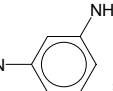
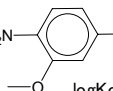
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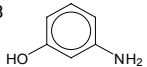
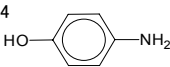
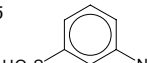
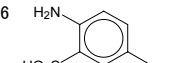
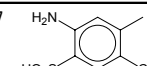
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Appendix: Summaries of the results of repeated dose toxicity tests of anilines

<p>1  logKow=1.62</p> <p>CAS: 100-61-8 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 5, 25, 125 mg/kg/day NOEL: <5 mg/kg/day</p> <p>【Clinical observation】cyanosis: 125 ♂ ♀ 【Urinalysis】ketone body ↑: >5 ♂ ♀, yellowish brown colored: 125 ♂ ♀ 【Hematology】Hgb ↓: >5 ♀, >25 ♂, Hct ↓: >25 ♂ ♀, RBC ↓: >25 ♂ ♀, Ret ↑: >25 ♂ ♀, MCV ↑: 125 ♂ ♀, MCH ↑: 125 ♂ ♀, MCHC ↑: 125 ♂ ♀ 【Blood Chemistry】Crn ↑: >5 ♀, 125 ♂, Bil ↑: 125 ♂ ♀, GOT ↑: 125 ♂ 【Organ weight】(Abs.) spleen ↑: 125 ♂ ♀, (Relat.) spleen ↑: 125 ♂ ♀ 【Histopathology】(spleen) congestion: >5 ♂, >25 ♀, deposit of pigment: >25 ♂, extramedullary-hematopoiesis: >25 ♂ ♀, (bone marrow) extramedullary-hematopoiesis: >25 ♀, 125 ♂, (liver) deposit of pigment: >25 ♂ ♀, extramedullary-hematopoiesis: >25 ♀, 125 ♂, (kidney) deposit of pigment: 125 ♂ ♀, hyaline droplet: >25 ♂</p>	<p>2  logKow=2.11</p> <p>CAS: 103-69-5 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 1, 5, 25, 125 mg/kg/day NOEL: 1 mg/kg/day</p> <p>【Body weight】↓: 125 ♂ ♀ 【Food consumption】↓: 125 ♂ ♀ 【Hematology】RBC ↓: >5 ♂ ♀, Hgb ↓: >5 ♀, >25 ♂, Hct ↓: >5 ♀, >25 ♂, PT ↓: >5 ♂, >25 ♀, Met-Hgb ↑: >25 ♂ ♀, Ret ↑: >25 ♂ ♀, MCV ↑: 125 ♂ ♀, MCH ↑: 125 ♂ ♀, MCHC ↓: 125 ♂ ♀, Heinz ↑: 125 ♂ ♀, Plt ↓: 125 ♂ 【Blood Chemistry】K ↑: >25 ♂, 125 ♀, Bil ↑: 125 ♂ ♀ 【Organ weight】(Abs.) spleen ↑: >25 ♂, 125 ♀, (Relat.) spleen ↑: >25 ♂, 125 ♀ 【Histopathology】(spleen) hemosiderosis and increased extramedullary erythropoiesis: >5 ♀, >25 ♂, congestion and decreased lymphocytes in marginal zone of lymph follicle: >25 ♂ ♀, (bone marrow) increased erythropoiesis: >25 ♂ ♀, (liver) hemosiderosis in Kupffer cell: >25 ♀, 125 ♂, extramedullary-erythropoiesis: 125 ♂ ♀, (kidney) brown pigmentation in cortical tubular epithelium: 125 ♂ ♀</p>
<p>3  logKow=1.62</p> <p>CAS: 108-44-1 OECD TG 422, 42 days, Gavage, ♂ Dose level: 30, 100, 300 mg/kg/day NOEL: <30 mg/kg/day</p> <p>【Clinical observation】brownish urine: >100 ♂, salivation: >100 ♂ 【Body weight】↓: 300 ♂ 【Food consumption】↓: 300 ♂ 【Hematology】Hgb ↓: >100 ♂, Hct ↓: >100 ♂, RBC ↓: >100 ♂ 【Blood Chemistry】Glu ↓: 300 ♂, Bil ↑: >100 ♂, Alb ↑: 300 ♂, S-GOT ↑: 300 ♂, Cho ↓: 300 ♂, A/G ↑: >100 ♂, Na ↑: >100 ♂, Cl ↑: >100 ♂ 【Organ weight】(Relat.) kidney: 300 ♂ 【Histopathology】(liver) deposit of pigment in Kupffer's cell: >100 ♂, extramedullary-hemopoiesis: >100 ♂, swelling of hepatocyte: >100 ♂, (kidney) deposit of pigment in tubular epithelium: >100 ♂, (spleen) deposit of pigment: >30 ♂, extramedullary-hemopoiesis: >30 ♂, congestion: >30 ♂</p>	<p>4  logKow=2.17</p> <p>CAS: 95-64-7 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 10, 50, 250 mg/kg/day NOEL: 50 mg/kg/day</p> <p>【Urinalysis】volume ↑: 250 ♂ ♀, pH ↓: 250 ♂ ♀, sp. gr ↓: 250 ♀ 【Hematology】Hgb ↓: 250 ♂ ♀, Hct ↓: 250 ♂ ♀, RBC ↓: 250 ♂ ♀, WBC ↑: 250 ♂, Ret ↑: 250 ♂ ♀, Plt ↑: 250 ♂ ♀ 【Blood Chemistry】Cho ↑: >50 ♀, 250 ♂ 【Organ weight】(Abs.) liver ↑: 250 ♂, spleen ↑: 250 ♂, (Relat.) liver ↑: 250 ♂ ♀, spleen ↑: 250 ♂ ♀, adrenal ↓: 250 ♀, testis ↑: 250 ♂ 【Histopathology】(kidney) hyaline droplet: >50 ♂, (bone marrow) hematopoiesis: 250 ♂ ♀, (spleen) congestion: 250 ♂ ♀, pigmentation: 250 ♂ ♀, hematopoiesis: 250 ♂ ♀, (liver) deposit of pigment: 250 ♂ ♀, single cell necrosis: 250 ♂ ♀, swelling of liver cell: 250 ♂ ♀, extramedullary-hematopoiesis: 250 ♂ ♀</p>
<p>5  logKow=2.17</p> <p>CAS: 95-68-1 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 2, 10, 50 mg/kg/day NOEL: <2 mg/kg/day</p> <p>【Clinical observation】vocalization with handling ↑: 50 ♀ 【Hematology】Hgb ↓: >10 ♂, 50 ♀, MCV ↓: 50 ♂, MCH ↓: 50 ♂, APTT ↑: >10 ♂, Plt ↑: >10 ♂ 【Blood Chemistry】Bil ↑: >2 ♂, 50 ♀, Cho ↑: >10 ♂, 50 ♀, TP ↑: 50 ♀, Alb ↑: 50 ♀, A/G ↑: 50 ♀, Ca ↑: 50 ♀ 【Organ weight】(Abs.) liver ↑: >10 ♂, 50 ♀, (Relat.) liver ↑: >10 ♂, 50 ♀, kidney ↑: 50 ♀ 【Histopathology】(liver) centrilobular-hypertrophy, hepatocyte: >10 ♂ ♀, (kidney) hyaline droplet, proximal tubular epithelium: >10 ♂</p>	<p>6  logKow=2.17</p> <p>CAS: 87-59-2 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 12, 60, 300 mg/kg/day NOEL: 12 mg/kg/day</p> <p>【Water consumption】↑: 300 ♀ ♂ 【Urinalysis】volume ↑: 300 ♀ ♂, sp. gr. ↓: 300 ♀ ♂, Bil ↑: 300 ♀ ♂, occult blood ↑: 300 ♀ ♂, RBC ↑: 300 ♀ ♂, WBC ↑: 300 ♀ ♂, small round epithelial cell ↑: 300 ♂ 【Hematology】Hgb ↓: >60 ♀ ♂, Hct ↓: >60 ♀, 300 ♂, MCV ↑: 300 ♀ ♂, MCH ↑: 300 ♀ ♂, MCHC ↓: 300 ♂, RBC ↓: >60 ♀, 300 ♂, WBC ↑: 300 ♀ ♂, Ret ↑: 300 ♀ ♂, Met-Hb ↑: >60 ♀, 300 ♂ 【Blood Chemistry】BUN ↑: 300 ♀ ♂, T-Bil ↑: 300 ♀ ♂, Cho ↑: 300 ♀, PL ↑: 300 ♀, GPT ↑: 300 ♀, indirect-Bil ↑: 300 ♂, γ-GTP ↑: 300 ♂, K ↑: 300 ♂ 【Organ weight】(Abs.) liver ↑: 300 ♀, kidney ↑: 300 ♀ ♂, spleen ↑: 300 ♀ ♂, thyroid ↑: >60, (Relat.) liver ↑: >60 ♂, 300 ♀, kidney ↑: 300 ♀ ♂, spleen ↑: 300 ♀ ♂, thyroid ↑: >12 ♀ 【Histopathology】(liver) centrilobular-hypertrophy, hepatocyte: 300 ♀ ♂, extramedullary-hematopoiesis: 300 ♀ ♂, proliferation, bile duct: 300 ♀ ♂, I deposit, hemosiderin, Kupffer's cell: 300 ♀ ♂, (kidney) necrosis, papillary: 300 ♀ ♂, dilatation, renal tubule: 300 ♀ ♂, cellular infiltration, interstitium, cortex: 300 ♀ ♂, hyaline cast: 300 ♀, basophilia, renal tubule: 300 ♂, eosinophilic body: 300 ♂, inner medulla-mineralization: 300 ♂, (spleen) red pulp-deposit, hemosiderin: >12 ♀, extramedullary-hematopoiesis: 300 ♀ ♂, congestion: 300 ♂, (bone marrow) hematopoiesis, increased (femur): >60 ♀ ♂, (urinary bladder) cellular infiltration, submucosa: 300 ♂</p>

