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REPORT OF THE EXPERT CONSULTATION TO EVALUATE AN ESTROGEN RECEPTOR BINDING AFFINITY MODEL FOR HAZARD IDENTIFICATION

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REPORT OF THE EXPERT CONSULTATION TO EVALUATE AN ESTROGEN RECEPTOR BINDING AFFINITY MODEL FOR HAZARD IDENTIFICATION

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries in North America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides and Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and the Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (http://www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

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, OECD, UNEP, UNIDO, UNITAR and WHO. The World Bank and UNDP are observers. 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.

FOREWORD

This document is a report of the expert consultation held on 17 February 2009 with the aim to evaluate a QSAR approach for estimating estrogen receptor binding affinity for chemicals in defined regulatory inventories developed by the United States Environmental Protection Agency. The expert consultation was held based on the key recommendation from the OECD Workshop on Structural Alerts for the OECD (Q)SAR Application Toolbox held in May 2008 to develop structural alerts for identifying estrogen receptor binders for inclusion in the Toolbox during phase 2 development which started in November 2008.

This document is published on 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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Background

1. The OECD (Q)SAR Application Toolbox is a stand-alone system intended to facilitate the formation of chemical categories and filling data gaps. The first version of the Toolbox released in March 2008 is already helpful to member countries and other stakeholders in forming categories and using existing data to fill data gaps. Phase 2 of the development of the Toolbox started in November 2008 and the aim is to ensure that the categories approach works uniformly for all discrete organic chemicals and for all regulatory endpoints. The 42nd Joint Meeting agreed that the main work item in the phase 2 project will be to gather and maintain additional categorisation methods [ENV/JM(2008)7]

2. A key recommendation from the OECD workshop on structural alerts on 15-16 May 2008 was to develop structural alerts for identifying Estrogen Receptor (ER) binders for inclusion in the OECD (Q)SAR Application Toolbox during phase 2 development [see ENV/JM/MONO(2009)4: http://www.olis.oecd.org/olis/2009doc.nsf/linkto/env-jm-mono(2009)4]. Unlike acute toxicity endpoints that are readily linked to the molecular initiating event the toxicological pathway for the more complex endpoints such as endocrine disruption have a more intricate decision tree. In order to use these complex pathways with confidence requires they undergo a review by experts.

Workshop

3. The expert consultation was held on 17 February 2009 in Paris at OECD headquarters. The agenda is outlined in Annex 1.

4. The consultation was attended by experts from Canada, the Czech Republic, Denmark, Germany, Italy, Japan, Sweden, the United States, the European Commission, BIAC, ICAPO. The list of the participants is attached to this document as Annex 2. The expert consultation was chaired by the OECD Secretariat.

Scope and Objectives

5. The Secretariat presented the scope and objectives of the expert consultation for setting the scene. The stated scope of the expert consultation was on evaluating a QSAR approach for estimating ER-binding affinity for chemicals in defined regulatory inventories developed by the United States Environmental Protection Agency (US-EPA).

6. The objectives of the expert consultation were:

- get a review from experts of the proposed approach to improve the scientific basis as well as regulatory acceptance of the approach;
- to establish a prototype for subsequent workshops on toxicological meaningful categorization mechanisms to be implemented in the OECD (Q)SAR Application Toolbox.

7. Within phase 2 of the development of the Toolbox, it has been agreed to examine complex endpoints such as reproductive toxicity with the express purpose of identifying structural alerts which taken collectively could assist in forming sub-categories for the same regulatory endpoint and be used for data gap filling. This work will increase confidence in predictions made with the help of the Toolbox.

8. The Secretariat indicated that the importance of alert-based expert systems (so-called profilers) in the Toolbox is to allow for the formation of toxicologically meaningful categories, e.g. based on protein binding. Such a category means that all the chemicals falling within it can be assessed when only a few

members are tested. This enables transparent and defensible categories to be formed. Version 1 of the Toolbox only contains a relatively small number of profilers. Incorporation of new profilers is seen as being essential to add new functionalities to the Toolbox. The better the profiler, the better and more precise the category. It is important to note that in the Toolbox profilers are not be used to predict adverse effects. Rather, the profilers are used to group chemicals to allow for read-across using existing experimental results.

9. At the *Workshop on Structural Alerts for the OECD (Q)SAR Application Toolbox* held on 15-16 May 2008 in Utrecht hosted by the Netherlands, a chemical sub-class approach to form a decision support system for estrogen receptor (ER) binding was presented by the United States. It was judged to have high relevance for regulatory decisions and a priority for inclusion into the OECD (Q)SAR Application Toolbox. However it was awaiting further peer-review.

Preparation for the Expert Consultation

10. In preperations for the expert consulation the US-EPA perpared a consultation document entitled: "(QUANTITATIVE) STRUCTURE ACTIVITY RELATIONSHIPS [(Q)SARs] OF ESTROGEN BINDING AFFINITY TO SUPPORT PRIORITIZATION OF HETEROGENOUS CHEMICALS WITHIN DEFINED INVENTORIES FOR SCREENING AND TESTING", which is reported in Annex 3. In addition, the US-EPA presented an overview of the system, which is reported in Annex 4.

Summary of the US-EPA approach

11. Hazard assessment of chemicals has traditionally relied on experimental data acquired using particular test guidelines, which are designed to identify hazards in different species and exposure scenarios. The ability to conduct hazard assessments is usually limited by the availability of experimental data. Because the test guidelines for reproductive effects can result in expensive and time consuming experimentation and review, a more streamlined approach is needed for hazard identification when reviewing large numbers of chemicals. In this regard the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) identified the need for screens and tests not requiring animals. Subsequently the OECD Validation Management Group for Non-Animal Testing (VMG-NA) was set up, whose main objective is to develop non-animal assays for endocrine testing, and in particular to develop and validate tools necessary for Level 1 (Sorting and Prioritization with existing data and/or (Q)SAR systems) and Level 2 (*In vitro* assays providing mechanistic data) of the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals [http://www.oecd.org/document/58/0,3343,en_2649_34377_2348794_1_1_1_1,00.html]. In the United States, legislative mandates require that chemicals within specified inventories be screened to determine if they can cause effects similar to that of estrogen (or other such endocrine effects).

12. There are two primary approaches to setting priorities for untested compounds. One approach is to develop high throughput screening test methods for each hazard and to then test all compounds for all hazards using such fast *in vitro* methods. A second approach is to use (Q)SAR methods to estimate missing endpoint values and prioritize chemicals with respect to hazard endpoints. OECD member countries have recognized the potential of (Q)SAR techniques to aid hazard assessment of untested chemicals. Principles to guide the validation of (Q)SAR models for regulatory purposes were developed. In an effort to enhance regulatory acceptance for using estimated values for hazard assessment, two critical characteristics of (Q)SAR models are being addressed by OECD. The first characteristic is transparency of the estimate, in terms of the methods used and in terms of the mechanistic plausibility and how the estimate is reasonable based on data for similar compounds. The second major characteristic for acceptance is usefulness of a particular approach for the chemicals being assessed. In other words, (Q)SAR methods must apply to

important regulatory endpoints, and they must be capable of making comparable estimates for all chemicals in the inventory being assessed by regulatory authorities.

13. The authors proposed the "subpocket" approach to subcategorizing ER-binders in the following manner. The structural domain of chemicals, which can bind to the ER is determined by the energy and steric constraints of the ER itself. It is generally accepted that ER-binding is much less a narrow lock-and-key interaction, which characterizes many other receptors. While only a small number of compounds have structures capable of ER-binding, the ER is flexible enough to permit binding with a limited range of chemical structures. Defining the boundaries of these chemical categories is the challenge for the expert system. There is sufficient experimental data available to hypothesize the nature of the chemical interaction in the various "subpockets" within the ER-binding domain(s). Three primary ER binding subpockets (referred to as sites A, B, and C) have been identified (Figure 1); each has different requirements for hydrogen bonding.

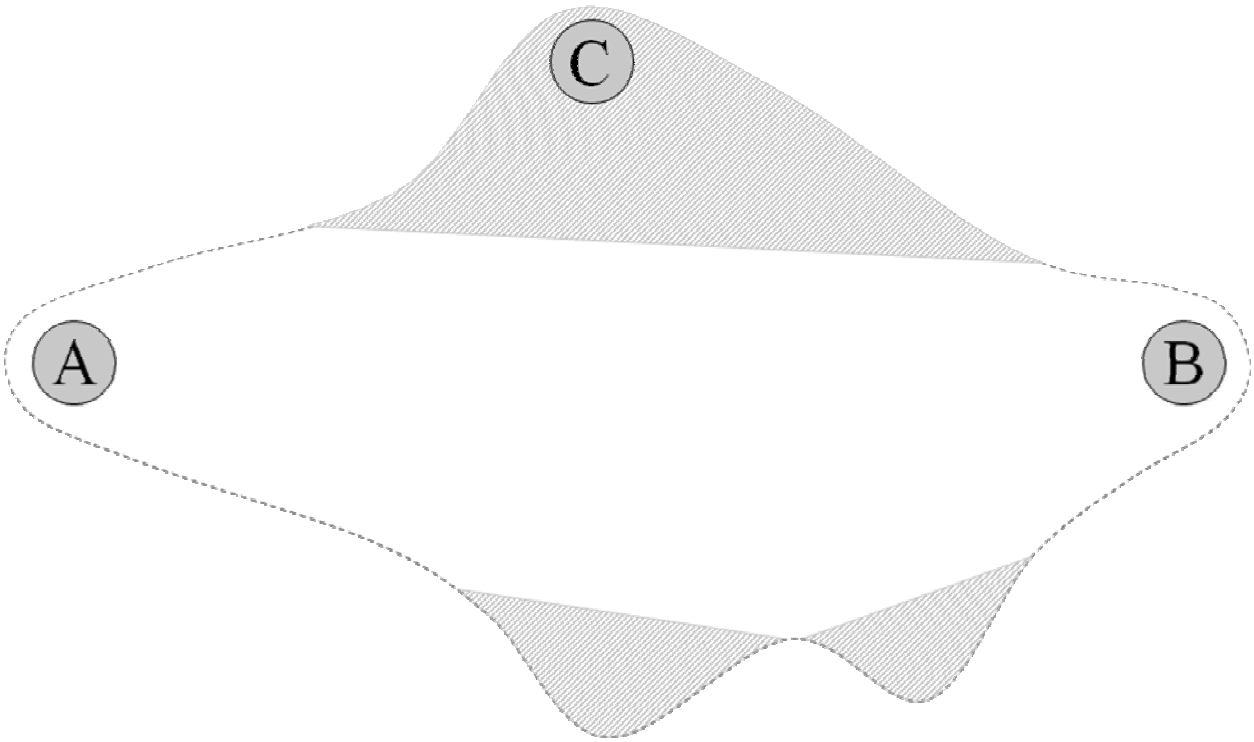

Figure 1. Estrogen Receptor Binding Subpockets (after Katzenellenbogen et al., 2003)

14. This information formed the basis of the proposed expert system. Specifically, in the proposed approach, rather than relying on statistical similarities between chemicals, which bind the receptor and the chemicals in the inventories, the decision tree described in detail by the authors uses basic structural features and simple properties to match inventory chemicals with "similar" chemical groups, and then assigns binding potential based on the similarity to tested structures. The expert rules related to various ER binding types are sequentially applied to address all chemicals in the inventories, thus a mechanistic plausibility lies behind each prediction.

15. The early recognition that chemicals interact with the ER and may affect the endocrine system has led to the development of large databases of ER binding affinity. While early work in this area focused on highly potent estrogen antagonists, later work included weaker binders in particular industrial organic

compounds (e.g., anilines, phenols, etc.). As noted by the authors, these new databases provided the essential information for the creation of structural boundaries for the domain(s) of the substances capable of binding to ER.

16. Since chemicals found within an inventory may be outside the range of previously tested compounds, the authors noted that the assays used to detect low affinity but measurable chemical-ER-interactions must be optimized to handle all the types of chemicals found in a specific regulatory inventory. The specific inventories used in the examples contained highly diverse structures with widely varying physical-chemical properties therefore chemical behavior in the assays used in the screening is an important consideration. Since it is most likely that compounds found in inventories will not be strong binders the authors demonstrated the importance of testing a chemical up to the limit of solubility in the assay media employed. This ensures that any potential for chemical-ER interaction is detected. The maximum test concentration is appropriately set on a chemical-by-chemical basis, as chemical availability in test media will vary. The authors further note that chemical effects on the assay components as well as assay effects on chemical availability are also important to consider to minimize misinterpretation of results. For binding determined in cell- or tissue-based assays, chemical-specific cytotoxicity and/or solubility should be used to set maximum test concentrations.

17. A more difficult question is whether all ER binders regardless of measured potency, produce adverse effects *in vivo*. Accordingly, the question of biological relevance of any degree of chemical ER binding detected using a cell-free assay is separate from the question whether a particular substance can bind to the ER. The authors state that the question of relevance is more appropriately addressed using more biologically relevant assays at a higher level of biological organization within the ER-mediated reproductive impairment pathway in a tiered testing approach. The pathway specific to ER-binding, which was presented by the authors is illustrated in Figure 2.