<p>7</p>  <p>logKow=2.17</p> <p>CAS: 108-69-0 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 10, 60, 30 mg/kg/day NOEL: 10 mg/kg/day</p> <p>【Clinical observation】cyanosis: 360 ♀ ♂, pale skin: 360 ♀ ♂, salivation: 360 ♀ ♂, exophthalmos: 360 ♀ ♂ 【Body weight】↓: 360 ♀ 【Food consumption】↓: 360 ♀ 【Urinalysis】volume ↑: 360 ♀ ♂, osmotic pressure ↓: 360 ♀ ♂, sp. gr. ↓: 360 ♀ ♂, Na ↑: 360 ♀ ♂, Cl ↑: 360 ♀ ♂, K ↑: 360 ♀ 【Hematology】Hgb ↓: >60 ♀ ♂, Hct ↓: 60 ♀, 360 ♀, RBC ↓: >60 ♀, 360 ♂, WBC ↑: 360 ♀, Ret ↑: >60 ♀ ♂, Neut ↑: 360 ♂, MCV ↑: >60 ♀, 360 ♂, MCHC ↓: >60 ♀, 360 ♂, MCH ↑: 360 ♀ ♂, Met-Hgb ↑: 360 ♀ ♂ 【Blood Chemistry】Bil ↑: 360 ♀ ♂, PL ↑: 360 ♀ ♂, Cho ↑: 360 ♀, Glu ↓: 360 ♂, GOT ↑: 360 ♀ ♂, GPT ↑: >60 ♀, 360 ♂, P ↑: 360 ♀ ♂, Ca ↑: 360 ♀, K ↓: 360 ♂, Cl ↓: 360 ♂ 【Organ weight】(Abs.) liver ↑: 360 ♀, kidney ↑: 360 ♀, spleen ↑: >60 ♀ ♂, thyroid ↑: 360 ♀ ♂, testis ↑: 360 ♂, (Relat.) liver ↑: 360 ♀ ♂, kidney ↑: 360 ♀ ♂, spleen ↑: 360 ♀ ♂, thyroid ↑: 360 ♀ ♂, testis ↑: 360 ♂ 【Histopathology】(liver)centrilobular-hypertrophy: >60 ♂, 360 ♀, deposit, hemosiderin, sinusoidal cell: >60 ♀ ♂, extramedullary-hematopoiesis: 360 ♀ ♂, periportal-deposit, hemosiderin, hepatocyte: 360 ♀, necrosis, massive: 360 ♀, (kidney) deposit, hemosiderin, tubular epithelium: 360 ♀ ♂, papilla-necrosis: 360 ♀ ♂</p>	<p>8</p>  <p>logKow=2.17</p> <p>CAS: 87-62-7 OECD TG 422, 42 days, Gavage, ♂ Dose level: 2, 10, 50, 250 mg/kg/day NOEL: 10 mg/kg/day</p> <p>【Clinical observation】locomotor activity ↓: >50 ♂, ptosis: >50 ♂ 【Body weight】↓: 250 ♂ 【FOB】forelimb and hindlimb, grip strength ↓: 250 ♂, locomotor activity ↓: 250 ♂ 【Urinalysis】volume ↑: 250 ♂, sp. gr. ↓: 250 ♂ 【Hematology】Hgb ↓: 250 ♂, RBC ↓: 250 ♂, MCV ↑: 250 ♂, MCHC ↓: 250 ♂, Ret ↑: 250 ♂, Met-HGB ↑: 250 ♂ 【Blood Chemistry】P ↑: 250 ♂ 【Organ weight】(Abs.)spleen ↑: 250 ♂, (Relat.) liver ↑: 250 ♂, spleen ↑: 250 ♂ 【Histopathology】(liver) centrilobular-hyperplasia, hepatocyte: >50 ♂, (kidney) eosinophilic, proximal: >50 ♂, hyaline droplet: 250 ♂, papilla-necrosis: 250 ♂ protein cast: 250 ♂, basophilic tubule: 250 ♂, (spleen) extramedullary-hematopoiesis: 250 ♂, extramedullary-hemosiderin deoisit: 250 ♂</p>
<p>9</p>  <p>logKow=1.16</p> <p>CAS: 536-90-3 OECD TG 422, 42 days, Gavage, ♂ Dose level: 2.4, 12, 60, 300 mg/kg/day NOEL: <2.4 mg/kg/day</p> <p>【Clinical observation】brown urine: 300 ♂ 【Body weight】↓: 300 ♂ 【Food consumption】↓: 300 ♂ 【Hematology】RBC ↓: >60 ♂, Hgb ↓: >60 ♂, Hct ↓: >60 ♂, Ret ↑: 300 ♂ 【Blood Chemistry】Bil ↑: >60 ♂, Glu ↓: 300 ♂, Na ↑: 300 ♂, Ca ↓: 300 ♂, P ↑: 300 ♂ 【Organ weight】(Abs.) spleen ↑: 300 ♂, (Relat.) liver ↑: 300 ♂, spleen ↑: 300 ♂, epididymis ↑: 300 ♂ 【Histopathology】(liver) extramedullary-hematopoiesis: 300 ♂, brown pigmentation in Kupffer's cell: 300 ♂, (kidney) brown pigmentation in proximal tubules: 300 ♂, eosinophilic change of urinary tubules: >60 ♂, (spleen) congestion: >60 ♂, brown pigmentation: >12 ♂, decreased cellularity of cell areas: >60 ♂</p>	<p>10</p>  <p>logKow=1.65</p> <p>CAS: 156-43-4 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 10, 40, 160 mg/kg/day NOEL: 10 mg/kg/day</p> <p>【Urinalysis】urobilinogen ↑: >40 ♂ ♀ 【Hematology】Hgb ↓: >40 ♂ ♀, Hct ↓: >40 ♂ ♀, RBC ↓: >40 ♂ ♀, Ret ↑: >40 ♂ ♀, MCH ↑: >40 ♀, 160 ♂, MCHC ↑: 160 ♂ ♀ 【Blood Chemistry】Cho ↓: 160 ♂ 【Organ weight】(Abs.) spleen ↑: >40 ♂ ♀, (Relat.) spleen ↑: >40 ♂ ♀ 【Histopathology】(spleen) hemosiderosis: 40 ♂ ♀, hemopoiesis ↑: >40 ♂ ♀, congestion: >40 ♂ ♀, (bone marrow) myeloid hyperplasia: >40 ♂ ♀</p>
<p>11</p>  <p>logKow=1.47</p> <p>CAS: 99-09-2 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 15, 50, 170 mg/kg/day NOEL: <15 mg/kg/day</p> <p>【Hematology】Hgb ↓: >15 ♂ ♀, Hct ↓: >15 ♂ ♀, RBC ↓: >15 ♂ ♀, WBC ↓: 170 ♀, E-Blast ↑: >15 ♂, >50 ♀, Ret ↑: >50 ♂ ♀, MCV ↑: >50 ♀, 170 ♂, MCH ↑: >50 ♀, 170 ♂, MCHC ↓: 170 ♂ ♀, Plt ↓: 170 ♀ 【Blood Chemistry】BUN ↑: >15 ♀, Cho ↑: >50 ♂, >15 ♀, TP ↑: >50 ♂, >15 ♀, Alb ↑: >50 ♂, >15 ♀, A/G ↑: >50 ♂, 170 ♀ 【Organ weight】(Abs.) liver ↑: >50 ♂ ♀, spleen ↑: >50 ♂ ♀, kidney ↑: 170 ♀, testis ↓: 170 ♂, (Relat.) spleen ↑: >15 ♂, >50 ♀, liver ↑: >50 ♂, >150 ♀, kidney ↑: 170 ♂ ♀, testis ↓: 170 ♂ 【Histopathology】(spleen) hemosiderosis: >15, extramedullary-hematopoiesis: >15, hyperemia and congestion: >15, (bone marrow) hematopoiesis: >15, erythroid hyperplasia: >50, (liver) swelling of hepatocyte: >50, hemosiderosis: 170, extramedullary-hematopoiesis: 170, (kidney) lipofuscin deposition: >50, (testis) derangement of spermatogenesis: >50, multinucleated giant cell formation: 170, increase in interstitial cell: 170, (epididymis) lack of sperm: >50</p>	<p>12</p>  <p>logKow=1.55</p> <p>CAS: 97-52-9 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 30, 100, 300 mg/kg/day NOEL: 30 mg/kg/day</p> <p>【Clinical observation】locomotor activity ↓: >100 ♂ ♀, hypothermia: 300 ♀ ♂, salivation: 300 ♀ ♂ 【Body weight】↓: 300 ♀ 【Food consumption】↓: 300 ♀ 【Urinalysis】pH ↓: 300 ♀ ♂, Bil ↑: 300 ♀ ♂, ketone body ↑: 300 ♂ 【Hematology】Hgb ↓: 300 ♀ ♂, Hct ↓: >100 ♀, 300 ♂, RBC ↓: 300 ♀ ♂, Ret ↑: 300 ♀ ♂, MCHC ↓: 300 ♀, Plt ↑: 300 ♀ ♂, fibrinogen ↑: 300 ♂ 【Blood Chemistry】Cho ↑: 300 ♂, Bil ↑: 300 ♀ ♂, GPT ↑: 300 ♂, GOT ↑: 300 ♀ ♂, K ↑: 300 ♂ 【Organ weight】(Abs.)spleen ↑: 300 ♂, (Relat.) liver ↑: 300 ♀ ♂, spleen ↑: 300 ♀ ♂ 【Histopathology】(liver) hypertrophy: 300 ♀ ♂, deposit, pigment: 300 ♀, extramedullary-hematopoiesis: 300 ♀, (heart) necrosis: 300 ♀ ♂, myocardial damage: 300 ♀ ♂, cellular infiltration: 300 ♀ ♂, pericarditis: 300 ♀, fibrosis, ventricle: 300 ♀, (spleen) deposit, pigment: 300 ♀ ♂, extramedullary-hematopoiesis: 300 ♀ ♂, (adrenal) degeneration, vacuolar: 300 ♂</p>

13	 logKow=0.24	CAS: 591-27-5 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 80, 240, 720 mg/kg/day NOEL: 80 mg/kg/day	14	 logKow=0.24	CAS: 123-30-8 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 4, 20, 100, 500 mg/kg/day NOEL: 20 mg/kg/day
<p>【Clinical observation】salivation: 720 ♀ ♂, tremor: 720 ♀ ♂</p> <p>【Body weight】↓ : 720 ♀ ♂</p> <p>【Food consumption】↑ : 720 ♀</p> <p>【Urinalysis】volume ↑ : 720 ♂ ♀, sp. gr. ↓ : 720 ♀</p> <p>【Hematology】Hgb ↓ : 720 ♀, RBC ↓ : 720 ♀, Ret ↑ : 720 ♀</p> <p>【Blood Chemistry】BUN ↑ : 720 ♀, Bil ↑ : 720 ♀ ♂, Cho ↑ : 720 ♂, TG ↓ : 720 ♂, GPT ↑ : 720 ♀ ♂, γ-GTP ↑ : 720 ♀</p> <p>【Organ weight】(Abs.)spleen ↑ : 720 ♀, thyroid ↑ : 720 ♀ ♂, (Relat.) liver: 720 ♀ ♂, kidney ↑ : 720 ♀ ♂, spleen ↑ : 720 ♀, thyroid: 720 ♀ ♂, testis ↑ : 720 ♂</p> <p>【Histopathology】(liver) deposit, brown pigment, Kupffer' s cell: 720 ♀ ♂, (kidney) deposit, brown pigment, proximal tubular epithelium: >240 ♀, 720 ♂, hyaline droplet, proximal tubular epithelium: 720 ♂, (spleen) deposit, hemosiderin: 720 ♂ ♀, (l pituitary) hypertrophy, basophil: 720 ♂, (thyroid) hypertrophy, follicular cell: 720 ♀ ♂</p>			<p>【Clinical observation】salivation: 500 ♀ ♂</p> <p>【Body weight】↓ : 500 ♂</p> <p>【Water consumption】↑ : 500 ♂</p> <p>【Urinalysis】epidermal cell ↑ : >100 ♀, 500 ♂</p> <p>【Hematology】Hgb ↓ : 500 ♀, Hct ↓ : 500 ♀, RBC ↓ : 500 ♀ ♂, Ret ↑ : 500 ♀</p> <p>【Blood Chemistry】Alb ↑ : 500 ♂</p> <p>【Organ weight】(Abs.)liver ↑ : >100 ♀, liver ↑ : 500 ♀, kidney ↑ : 500 ♀ ♂, (Relat.) spleen ↑ : 500 ♀, liver ↑ : 500 ♀ ♂</p> <p>【Histopathology】spleen-fibrosis, capsule: >100 ♂, spleen-increase in extramedullary hematopoiesis: 500 ♀ ♂, spleen-increase in hemosiderin pigment: 500 ♀, kidneys-basophilic tubule, proximal: >100 ♀ ♂, kidney-inflammation cell infiltration, interstitium: 500 ♂</p>		
15	 logKow=-2.08	CAS: 121-47-1 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 100, 300, 1000 mg/kg/day NOEL: 300mg/kg/day	16	 logKow=-1.53	CAS: 88-44-8 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 100, 300, 1000 mg/kg/day NOEL: 300 mg/kg/day
<p>【Water consumption】↑ : 300 ♂</p> <p>【Urinalysis】pH ↓ : 1000 ♂ ♀</p>			<p>【Clinical observation】sp. gr. ↑ : 1000 ♂, pH ↓ : 1000 ♂</p> <p>【Blood Chemistry】Cho ↓ : 1000 ♂, GPT ↑ : 1000 ♀, Glu ↓ : 1000 ♀</p> <p>【Necropsy】cecum-distention: 1000 ♀ ♂</p>		
17	 logKow=-0.89	CAS: 88-53-9 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 100, 300, 1000 mg/kg/day NOEL: 1000 mg/kg/day			