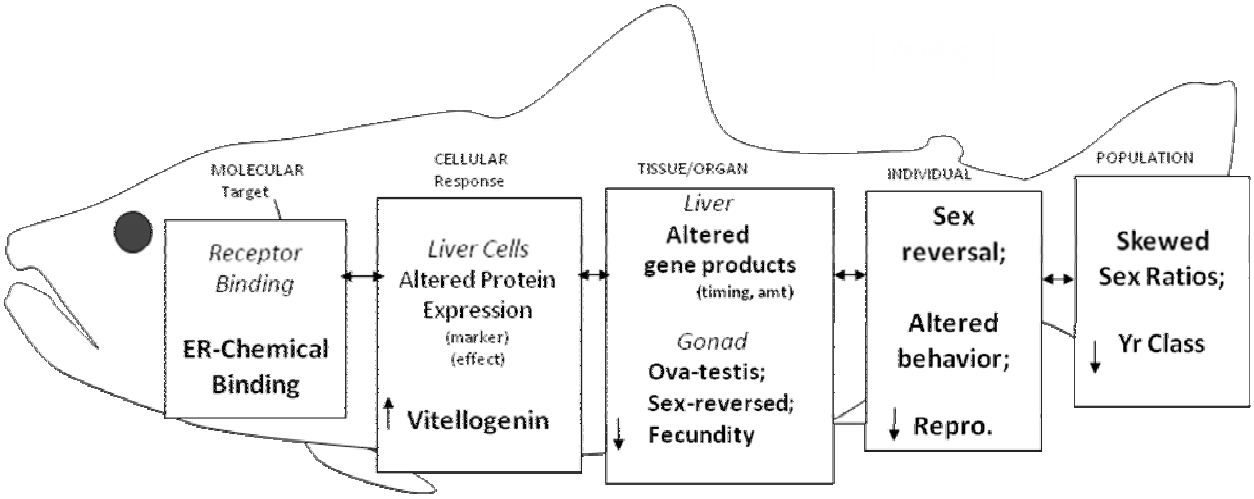

Figure 2 Fish ER-Binding to *In Vivo* Outcome Pathway

18. The example demonstrated by the authors was a second *in vitro* assay to confirm ER-mediated gene activation within metabolically-competent liver tissue. Such an assay increases relevance but also biological complexity. As noted by the authors, research is in progress, which evaluates the extent to which chemicals with very low ER-binding affinities can cause adverse effects in fish *in vivo*.

19. As stated by the authors, a chief concern is how well the domain of the expert system covers the breadth of chemical structures included in a regulatory inventory (i.e., the regulatory domain). The authors addressed the issue of regulatory applicability with two examples, which used the knowledge in the expert system to subdivide lists of chemicals into groups, which have specific chemical features that are mechanistically related to the hazard endpoint. The expert knowledge was synthesized into testable rules (Figure 3) and then tested against specific regulatory inventories. Specifically, the authors focused on estimating the ER-binding affinity of 211 antimicrobial active ingredients (AMA) and 393 inert ingredients in pesticides used on crops (PI) from their chemical structures. The chemical sub-classes represented in these lists are numerous, including acyclic and cyclic chemical; the latter included alkylphenols, alkyloxyphenols and parabens, which are known ER-binders. The authors tested a wide variety of additional chemicals to cover the groups found on the AMA and PI lists. By focusing on the two inventories the authors did not describe all the subclasses of chemicals tested to develop the expert rules for estimating ER-binding affinity. While the expert system for ER-binding affinity was created with specific chemical inventories and domains in mind, the expert system can be easily expanded as additional data become available on chemicals from other regulatory inventories.

20. The expert system model was also developed with the belief that the model could be used manually for individual chemicals, or computer automated for evaluating large lists of chemicals. As emphasized by the authors, the query language for the logical grouping of chemicals as well as the estimation of ER-binding affinity is written in a syntax that is understandable and transferable to computer languages. This is most relevant to the OECD (Q)SAR Application Toolbox.

21. As outlined in figure 3, the system examines each chemical and places them into groups of inactive chemicals, into “drug-like” groups of chemicals, which have the potential for strong ER binding affinity, or into groups of chemicals which may have weak-to-moderate binding affinity depending on specific properties or structural features. The authors noted in detail the rationale for queries I through VI (see Annex 3 & 4). When the expert system was applied to the inventories, favorable results were observed, demonstrating the value of using the methodology before testing. Moreover, the results show that only a few chemicals included in these inventories are likely to bind to the ER.

22. An important facet of the reported work is that if the ER-binding affinity for a given chemical cannot be estimated, it means that the chemical contains structural features which are not included in the chemicals that have been tested in the current training set. The approach allows for expansion to include new and different structures by strategic testing. This hypothesis-based, strategic expansion of the expert system results in fewer and fewer chemicals passing through the system and being relegated to a chemical group that has not yet been studied.

23. It was noted by the authors that the ER binding affinity expert-system can be distributed to all OECD member countries and other stakeholders either as a series of scientific papers or as one of the profilers in the (Q)SAR Application Toolbox. The advantages of distributing the ER binding expert system as a profiler in the Toolbox are that the knowledge base can be preserved and expanded over time to encompass larger inventories while facilitating the identification of untested chemicals with a high probability of ER-binding.

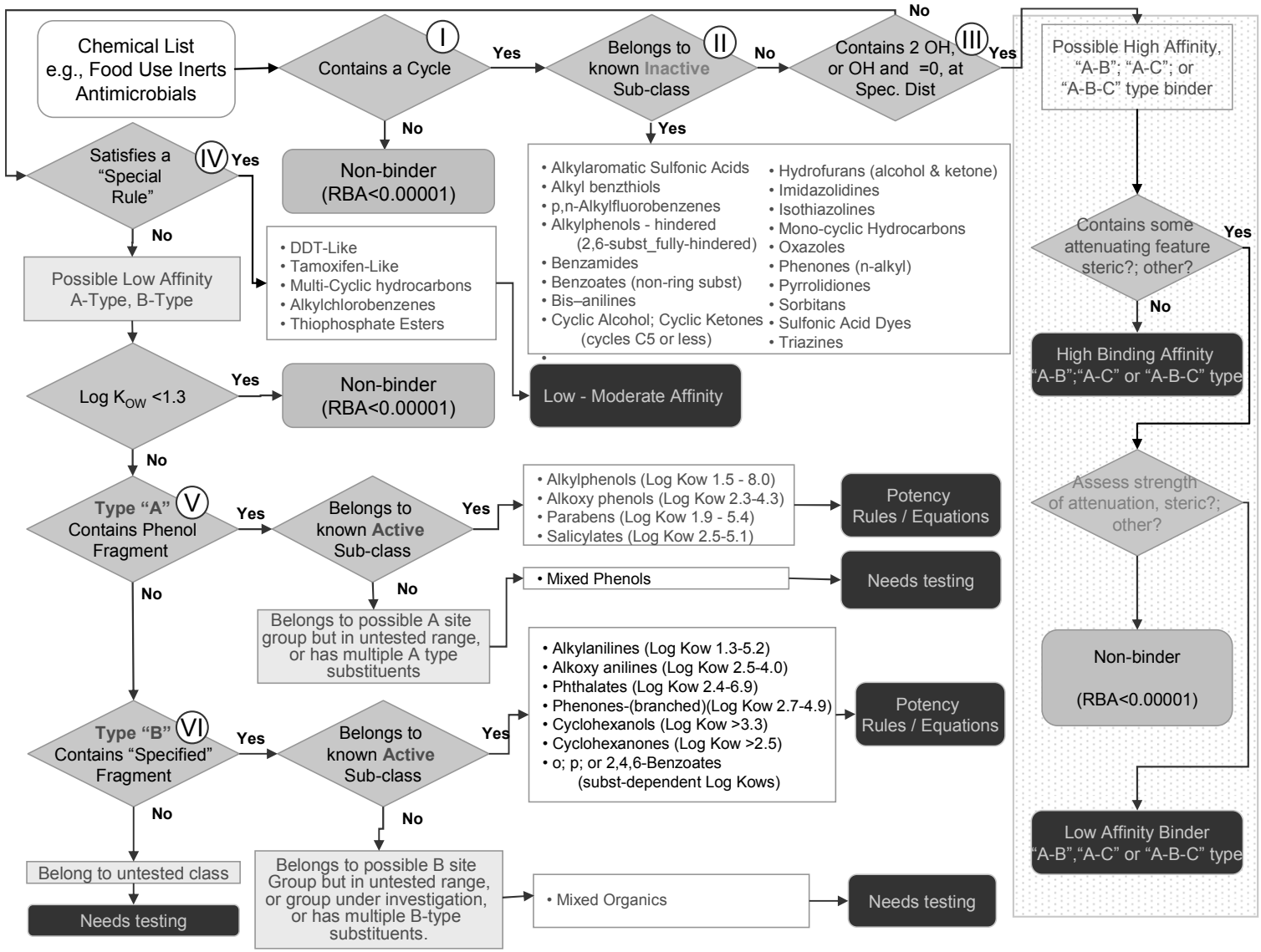

Figure 3. The Expert-System for Estrogen Binding Affinity.

Preparatory work by three experts

24. Three expert reviewers were selected by the Secretariat. These were Dr Atsushi Ono of the Division of Risk Assessment, National Institute of Health Sciences, Japan; Dr Joel Coats of Iowa State University in the United States and Dr Mark Bonnell of the Ecological Assessments Division, Environmental Canada. The reviewers were provided with the consultation documents.

25. In an effort to provide guidance to the reviewers, a series of questions were drafted by the OECD Secretariat and submitted to the reviewers. These questions are listed in Annex 5.

26. The review report of Dr. Ono is reported in Annex 6.

27. The review report of Dr. Coats is reported in Annex 7.

28. The review report of Dr. Bonnell is reported in Annex 8.

Proceedings of the Expert Consultations

29. The Consultations was conducted as described in the agenda reported in Annex 1. Briefly, the US-EPA presented an overview of the expert-system, which was followed by the reviews of Drs. Ono, Coats, and Bonnell. The US-EPA then provided clarification and response to the reviews. The clarifications were followed by a general discussion by all participants.

Outcome of the Expert Consultation

30. Overall, the participants agreed with the reports presented by the reviewers, as outlined in Annexes 6-8. The meeting specifically highlighted some issues:

- Regarding the applicability of the ER binding affinity methodology to determine a wide range of potential binding affinity for diverse chemicals (see questions 1 in Annex 5), the meeting agreed that this was indeed demonstrated for two specific inventories, but that the applicability to other inventories was uncertain.

- The meeting agreed that expert system can be used to group chemicals into toxicologically meaningful groups and that within these groups it is possible to fill data gaps (see question 2 in Annex 5).

- The meeting agreed in general that the expert system outputs clearly and effectively provide explanatory information of the basis for the estimate as well as how it compares to measured data for other members of the same subgroup (see question 3 in Annex 5). The meeting furthermore agreed with the suggestions from the reviewers for more background information and explanations.

- Regarding the strength of this approach (see question 3 in Annex 5), the meeting highlighted its mechanistic perspective, the straightforward decision tree as well as the fact that the system can be expanded. The lack of background information on definition of relative binding affinity, on the rationale for specific values as well as the uncertainties regarding the degree of overlap with other regulatory inventories were identified as limitations.

- Regarding the compliance of the expert system with the OECD principles for validation of QSAR models (see question 5 in Annex 5), the meeting agreed that the endpoint was well defined (trout

ER-binding), although not well understood, that the algorithm is unambiguous, that it has excellent mechanistic plausibility, that the domains appear to be well-defined but that the boundaries need to be clarified and that summary statistics may not be appropriate to evaluate the system.

- The meeting agreed that it is uncertain if the QSAR Model Reporting Format (QMRF) based on the OECD Guidance Document on the Validation of (Q)SAR Models is appropriate to the ER binding algorithm (see question 6 in Annex 5). In general the QMRF can be applied to such expert systems, but some QMRF items may be difficult to address or inappropriate including the statistical ones.

31. A summary of agreed conclusions and recommendations is:

a. While not all the data is reported there is a strong experimental basis for the system with even more experimental evidence for the pathway to be presented in the near future.

b. Grouping of chemicals using the proposed expert system looks promising and provides the most transparent mechanistic explanation system to date.

c. As an expert system it can be expanded as described in the consultation document for broader regulatory domains.

d. The tool was developed to be consistent with level 1 of the OECD EDTA Conceptual Framework.

e. The tool is useful to build chemical categories.

f. ER-binding will not automatically translate to reproductive toxicity. Therefore there would be greater risk assessment relevance if the system was assessed for its ability to use ER-binding affinity predictions to predict effects at higher levels of biological organization.

g. The expert system on ER-binding affinity should be automated.

h. It is recommended that the tool should be implemented in the OECD QSAR Application Toolbox.

i. Further discussions are necessary on how to implement such an expert system in the Toolbox.

ANNEX 1: AGENDA OF THE EXPERT CONSULTATION ON EVALUATION OF A QSAR APPROACH FOR ESTIMATING ESTROGEN RECEPTOR BINDING AFFINITY

To be held at Room 4, OECD Conference Center, Paris, France

17 February 2009

The meeting starts at 09h00 and closes at 17h30 on Tuesday, 17 February 2009.

Tuesday, 17 February 2009

09h00 **1 Opening and the adoption of the agenda (10min)**
The meeting will be opened by the OECD Secretariat. The Secretariat will explain the purpose of the Expert Consultation and housekeeping items. The Secretariat will also confirm that the participants have all meeting documents. The participants will briefly introduce themselves to the meeting (Tour de Table). The participants will be asked to approve the agenda, and discuss changes in meeting papers and scheduling of the agenda items if necessary.

09h10 **2 Overview of A QSAR Approach for Estimating Estrogen Receptor Binding Affinity for Chemicals in Defined Regulatory Inventories (50min)**
The OECD Secretariat will ask Dr Patricia Schmieder of the US-EPA to present an overview of the A QSAR Approach for Estimating Estrogen Receptor Binding Affinity for Chemicals in Defined Regulatory Inventories. The participants will be invited to take note of this activity and to ask questions as appropriate.

10h00 **3 First review of the QSAR Approach for Estimating Estrogen Receptor Binding Affinity for Chemicals in Defined Regulatory Inventories (30min)**
The Secretariat will ask Dr Atsushi Ono of the National Institute of Health Sciences (NIHS), Japan to present his review of the approach described by the US-EPA. The participants will be invited to take note of this review and to ask questions as appropriate.

10h30 **4 Second review of the QSAR Approach for Estimating Estrogen Receptor Binding Affinity for Chemicals in Defined Regulatory Inventories (30min)**
The Secretariat will ask Dr Joel Coats of the Iowa State University to present his review of the approach described by the US-EPA. The participants will be invited to take note of this review and to ask questions as appropriate.

11h00 *Coffee Break (30min)*

11h30 **5 Third review of the QSAR Approach for Estimating Estrogen Receptor Binding Affinity for Chemicals in Defined Regulatory Inventories (30min)**
The Secretariat will ask Dr Mark Bonnell of Environment Canada to present his review of the approach described by the US-EPA. The participants will be invited to take note of this review and to ask questions as appropriate.

12h00 **6 Clarification and Responses to the Review's Comments (30min)**

The Secretariat will ask Dr Schmieder of the USEPA to provide clarification of the approach and respond to the reviewers' comments as necessary. The participants will be invited to take note and to ask questions as appropriate.