*The values of LogKow were calculated by KOWWIN.

-Case Study-

Hemolytic Anemia Induced by Anilines and Nephrotoxicity Induced by 4-Aminophenols in Repeated Dose Toxicity

Yuki SAKURATANI

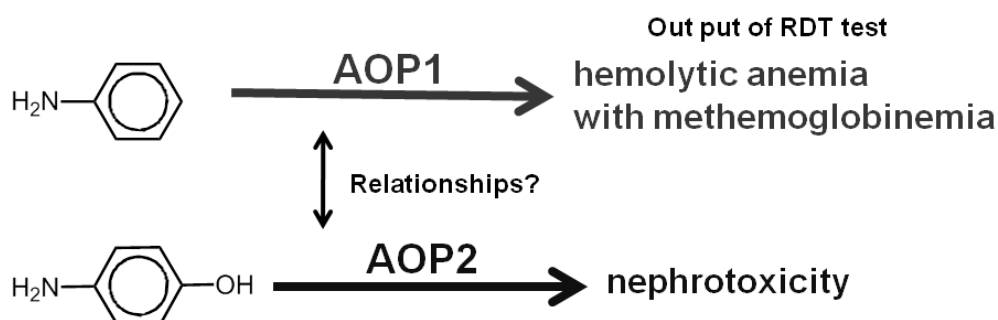
National Institute of Technology and Evaluation, Japan

Workshop on Using Mechanistic Information in Forming Chemical Categories to Fill Data Gaps
for Regulatory Purposes, 8-10 December 2010, Washington DC

1

Introduction

In this case study, categorization of anilines for the repeated dose toxicity (RDT) is discussed, based on two AOPs related to the hemolytic anemia (AOP1) and the nephrotoxicity (AOP2).

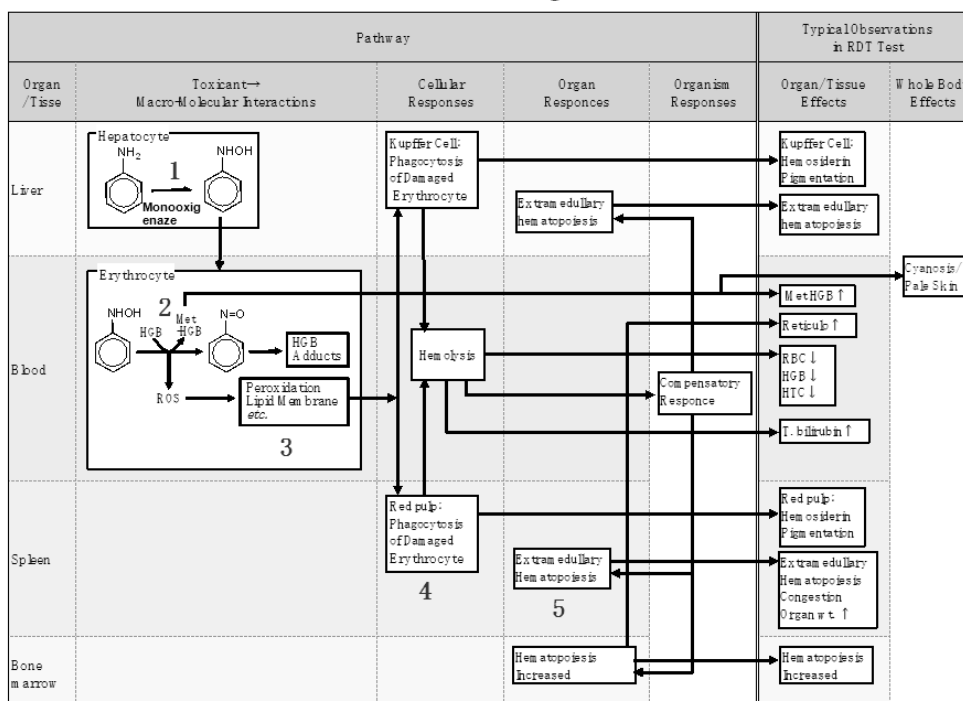


2

Part I: Building An Adverse Outcome Pathway

3

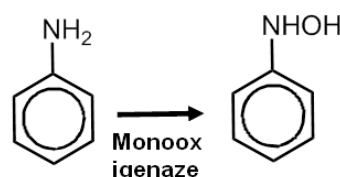
Brief Summary of AOP1



4

Key Event 1 (AOP1)

Anilines are metabolized in hepatocytes by monooxygenase such as P450 to N-hydroxyl anilines.



Evidence:

N-hydroxylation of various anilines was observed in vitro tests using liver microsome of mammals such as rats and rabbits (Kiese, 1966).

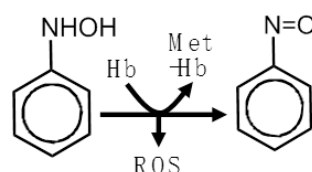
Nitroso benzenes were observed as metabolite of anilines (N-hydroxyanilines was rapidly oxidized to nitrosobenzene) in vivo test using mammals such as dogs and cats (Kiese, 1966).

[Strength of evidence: Strong]

5

Key Event 2 (AOP1)

N-Hydroxyl anilines react with hemoglobin (Hb) in erythrocyte to produce nitrosoaniline and methaemoglobin (Met-Hb).



Evidence:

Anilines themselves do not cause hemoglobin oxidation by the incubation with erythrocyte suspension. On the other hand, production of methaemoglobin and nitrosobenzene were observed in case N-hydroxyl anilines were incubated with erythrocyte suspension (Kiese, 1966).

[Strength of evidence: Strong]

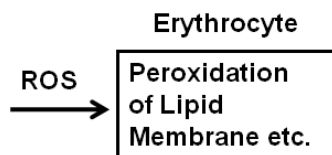
Typical Observation in RDT Test (Methemoglobinemia):

【Hematological Examination】 Met-HGB↑
 【Clinical observation】 Cyanosis

6

Key Event 3 (AOP1)

Erythrocytes are degenerated (peroxidation of lipid membrane etc.) by reactive oxygen species (ROS) produced in the above reaction.



Evidence:

Production of free radicals were identified in rat erythrocyte exposed to hemolytic concentration of N-hydroxylanilines (Bradshaw et al., 1995). However, it is unclear how the internal oxidative damage in the erythrocyte is transmitted across the cell membrane to activate signaling mechanism for macrophage recognition (McMillan et al., 2005).

[Strength of evidence : Moderate]

7

Key Event 4 (AOP1)

Phagocytosis of degenerated erythrocytes mainly in the spleen results in hemolysis.

Evidence:

Hemosiderin pigmentation was observed in spleen in a dose-dependent manner in a repeated dose toxicity test of anilines in rats (Pauluhn, 2004) .

[Strength of evidence: Strong]

Typical Observation in RDT Test (hemolytic anemia):

【Hematological Examination】 RBC↓, HGB↓, HTC↓

【Histopathological Examination】

(Spleen), Pigmentation of hemosiderin, Congestion ,

【Organ weight】 Spleen ↑

8

Key Event 5 (AOP1)

Extramedullary hematopoiesis occurs in the spleen and/or liver as a compensatory response.

Evidence:

Extramedullary hematopoiesis in the spleen and/or liver was observed in histopathological examination.

[Strength of evidence: Strong].

Typical Observation in RDT Test:

【Histopathological Examination】

(spleen) Extramedullary hematopoiesis

(liver) Extramedullary hematopoiesis

9

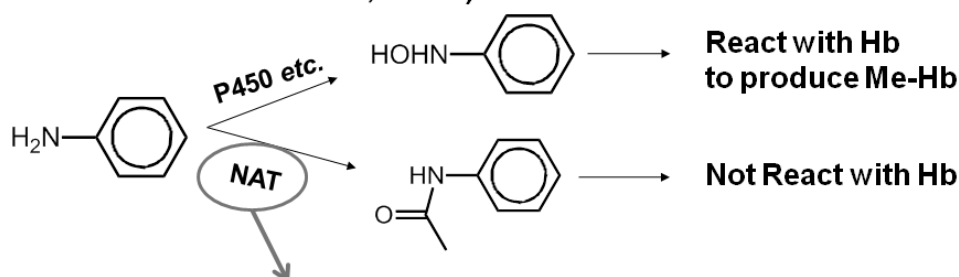
Summary (AOP 1)

There is enough evidence to support the AOP. It can be explained by linking with the molecular initiating event and observations in repeated dose toxicity test.

10

Species Differences (AOP1)

- The AOP1 can be applied to mammals and birds. It is known that there are large species differences in the susceptibility to methemoglobinemia after administration of aniline due to their differences in metabolism. (Kiese 1974; Blaauboer et al.; 1979; Brodie and Axelrod, 1948).



Dogs do not have N-acetyltransferase: NAT (Trepanier 1997). Therefore, dogs show higher susceptibility to methemoglobinemia by anilines in comparison with other mammals having NAT.

11

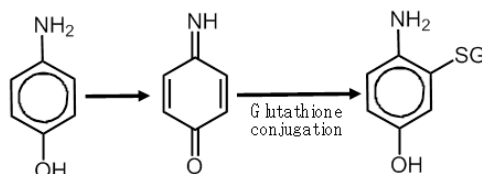
Brief Summary of AOP2

Pathway					Typical Observations in RDT Test	
Organ / Tissue	Toxicant → Macro-Molecular Interactions	Cellular Responses	Organ Responses	Organism Responses	Organ/Tissue Effects	Whole Body Effects
Liver	Hepatocyte 					
Blood			BUN ↑ Creatinine ↑		BUN ↑ Creatinine ↑	
Kidney	Renal proximal tubules 	Renal proximal tubules Degeneration / Necrosis	Impairment of Renal Function		Renal proximal tubules Degeneration / Necrosis	

12

Key Event 1 (AOP2)

4-aminophenols are metabolized by oxidase in hepatocyte to benzoquinoneimine, and it follows to form 4-aminophenol glutathione S-conjugates.



Evidence:

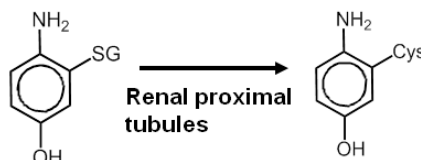
It was observed that 4-aminophenols were metabolized by oxidase in hepatocyte to benzoquinoneimine (Calder et al., 1979). Synthesized quinoneimine reacts non-enzymatically with glutathione (Eckert et al., 1990).

[Strength of evidence: Strong].

13

Key Event 2 (AOP2)

The glutathione S-conjugates are excreted with bile and reabsorbed in the small intestine. And then, the glutathione S-conjugates are hydrolysed to cysteine S-conjugates to be absorbed and concentrated in the renal proximal tubules.



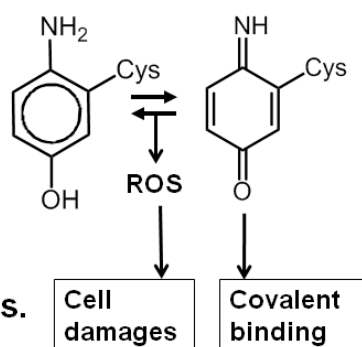
Evidence:

The glutathione S-conjugates as a metabolite of aminophenols were detected in bile of rats (Klos et al., 1992). Biliary cannulation partially protected rats from 4-aminophenol nephrotoxicity (Gartland et al 1990). Depletion of glutathione by buthionine sulfoximine, which inhibits glutathione synthesis, completely protected rats against 4-aminophenol induced nephrotoxicity (Gartland et al 1990). [Strength of evidence: Strong].

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Key Event 3 (AOP2)

The cysteine S-conjugates are metabolized in renal proximal tubules to benzoquinoneimines. The benzoquinoneimines induce oxidative stress by redox cycling and/or form covalent binding to cellular macromolecules in renal proximal tubules. These cause cytotoxicity in renal proximal tubules.



Evidence:

It was observed that 4-aminophenol produced a dose-dependent decrease in renal glutathione and covalently bound to renal proteins, both in vitro and in vivo (Crowe et al., 1979; Calder et al., 1979)

[Strength of evidence: Moderate].