12h30 *Lunch Break (90min)*

14h00 **7 Discussion of the QSAR Approach for Estimating Estrogen Receptor Binding Affinity for Chemicals in Defined Regulatory Inventories (90min)**
The participants will be invited to discuss the approach and to provide comments as appropriate.

15h30 *Coffee Break (30min)*

16h00 **8 Finding of the Expert Consultation (60min)**
The Secretariat will present the findings of the Expert Consultation. The participants will be invited to provide comments as appropriate.

17h00 **9 Other issues relating to the Expert Consultation (30min)**
The Secretariat will consider any other issues related to the Expert Consultation raised by the participants.

17h30 *Meeting adjourns*

ANNEX 2: LIST OF PARTICIPANTS

Canada/Canada	Mark BONNELL Senior Science Advisor Environment Canada
Czech Republic/République Tchèque	Dr. Marian RUCKI Expert National Institute of Public Health
	Mr. Milon TICHY Expert National Institute of Public Health
Denmark/Danemark	Mr. Jay NIELEMÄ Chief Advisor Food Inst.
	Mr. Henrik TYLE Chief Adviser Chemicals Division Danish Environmental Protection Agency
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	Ms. Frauke STOCK Federal Environment Agency (Umweltbundesamt)
Italy/Italie	Mr. Romualdo BENIGNI Experimental and Computational Carcinogenesis Environment and Primary Prevention Department Istituto Superiore di Sanita
Japan/Japon	Ms. Yuri ISHII Technical official Ministry of Health, Labour and Welfare
	Dr. Atsushi ONO Senior Researcher National Institute of Health Sciences
	Dr. Hiroaki SHIRAISHI Director National Institute for Environmental Studies

Sweden/Suède	Dr. Jörgen HENRIKSSON Principal Scientific Officer Swedish Chemicals Agency (KEMI)
United States/États-Unis	Dr. Steven BRADBURY Deputy Director Environmental Fate and Effects Division US Environmental Protection Agency Office of Pesticide Programs Dr. Tala HENRY Toxicologist US Environmental Protection Agency Office of Pollution Prevention and Toxic Substance Dr. Patricia SCHMIEDER ORD/Nat'l Health & Environ Effect Res Lab/Mid-Continent Ecology Division USEPA ORD National Health and Environmental Effects Research Agency
EC/CE	Dr. Tatiana NETZEVA Senior Scientific Officer Unit C2 - Registration European Chemicals Agency Mr. Andrew WORTH Project Leader - Toxicology Institute for Health & Consumer Protection European Commission Joint Research Centre Doris HIRMANN Scientific Officer Unit for Scientific IT Tools European Chemicals Agency
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Mr. Terry SCHULTZ
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ANNEX 3: "(QUANTITATIVE) STRUCTURE ACTIVITY RELATIONSHIPS [(Q)SARS] OF ESTROGEN BINDING AFFINITY TO SUPPORT PRIORITIZATION OF HETEROGENOUS CHEMICALS WITHIN DEFINED INVENTORIES FOR SCREENING AND TESTING

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Chapter I. Identifying Hazards to ER-Mediated Pathways

This section briefly describes the context for prioritizing chemicals in commerce for subsequent in vitro and/or in vivo screening of their potential to interact with endocrine systems and the importance of mechanistic understanding of chemical interactions with key proteins, such as the estrogen receptor (ER).

Background

Hazard assessment of chemicals has historically relied on empirical data from specialized test guidelines which are designed to identify hazards in different species and possible exposure scenarios. The pace of conducting hazard assessments is generally limited by the availability of test data as well as experts who interpret the test data. Since test data for the majority of industrial chemicals have never been reported in the literature and, since there are more new chemicals being produced each year than can be assessed annually for hazards through the development and review of new test data, there are a limited number of chemicals that can be assessed by safety experts. This trend is likely to continue unless a more strategic approach is adopted to set priorities for testing and assessment so the chemicals with greatest potential for impacts in our ecosystems and human populations are evaluated (OECD Workshop Report on Integrated Approaches to Testing and Assessment, December, 2007).

The Screening Information Data Sets (SIDS) established by the OECD contains summary information for major hazard endpoints (http://www.oecd.org/document/7/0,3343,en_2649_34379_1947463_1_1_1_1,00.html). One reason to avoid testing all chemicals for all SIDS endpoints is the high cost of testing. A second reason to avoid testing all chemicals for all SIDS endpoints is that only a small percentage of industrial chemicals are likely to be identified to pose a significant hazard in any given test guideline.

Because the test guidelines for reproductive effects can result in expensive and time consuming data development and review, a more strategic approach is needed to set priorities for hazard identification. In this regard the EDTA identified the great importance and urgent need for relatively cheap and quick screens and tests not requiring animals. The EDTA subsequently created the VMG-NA, whose main objective is to identify or propose validated or promising non-animal assays for endocrine testing, and in particular to develop and validate tools necessary for Level 1 (Sorting and Prioritization with existing data and/or (Q)SAR systems) and Level 2 (*In vitro* assays providing mechanistic data) of the EDTA Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals. In some regulatory agencies such as US EPA, legislative mandates established a requirement that chemicals within specified inventories be screened to determine if they can cause effects similar to that of estrogen or other such endocrine effects. While specifying the very broad hazard of endocrine disruption in terms of well-defined processes, such as binding to the ER, for regulatory purposes simplifies some aspects of hazard identification, there remains a great need to develop sorting and prioritization tools for large numbers of untested chemicals.

Broadly, there are two primary approaches to setting priorities for initial hazard identification of untested chemicals. One approach is to develop high throughput screening methods for each hazard and to then test all chemicals for all hazards using much faster *in vitro* methods. Enormous efforts are underway to develop these alternative test methods. A second approach is to use (Q)SAR methods to estimate missing endpoint values and prioritize chemicals with respect to the hazard endpoints. This document focuses on the use of (Q)SAR methods to estimate an important SIDS hazard without testing, and illustrates how expert systems can be used to prioritize large heterogeneous inventories of chemicals for potential hazards.

OECD member countries have already recognized the potential of (Q)SAR to accelerate the initial hazard assessment for thousands of untested chemicals. OECD member countries have developed principles to guide the validation of (Q)SAR models for regulatory purposes, and have compiled case studies to illustrate how member countries are using, or anticipate using, (Q)SAR methods within national regulatory contexts.

Beyond the importance of validating models for national regulatory needs, two important characteristics of (Q)SAR models are being addressed by OECD to enhance regulatory acceptance for using estimated values for priority-setting . The first characteristic is **transparency** of the (Q)SAR estimate, not so much in terms of the methods used, but rather in terms of explaining how the estimate can be explained mechanistically and how the estimate is reasonable based on data for comparable chemicals. The second major characteristic for (Q)SAR acceptance is **usefulness** of a particular (Q)SAR model for the specific chemicals being assessed. In short, (Q)SAR methods must inform important SIDS endpoints, and they must be capable of making comparable estimates for all chemicals in the inventory being assessed by different regulatory authorities.