15

Summary (AOP 2)

- As mentioned above, there are enough evidence to support the AOP2. This AOP was well established in rat.

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Part II: Building a Category for the AOP Pathway

17

Identification of the anilines to induce hemolytic anemia by the AOP1

Research Papers

It was reported that normal aniline, alkylanilines, haloanilines, alkoxyanilines and N-alkylanilines produced methaemoglobin in blood in vitro tests or single dose toxicity tests focusing on methemoglobin anemia.

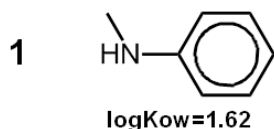
Repeated Dose Toxicity (RDT) Tests

The test reports for 17 mono-cyclic aniline derivatives were selected from the test reports for about 300 chemicals under Japanese Chemical Substances Control Law (See the annex).

The findings (symptoms) related to the AOP1 were investigated.

18

Validation of AOP1 for RDT test (N-Methyl aniline)



CAS: 100-61-8
OECD TG 407, 28 days, Gavage, ♂ ♀
Dose level: 5, 25, 125 mg/kg/day
NOEL: <5 mg/kg/day

Red: AOP1

【Clinical observation】 cyanosis: 125♂♀
【Urinalysis】 ketone body↑: >5♂♀, yellowish brown colored: 125♂♀
【Hematology】 Hgb↓: >5♀, >25♂, Hct↓: >25♂♀, RBC↓: >25♂♀, Ret↑: >25♂♀,
 MCV↑: 125♂♀, MCH↑: 125♂♀, MCHC↑: 125♂♀
【Blood Chemistry】 Crn↑: >5♀, 125♂, Bil↑: 125♂♀, GOT↑: 125♂
【Organ weight】 (Abs.) spleen↑: 125♂♀, (Relat.) spleen↑: 125♂♀
【Histopathology】
 (spleen) congestion: >5♂, >25♀, deposit of pigment: >25♂, extramedullary-hematopoiesis: >25♂♀
 (bone marrow) extramedullary-hematopoiesis: >25♀, 125♂
 (liver) deposit of pigment: >25♂♀, extramedullary-hematopoiesis: >25♀, 125♂
 (kidney) deposit of pigment: 125♂♀, hyaline droplet: >25♂

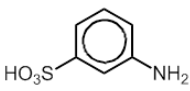
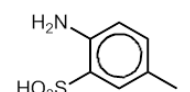
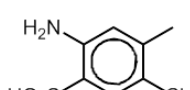
Many findings related to the AOP1 were observed as the associated reasons of the NOELs (predominant adverse effect) in the RDT test.

Validation of AOP1 for RDT test (Anilines with logKow>1)

[illegible]

We can identify that these chemicals are the category members of AOP1 on RDT.

Consideration of the Mitigating Factor of AOP1

15	 <p>logKow = -2.08</p> <p>[Water consumption] ↑: 300♂ [Urinalysis] pH↓: 1000♂♀</p>	CAS: 121-47-1 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 100, 300, 1000 mg/kg/day NOEL: 300mg/kg/day	Three chemicals, amino benzene sulfonic acids that had lower log Kows (negative values) lack the potential to induce hemolytic anemia even at high dose levels. One reason is the low bioavailability due to their relatively high water solubility.
16	 <p>logKow = -1.53</p> <p>[Clinical observation] sp. gr.↑: 1000♂, pH↓: 1000♂ [Blood Chemistry] Cho↓: 1000♂, GPT↑: 1000♀, Glu↓: 1000♀ [Necropsy] cecum-distention: 1000♂♀</p>	CAS: 88-44-8 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 100, 300, 1000 mg/kg/day NOEL: 300 mg/kg/day	
17	 <p>logKow = -0.89</p>	CAS: 88-53-9 OECD TG 407, 28 days, Gavage, ♂ ♀ Dose level: 100, 300, 1000 mg/kg/day NOEL: 1000 mg/kg/day	

No adverse effect was observed in the test.

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Chemical Space of the AOP1 on RDT test

We can define the chemical space of the category for AOP 1 as the log Kow>1 for the chemicals such as anilines normal aniline, alkyanilines, haloanilines, alkoxyanilines and N-alkylanilines.

However, the nitro-substituted anilines (Nos. 11,12) need to be further subcategorized because it is known that a different pathway of hemolytic anemia may occur (Akintonwa, D. A. A. 2000).

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Identification of the Anilines to Induce Nephrotoxicity by the AOP2

Research Papers

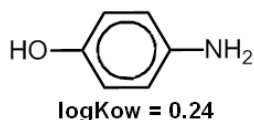
Nephrotoxicity was observed in the single dose toxicity tests of 2-aminophenol, 4-aminophenol and halogenated anilines.

Repeated Dose Toxicity (RDT) Tests

Only 4-aminophenol was found to induce nephrotoxicity by AOP2.

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Validation of AOP2 for RDT test (p-Amino phenol)



CAS: 123-30-8
OECD TG 407, 28 days, Gavage, ♂ ♀
Dose level: 4, 20, 100, 500 mg/kg/day
NOEL: 20 mg/kg/day

[Clinical observation] salivation: 500♀♂
 [Body weight] ↓: 500♂
 [Water consumption] ↑: 500♂
 [Urinalysis] epidermal cell↑: >100♀, 500♂
 [Hematology] Hgb↓: 500♀, Hct↓: 500♀, RBC↓: 500♀♂, Ret↑: 500♀
 [Blood Chemistry] Alb↑: 500♂
 [Organ weight] (Abs.)liver↑: >100♀, liver↑: 500♀, kidney↑: 500♀♂,
 (Relat.) spleen↑: 500♀, liver↑: 500♀♂
 [Histopathology]
 (spleen) fibrosis, capsule: >100♂, extramedullary hematopoiesis: 500♀♂, hemosiderin pigment: 500♀,
 (kidney) basophilic tubule, proximal: >100♀♂, inflammation cell infiltration, interstitium: 500♂

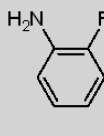
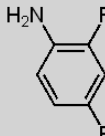
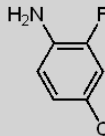
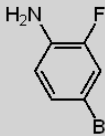
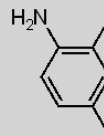
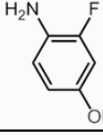
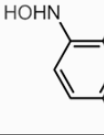
Red: AOP1
Blue: AOP2

The findings related to AOP2 were observed as the associated reasons of the NOELs in the RDT test.

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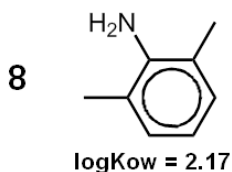
Consideration of Metabolite

- It is reported that the predominant endpoint exerted by anilines is considered to depend on the metabolism of anilines (Cnubben et al. 1996).

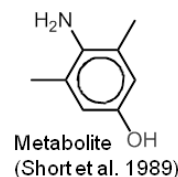
Tested Chemicals					
Nephrotoxicity	High				Low
Methemoglobinemia	Low				High
Rate of metabolite		High			Low
		Low			High

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Validation of AOP2 for RDT test (2,6-Dimethyl aniline)



CAS: 87-62-7
OECD TG 422, 42 days, Gavage, ♂
Dose level: 2, 10, 50, 250 mg/kg/day
NOEL: 10 mg/kg/day



【Clinical observation】 locomotor activity↓: >50σ, ptosis: >50σ

【Body weight】 ↓: 250σ

【FOB】 forelimb and hindlimb, grip strength↓: 250σ, locomotor activity↓: 250σ

【Urinalysis】 volume ↑: 250σ, sp. gr.↓: 250σ

【Hematology】 Hgb↓: 250σ, RBC↓: 250σ, MCV↑: 250σ, MCHC↓: 250σ, Ret↑: 250σ,

Met-HGB↑: 250σ

【Blood Chemistry】 P↑: 250σ

【Organ weight】 (Abs.)spleen↑: 250σ, (Relat.) liver ↑: 250σ, spleen↑: 250σ

【Histopathology】

(liver) centrilobular-hyperplasia, hepatocyte: >50σ

(kidney) eosinophilic, proximal: >50σ, hyaline droplet: 250σ, papilla-necrosis: 250σ, protein cast: 250σ, basophilic tubule: 250σ

(spleen) extramedullary-hematopoiesis: 250σ, extramedullary-hemosiderin deosit:250σ

Red: AOP1
Blue: AOP2

Findings related to the AOP2 were observed in the RDT test as well as the AOP1.

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Chemical Space of AOP2 for RDT test

The chemical space of the AOP2 is not clear, due to the small category members validated in RDT test. Even AOP2 is not major pathway for most of anilines, AOP2 can still give the indication of different pathway from AOP1, for aniline with a specific substructure.

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Summary of the Categorization

	AOP1	AOP2	logKow	Category Member	LOEL in RDT Test (mg/kg/days)		
					Total	Anemia	Kidney Effects
1	Relevance	/	>1		2-250	2-250	>60
2	Relevance	Possible			50	250	250
3	Possible	/			15, 100	15, 300	15, -
4	Possible	/			240	720	720
5	Possible	Relevance	0-1		100	500	100
6	/	/	<0		>1000	/	/

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Part III: Conclusions about the AOP Pathway and Conclusions

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Conclusions (1)

How strong is the relationship that links the chemical stressor, in a causative manner, to the adverse outcome?

There is enough mechanistic evidence to support the AOP1 and AOP2 (Part I). Both AOPs are validated to be consistent with the results of repeated dose toxicity test of anilines (Part II). Therefore, the confidence of the AOP1 and AOP2 could be considered high.

What is the overall level of Confidence with the chemical category?

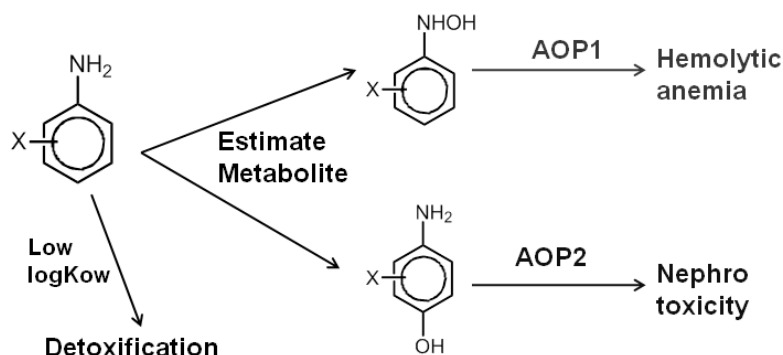
All the category members of AOP1 defined in Part II showed the hemolytic anemia in the RDT test with the dose rate of less than 250mg/kg and the primary reason of the decision of NOEL values in these RDT test for almost all the category members are based on the hemolytic anemia.

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Conclusions (2)

To add a new substance/chemical to this category – what are the necessary data?

In order to add new category member, the information such as log Kow, metabolite and the reactivity to target molecules are important. It is very effective to utilize QSAR prediction for logKow and the metabolites for the compounds with no experimental data.



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Conclusions (3)

Are there data deficiencies at any step along the AOP that would limit the utility of the AOP concept being applied for a specific purpose ?

It would be possible to use the AOP1 category for evaluating NOEL values for the screening purpose in the risk assessment of anilines.

It might be possible to fill the data gaps of NOELs by read-across, in case the target chemical is very similar to category members with RDT test data.

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Part IV: Lessons Illustrated

Discuss the lessons learned from this case study regarding the concept of the Adverse Outcome Pathway and its utility in forming toxicologically meaningful chemical categories for filling data gaps by non-test methods (e.g., read-across and structure-activity relationships) for regulatory purposes.

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The outcomes from the “NEDO Development of Hazard Assessment Techniques Using Structure-activity Relationship Methods.” were used for preparing the case study.

Project leader: Makoto Hayashi, Biosafety Research Center, Foods, Drugs, and Pesticides

Project Team:

Prof. Ovanes Mekenyan, Bourgas “Prof. Assen Zlatarov” Univ.

Prof. Yasushi Yamazoe, Tohoku Univ.

Prof. Takashi Okada, Kwansei gakuin Univ.

Fujitsu Limited

National Institute of Health Sciences

National Institute of Technology and Evaluation

ANNEX 9

**Participants list for Workshop on Using Mechanistic Information in Forming
Chemical Categories**

Washington , United States

8/12/2010 - 10/12/2010

Australia/Australie

Ms. Simone WARD
Environmental Risk Assessor
Chemical Assessment Section
Department of Sustainability, Environment, Water, Populations and
Communities
Jonh Gorton Building
Kind Edward Tce
2600 Parkes
Australia

Canada

Dr. Tara S. BARTON-MACLAREN
A/ Manager
Existing Substances Risk Assessment Bureau
Health Canada - Safe Environment Directorate
Existing Substances Risk Assessment Bureau
269 Laurier Avenue W.
K1A 0KA Ottawa
Canada

*** Chair**

Ms. Kathy HUGHES
Chief, Hazard Methodology Division
Safe Environments Directorate
Health Canada
4-096
269 Laurier Avenue West
K1A 0K9 Ottawa
Canada

Dr. Sean KENNEDY
Research Scientist
National Wildlife Research Centre
Environment Canada,
National Wildlife Research Centre, 1125 Colonel By Drive
K1A 0H3 Ottawa
Canada

Mr. Benny LING
Evaluator
PMRA
Health Canada
2720 Riverside Drive
6606E Ottawa

Denmark/Danemark

Mrs. Marianne DYBDAHL
Project scientist, Toxicology and Risk Assessment
Division of Toxicology and Risk Assessment
National food Institute
DTU Food, National Food Institute
Technical University of Denmark Mørkhøj Bygade 19, building G
2860 Søborg
Denmark

Mr. Magnus LOFSTEDT
Chemicals Division
Danish EPA
Strandgade 29
1401 Copenhagen K
Denmark

France

Mme Laure GEOFFROY
Ecotoxicologist
Bureau d'Evaluation des Risques des Produits et agents Chimiques
Institut National de l'Environnement Industriel et des Risques (INERIS)
INERIS
BP2
60500 Verneuil en Halatte
France

Japan/Japon

Dr. Yumi AKAHORI
Research Scientist
Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute, Japan (CERI)
1-4-25
Kouraku
112-0004 Bunkyo-ku
Japan

Dr. Atsushi ONO
Senior Researcher
Division of Risk Assessment
National Institute of Health Sciences
Kamiyoga 1-18-1
Setagaya-ku
158-8501 Tokyo
Japan