Since the OECD principles of validation for (Q)SAR seek to describe the domain of the (Q)SAR model in terms of the chemical structures used to create the model, usefulness can be evaluated by comparing the domain of the (Q)SAR methods and the domain of the regulatory inventory assessed in a specific regulatory context. Unfortunately, most developers of (Q)SAR models do not use a specific regulatory inventory as a target for the model domain. This document will discuss an (Q)SAR-based hazard identification approach for estrogenic activity which maintains mechanistic transparency and allows the domain of the knowledge base to be aligned with the domains of specific regulatory inventories.

ER Binding Affinity: An Important Indicator of Reproductive Hazards

To ensure a strong mechanistic linkage between the SIDS and a hazard identification endpoint for (Q)SAR modeling, it is important to outline the importance of ER binding to the SIDS. Impaired reproduction is an important SIDS endpoint, and a variety of test methods may be used to inform the hazard identification process. Since reproductive test methods are very costly, many *in vitro* screening test methods, such as ER binding are being developed. Chemicals interact with proteins such as the ER to initiate a cascade of biological effects called a toxicity pathway. There are multiple proteins and toxicity 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. Much of the research effort of the past two decades in the area of endocrine disruption has focused on increasing our understanding of how chemicals perturb these endogenous hormone systems.

Despite the complexity of the endpoints for reproductive impairment, it has long been appreciated that chemical binding to the ER is one important mechanism of interfering with processes involved in reproduction. The ER-mediated adverse outcome pathway (Figure 1 in Schmieder et al., 2004)) 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 the ER-mediated pathway

are identified, the conceptual model is useful for generating testable hypotheses at various levels along the pathway and providing the regulatory community with a rationale for decision making that can be readily articulated and tested.

The energy and steric constraints dictated by the ER itself, shapes the domain of the chemical structures which can bind to the receptor. It is also known that the ER is much less of a lock-and-key interaction than some highly specific receptors. Although only a small percentage of chemicals have structures capable of binding, the ER is nonspecific enough to permit binding with a diverse array of chemical structures. Defining the boundaries of the diverse chemical array is the challenge for (Q)SAR. By grouping chemicals that interact with ER separately from those that cannot, one can start to determine what is common about chemical structures producing the activity.

Summary of the ER Binding Domain

Theories of chemical interaction in the various "subpockets" within the ER ligand binding domain have been presented by numerous investigators. Theories for chemical interaction with the ER were typically based on information gained from steroidal structures which interact at two points within the ER e.g., two hydrogen-bonding groups of a chemical interacting at different ER sub-pockets. However, it is also known that chemicals that contain only one hydrogen-bonding group, such as nonylphenols, bind ER and cause subsequent gene activation and *in vivo* effects. Katzenellenbogen et al., (2003) describe the "dynamic and plastic character of ER" in their description of ER binding subpockets. Through their larger body of work they have come to identify three primary ER binding subpockets (referred to further as sites A, B, and C) each with different requirements for hydrogen bonding. This information proves useful for formulating and testing hypotheses of how chemicals with only one potential H-bonding group may interact with the ER, and how this may vary dependent upon the character of the H-bonding group.

Various QSAR, docking, and conformational models have also provided insights as to how chemicals may interact with the ER, however, steric models do not currently appear adequate for explaining interaction of the lowest affinity compounds. In the current approach, rather than relying on statistical similarities between chemicals that bind the receptor and the chemicals in the inventories, the decision tree uses basic structural features and simple properties to match inventory chemicals with like chemical groups, and then assigns binding potential based on the similarity to tested structures. Expert rules related to various ER binding types are sequentially applied to address all chemicals in the specific inventories, thus a mechanistic basis underlies each assignment.

Chapter II. Tailoring ER Binding Domains to Regulatory Inventories

This section illustrates why many databases developed for drug design are often inadequate to develop screening models for hazards of environmental contaminants to human or other populations. This section describes how (Q)SAR models which do not fully encompass the large domains of regulatory inventories are often considered "good but not-useful science". Finally, this section illustrates how the knowledge base of the ER binding model can be strategically expanded to encompass specific regulatory inventories.

Measuring ER Binding Affinity for Diverse Chemicals

The early recognition that chemicals interact with the ER and alter the endocrine system has led to the development of large databases of ER binding affinity. The majority of early work in this area came from drug design and the search for highly potent estrogen antagonists as breast cancer therapies. This early work involving is of limited use in developing a (Q)SAR model to estimate ER binding affinity for regulatory inventories for several important reasons. First, to minimize non-target toxic effects of drugs,

chemicals with binding affinities comparable to the very strong binding of the natural ligand are sought for use as drugs. This limited the diversity of the chemicals tested in these early databases and left them with little or no useful information with respect to building a (Q)SAR domain for use with chemicals not designed *a priori* to be biologically active (e.g., industrial chemicals) or with chemicals designed for a primary biological activity not associated with endocrine effects (e.g., many classes of pesticides).

The focus on chemical structures with strong binding affinities also caused much of the early data to be generated with cutoffs in concentrations used in testing, with the result that many classes of chemicals with lower binding potential were listed as "nonbinders". Consequently, initial (Q)SAR models for ER binding that incorporated these data in their training sets produced erroneous predictions. However, the greater error was that these data created erroneous boundary conditions for the domain of the chemicals which are capable of binding to ER sufficiently to produce a reproductive or developmental effect. .

Related to these limitations, the assays to measure binding in the drug design context are optimized for the highest affinity chemicals and do not attempt to measure low affinity interactions. Shifting to lower binding affinity measurements requires testing at higher concentrations and leads to questions of supersaturation and the experimental need for interpretation of binding curves using higher order assays. These questions were minimized using ER binding assays optimized for detecting low affinity effects (for details see: Schmieder et al., 2004; Tapper et al., 2009; Hornung et al., 2009; Sheedy et al., 2009).

A more complex question, i.e., whether all ER binding chemicals regardless of measured potency, produce adverse effects in whole organisms or populations, is an important risk assessment question but is not a goal of the prioritization efforts. Assays used to answer the first question, to detect low affinity but measurable chemical-ER-interactions, must be optimized to handle all the types of chemicals found in a specific regulatory inventory. The specific inventories of current concern contain highly diverse structures with widely varying physical-chemical properties (e.g., Log Kow of <-1.0 to >8.0; neutral organics; weak acids; organometallics, etc), thus chemical behavior in the assays used in the screening is an important consideration.

To ensure that any potential for chemical-ER interaction is detected, a chemical should be tested up to the limit of solubility in the assay media employed (Schmieder et al., 2004; Tapper et al., 2009; Hornung et al., 2009; Sheedy et al., 2009). Thus the maximum test concentrations is appropriately set on a chemical-by-chemical basis, as chemical availability in test media will vary with structure, aqueous solubility, Log Kow, non-specific protein binding, pKa, etc. For example, the maximum solubility of a chemical of Log Kow 1.3 might be 1×10^{-1} M, while a chemical of Log Kow 6 may only be soluble to 1×10^{-4} M. Chemical effects on the assay components as well as assay effects on chemical availability (e.g, pH, total protein content of media, chemical availability, hydrolysis, reactivity, protein denaturation, etc) are also important to consider to minimize misinterpretation of results especially in cell-free binding assays. For binding determined in whole-cell or tissue assays, chemical-specific cytotoxicity or solubility (in absence of cytotoxicity) are used to set maximum test concentrations.