Dr. Yuki SAKURATANI
Chief
Safety Assessment Division, Chemical Management Center
National Institute of Technology and Evaluation
2-49-10 Nishihara, Shibuya-ku
151-0066 Tokyo
Japan

Dr. Takashi YAMADA
Safety Assessment Division, Chemical Management Center
National Institute of Technology and Evaluation
2-49-10, Nishihara, Shibuya-ku
151-0066 Tokyo
Japan

Korea/Corée

Dr. Seokjoo YOON
R&D Division
Korea institute of toxicology
100 Jang-Dong
Daejeon
Korea

Netherlands/Pays-Bas
*** Chair**

Dr. Emiel RORIJE
SEC
RIVM-National Institute for Public Health and the Environment
P.O.Box 1
3720 BA Bilthoven
Netherlands

Dr. Rob STIERUM
Head Toxicogenomics
Business unit Biosciences
TNO Quality of Life
Physiological Genomics
PO Box 360 (PP8)
3700
Netherlands

Norway/Norvège

Mr. Knut-Erik TOLLEFSEN
Professor/Senior Researcher
Norwegian Institute for Water Research (NIVA)
Gaustadalleen 21
N00349
Oslo
Norway

United States/États-Unis

Dr. Gary ANKLEY
US Environmental Protection Agency
Mid-Continent Ecology Division
6201 Congdon Boulevard
MN 55804 Duluth
United States

Ms. Kelly MAYO BEAN
US Environmental Protection Agency
1200 Pennsylvania Ave., NW
20460 Washington
United States

Dr. Kevin CROFTON
Neurotoxicity Division
U.S. Environmental Protection Agency
109 TW Alexander Dr,
MD-B105-04
27711 Research Triangle Park
United States

***Chair**

Dr. Vicki DELLARCO
Science Advisor
US Environmental Protection Agency
Office of Pesticide Programs
Office of Pesticide Programs (MC 7509P)
1200 Pennsylvania Ave., NW
DC 20460 Washington
United States

Dr. Tala HENRY
Toxicologist
US Environmental Protection Agency
Office of Pollution Prevention and Toxic Substance
1200 Pennsylvania Ave., NW (7403M)
DC 20460 Washington
United States

Dr. Mike HORNUNG
U.S. Environmental Protection Agency
6201 Congdon Blvd
55804 Duluth
United States

Mr. Richard JUDSON
Research Chemist
National Center for Computational Toxicology
US Environmental Protection Agency
1200 Pennsylvania Ave., NW
20460 Washington
United States

Ms. Mary MANIBUSAN
Branch Chief
Health Effects Division
US Environmental Protection Agency
Risk Assessment
1200 Pennsylvania Ave, NW
7509P
20460 Washington
United States

Mr. Edward J. PERKINS
Environmental Laboratory
US Army Engineer research and Development Center
1025 Joe Keeley Drive
73072 NORMAN
United States

Ms. Chris RUSSOM
MED
USEPA ORD NHEERL
6201 Congdon Blvd
55804 Duluth
United States

*** Rapporteur**

Dr. Louis (Gino) SCARANO
Risk Assessment Division (7403 M)
U.S. Environmental Protection Agency
1200 Pennsylvania Ave, NW
Mailcode -7403M
20460 Washington DC
United States

Dr. Patricia SCHMIEDER
Mid-Continent Ecology Division
USEPA, ORD, National Health and Environmental Effects Research
Laboratory
6201 Congdon Blvd
55804 Duluth, Minnesota
United States

Dr. Jennifer SEED
Risk Assessment Division (7403 M)
US Environmental Protection Agency
1200 Pennsylvania Ave., NW
Mailcode – 7403M
20460 Washington DC
United States

Dr. Timothy SHAFER
Integrated Systems Toxicology Division
U.S. Environmental Protection Agency
109 Alexander Dr
MD B105-03
27711 Research Triangle Park
United States

Mr. Thomas STEEGER
Senior Biologist
Environment Fate and Effects Division
US Environmental Protection Agency
1200 Pennsylvania Ave.
20460 Washington
United States

Dr. Daniel VILLENEUVE
Mid-Continent Ecology Division
U.S. Environmental Protection Agency
US EPA Mid-Continent Ecology Division
6201 Congdon Blvd
55804 Duluth
United States

EU/UE

Mr. Manuel DOMINGUEZ ESTEVEZ
Senior Scientific Officer – Toxicology
European Chemicals Agency
Annankatu 18 PO Box 400
00121 Helsinki
Finland

Dr. George FOTAKIS
Junior Scientific Officer
European Chemical Agency
Annankatu 18 PO Box 400
00121 Helsinki
Finland

*** Chair**

Mr. Maurice WHELAN
Joint Research Centre
Institute for Health and Consumer Protection
Ispra
Italy

**Business and Industry
Advisory Committee
(BIAC)/Comité consultatif
économique et industriel
(BIAC)**

Dr. Karen BLACKBURN
Research Fellow
Central Product Safety
The Procter and Gamble Co
Central Product Safety
Miami Valley Innovation Center
The Procter and Gamble Co.
45253-8707 Cincinnati
United States

Dr. Hennicke KAMP
Toxicologist (ERT, DGPT), Group Leader
BASF SE GV/TE – Z470
67056 Ludwigshafen
Germany

Dr. David STEUP
Senior Toxicologist, Shell Health REACH Coordinator
Environmental and Product Health
Shell Oil Company
P O Box 2463
77252-2463 Houston
United States

*** Rapporteur**

Ms. Grace TIER
DuPont
1090 Elkton Rd
19711 Newark
United States

Dr. Satoshi UWAGAWA
General Manager Chemicals Control Dep
Japan Chemical Industry Association
Japan

**International Council on
Animal Protection in OECD
Programmes
(ICAPO)**

Dr. Nancy BECK
Science and Policy Advisor
Dept of Toxicology and Research
Physicians Committee for Responsible Medicine
5100 Wisconsin Ave NW, Suite 400
20010 Washington DC
United States

Dr. Chad SANDUSKY
Director of Toxicology and Research
Physicians Committee for Responsible Medicine
5100 Wisconsin Ave, NW Suite 400
DC 20016 Washington
United States

OECD/OCDE

M. Bob DIDERICH
Principal Administrator
ENV/EHS
OECD
Marshall Building 0352
2 rue André-Pascal
75016 Paris
France

Mr. Terry SCHULTZ
Administrator, (Q)SARs
ENV/EHS
OECD
Marshall Building 0332
2 rue André-Pascal
75016 Paris
France

Hristo ALADJOV
41 Evlogi Georgiev Blvd. Ap.9
1442 Sofia
Bulgaria

*** Rapporteur**

Dr. Mark CRONIN
School of Pharmacy and Chemistry
Liverpool John Moores University,
Byrom Street
L3 3AF Liverpool
United Kingdom

ILSI/HESI

Michelle R. EMBRY
Senior Scientific Program Manager
ILSI Health and Environmental Sciences Institute (HESI)
1156 15th Street, NW, 2nd floor
DC 20005 Washington
United States

Other

Dr. Philip JUDSON
Scientific Advisor
Lhasa Limited
22-23 Blenheims Terrace
Woodhouse Lane
LS2 9HD Leeds
United Kingdom