The biological relevance of any degree of chemical ER binding detected using a cell-free assay is a separate question from "Can the chemical bind ER?". The question of relevance is more appropriately addressed using more biologically relevant assays at a higher level of biological organization within the ER-mediated reproductive impairment pathway (Figure 1 in Schmieder et al., 2004). For example, using a second *in vitro* assay to confirm ER-mediated gene activation within metabolically-competent tissue increases relevance but also complexity (Schmieder et al., 2004; Tapper et al., 2009; Hornung et al., 2009). Additionally testing a subset of prioritized chemicals *in vivo* increases the regulatory relevance even further. Research in progress is evaluating the extent to which chemicals with very low apparent ER binding affinities (e.g., five to six orders of magnitude less than that of estradiol) can cause adverse effects

in fish *in vivo* (R. Johnson, personal communication, 3rd Annual McKim Conference on Predictive Toxicology, Duluth, MN, USA, Sept 16-18, 2008).

Overlaying Model Domains and Regulatory Inventories

The majority of (Q)SAR models reported in the literature (more than 15,000 models) are simple statistical relationships between endpoint data collections and molecular descriptors. The primary limitation of these statistical models is that chemicals can produce the same endpoint activity even though the toxicity mechanisms by which they interact with biological systems are completely different. For example, one chemical may bind to the ER via a hydrogen bond donating group and another may bind via a hydrogen bond accepting group or hydrophobic forces. When data from different mechanisms involving a common biological effect are combined statistically in a single (Q)SAR model, the model is likely to give spurious results unless the domain is narrowly defined.

Evaluation of a (Q)SAR model for specific regulatory purposes, therefore, must include an evaluation of the mechanism of chemical interaction as well as the evaluation of how well the endpoint value is predicted by the (Q)SAR model. In general, statistical evaluations have not been successful in characterizing the knowledge that discriminates interaction mechanisms. The OECD Guidance Document on the Validation of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models offers several examples of statistical clustering approaches for defining the domain of (Q)SAR models using simplified structure spaces. However, these methods should be used with caution because the dimensionality of chemical structure space is still not well defined.

As stated previously, a primary concern of the decision makers is likely to be how well the domain of a (Q)SAR model covers the breadth of chemical structures included in the regulatory inventory (regulatory domain). We propose to address the regulatory applicability issues with an expert knowledge method that subdivides lists of chemicals into groups which have specific chemical attributes mechanistically related to the hazard endpoint. Expert knowledge is synthesized into "testable rules" much like the structural alerts and other SAR techniques. At any time in the development of the knowledge base, the domain of the expert "rules" can be evaluated with a list of inventory chemicals to determine if the knowledge base has sufficient test data and "rules" to include all chemicals in at least one subgroup. If some of the inventory chemicals are not defined by the subgroups, these chemicals are clearly outside the domain and the knowledge bases must be expanded to cover the regulatory domain for specific kinds of structures.

In the expert system approach, a first-generation logic tree is developed from the available data, and then tested against a specific regulatory inventory. From the chemicals not addressed by the current knowledge base in the logic tree, representative chemicals are tested *in vitro* to expand the expert "rule" base. With each iteration, the number of inventory chemicals outside the domain decreases until the decision tree domain covers the regulatory inventory.

For example, the USEPA Office of Pesticide Programs (OPP) sought to set screening and testing priorities for estrogen mimics for chemicals which are used as the inert ingredients (other than active ingredient) in pesticides used on crops (FI) and as antimicrobial active ingredients (AM). The FI inventory contained 893 total entries, of which only 393 were discrete chemical structures (93% organics, 6% inorganics, 1% organometallics). The 500 remaining listings included polymers of mixed chain length, chemical mixtures, and undefined substances (e.g., sand, blood, tannins, cod liver oil). The AM list contained a total of 299 substances of which 211 were discrete chemicals (72% organics, 25% inorganics, 3% organometallics) and 88 were polymers, mixtures and undefined substances.

To demonstrate the proof-of-concept for our approach, we concentrated on estimating the estrogen receptor (ER) binding affinity of the 211 AM and 393 FI chemical structures. The chemical sub-classes

represented in these lists included acyclic chemical including alcohols, amides, quaternary amines, acyclic sulfates, carboxy esters and ethers, etc. Cyclic chemicals included alkybenzenesulfonic acids, alkylphenols, alkyloxyphenols, chlorobenzenes, furans, cyclic hexanones and hexanols, imidazolidines, isothiazolines, oxazoles, parabens, cyclic pentanones, phenones, pyrrolidines, phthalates, salicylates, sorbitans, sulfonic acid dyes, triazines, and mixed functional groups.

When the available data for ER binding affinity was first compared to the specific chemical groups in the FI and AM inventories, the overlap was minimal. This results from chemicals studied in the literature being mostly steroids and high binding affinity pharmaceuticals often designed to interact with the ER, with relatively few pesticides and industrial chemicals represented in the literature. The chemical found with measured ER binding activity represented less than 4% of chemical in the regulatory inventories. For example, the FI and AM inventories contain greater than 50% acyclic chemicals. Consequently, any (Q)SAR model based on literature data would not have a applicability domain covering these regulatory inventories.

Expanding the Knowledge Base for ER Binding Affinity

It is difficult to separate the experiments for expanding the ER database to understand the mechanisms of ER binding from those intended to expand the domain to overlap with the regulatory inventories. Expert rules were written to address structural features or chemical properties associated with binding types, as previously described. Early decision tree assessment of the specific inventories with regard to potential binding mechanism quickly revealed that none of the inventory chemicals contained two possible hydrogen bonding groups at the steroidal distances required for high affinity interactions (i.e., A-B binding). Thus, no further testing was conducted for chemicals with these structural characteristics. Instead, testing efforts were concentrated on better defining ER binding within one subpocket, Site A or Site B.

The inventory chemicals contained a significant number of chemicals with at least one possible hydrogen bonding site. Due to the importance of hydrogen bond donating phenolic groups for Site A interactions as previously described, a series of alkylphenols were tested for ER binding to ensure that the domain covered these phenols. While mostly para-substituted alkylphenols were tested, a sufficient diversity of other phenols was included to allow sub-grouping with respect to branched versus n-chain, and ortho-, meta and parasubstitution. Alkylphenols from Log Kow 1.5 to 8 (phenol to dodecylphenol) were examined and all chemicals within the series bound ER (Tapper et al., 2009).

The potential for steric hinderance was also investigated with only the 2,6-ditertbutyl substituted compound shown to be incapable of binding, presumably due to inability of the phenol to get close enough to the A site to hydrogen bond. Additionally, a sub-group of alkylphenols were tested where a para-alkoxy substituent replaced the p-alkyl chain and all were determined to bind. The ER binding affinity of alkylphenols was found to increase linearly with Log Kow from 1.5 to ~4 to 4.5 and then remain nearly constant at greater lipophilicity (to Log Kow 7.9). The bilinear relationship between ER binding affinity and Log Kow is consistent with the hypothesis that the binding involves a hydrogen bonding group which can be increased through hydrophobic forces in the center of the ER subpockets (Figure 1). Several groups were investigated for their effectiveness as hydrogen bond acceptors, presumably interacting at ER subpocket Site B. An extensive series of p-alkylanilines were tested, similar to that of alkylphenols, to further increase mechanistic understanding and importance of lipophilicity in receptor binding. The alkylanilines from Log Kow of 1.3 to 5.2 were found to bind ER in a relationship similar to that found with alkylphenols. Similar alkoxyanilines also bound to the ER. The alkylanilines and the alkoxyanilines, however, show less affinity for ER when compared to phenols of the same alkyl chain length (Figure 1). In fact, aniline itself was determined to be a non-binder.

Although details will not be presented here, many additional groups examined (e.g., phenones, phthalates, ring-subst benzoates) had chemical parameters and measured ER affinities consistent with B-site interactions (Hornung et al., 2009). As with the alkylphenols, parabens and salicylates also have been identified in the literature to be capable of binding to the ER and inducing vitellogenin. Sufficient testing was conducted within these groups to suggest that parabens and salicylates have the potential to bind to the ER, at least within the tested Log Kow range of 1.8 to 5.5.

A wide variety of additional chemicals were tested to cover the types of groups found on the FI and AM lists and were found incapable of binding to the ER. These included charged organics, neutral organics, acyclics, and organometallics, among other classes. The results suggest that these chemicals do not have any moieties with sufficient hydrogen bonding capability to interact with ER or are excluded from the binding site with hydrophilic moieties. Some of these groups contained a large number of chemical structures. For instance, there were more than 70 alkylaromatic sulfonic acids in the inventories, which included many salts and other variations of a basic structure. It was found useful to test selected members of the group to cover the Log Kow range and structural complexity of the compounds. Figure 2 shows the tested chemicals (training set structures listed near top of the figure) and a sampling of the inventory for the Sulfonic Acid Dyes group. This limited testing was determined to be sufficient to predict that additional members of the group have a low potential to interact with the ER. This figure is included to illustrate how tested chemicals are compared to inventory structures for transparency, allowing users to evaluate the adequacy of coverage for themselves.

It is beyond the scope of this summary to recount all the subclasses of chemicals tested and to illustrate how the expert rules for estimating ER binding affinity can be introduced into a logic tree. Nonetheless, the iterative process was continued by examining chemicals with mixed functional groups referred to as Mixed Phenols and Mixed Organics which were not grouped otherwise by the decision tree. Presently, the majority of Mixed Phenols and Mixed Organics that have been tested do not bind.

A Rule-Based Expert System to Predict ER Binding Affinity

Chemical structures in heterogeneous inventories are inherently hierarchical. When some chemical groups behave quite differently from others, or the information known about each group varies substantially, rule-based expert systems offer one approach to estimating specific properties of an entire array of chemicals. Creating logic trees that reflect the hierarchy and apply expert rules and localized (Q)SAR models for specific chemical structures has numerous advantages in hazard identification. Perhaps most important is the advantage of maintaining transparency of the estimates by describing the logic for grouping the chemicals, providing the localized (Q)SAR model or expert rules that were used to make the estimate, providing literature values for other chemicals in the same group, and providing complete literature citations to support the estimate. These data enable hazard assessors to discuss the scientific basis for the all estimates, and justify modifying a specific estimate if new knowledge is available to the assessor.

The expert system for ER binding affinity was created with specific chemical inventories and domains in mind. Consequently, the expert system can be easily expanded as additional data become available on chemicals from even larger domains. The expert system model was also developed with the presumption that the model could be used manually for individual chemicals, or totally automated on a small personal computer for ranking larger lists of chemicals. The query language for the logical grouping of chemicals as well as the estimation of ER binding affinity is written in a syntax that is understandable and transferable to computer languages.

Figure 3 presents an overview of the decision tree model for ER binding affinity. The current version examines each chemical and places them into groups of inactive chemicals, into “drug-like” groups of

chemicals which have the potential for significant ER binding affinity, or into groups of chemicals which may have weak-to-moderate binding affinity depending on specific properties or structural features. Each of these major groups is then further evaluated as needed to present an estimate of the ER binding affinity.

Numerous investigators have noted that chemicals must have at least one cycle in the structure for the chemical to competitively bind at the ER. This general "testable rule" is mechanistically reasonable because flexibility and binding affinity, in general, vary inversely. Cyclic and multi-cyclic chemicals tend to be less flexible, at least in the absence of long alkyl chains, and the literature suggests that at least one cycle in chemical structure is needed to enable the chemicals to bind. We tested numerous acyclic chemicals in the inventories and none had measureable binding. These included both ionic (carboxylic acids, quaternary amines, sulfates, and phosphates) and non-ionic acyclic structures (alcohols, amines, esters) Table 1. Therefore, the first query (I) in the expert system in Figure 3 is whether the chemical contains a cycle. If not, the chemical is considered within the domain of the ER binding model, but the chemical is predicted to be inactive in the ER-mediated pathway.

The second query (II) in Figure 3 is to determine if the chemical belongs to one of the subgroups of chemicals for which all members tested so far did not competitively bind to ER. There are a variety of steric and electrostatic reasons why these groups cannot bind to the ER, but the discussion of each is beyond the scope of this review. For the majority, the presence of a charge in the molecule, absence of hydrogen bonding groups, or inappropriate geometry explains the failure of these chemicals to bind to ER. Suffice it to say, these inactive groups are well-defined to ensure the structures in the specific inventories under study are adequately covered, and no tested members of these groups have been shown to be active in the ER-mediated pathway. The "known inactive" subgroups" include: alkylaromatic sulfonic acids, alkyl benzthiols, p,n-alkyl fluorobenzenes, hindered alkylphenol, benzamides, benzoates missing any additional ring substituents, bis-anilines, hydrofurans (alcohol and ketone), imidazolidines, isothiazolines, mono-cyclic hydrocarbons, oxazoles, n-alkyl phenones, pyrrolidiones, sorbitans, sulfonic acid dyes, and triazines (Figure 3; Table 1).

The chemicals that do not meet the requirements of the first two queries belong to groups of chemicals for which some members bind to the ER. The chemicals with the greatest binding affinity to the ER receptors are chemicals that mimic steroids, and the primary feature for large ER binding affinity is to have two oxygen atoms available for hydrogen bonding at the specific distances dictated by the ER receptor. Chemicals which meet these requirements (III) are removed from the other groups for further evaluation. However, if the chemicals do not meet this requirement, the chemicals may have binding affinity anywhere from moderate to unmeasureable and are further evaluated by the expert system.

To begin evaluating these remaining groups, the chemical is next queried against a set of "special rules". For example, the literature contains examples of DDT-like chemicals that appear to have the proper shape to fit in the hydrophobic pocket of the receptor with pi-bonding interactions strong enough to make these chemicals active in the ER toxicity pathway. There are only a few such chemicals and a query set (IV) was established to compare them to the inventory chemicals to determine if the chemicals can reasonably be expected to bind to the ER through any of the interactions unique to these groups (Table 2).

If the chemical does not meet any of the requirements for the first four queries, it belongs to a very large group that has some members which bind to the ER. However, water soluble chemicals are excluded from the binding site. Of over 350 chemicals tested thus far, no chemical with a Log Kow less than 1.3 has been shown to competitively bind with ER. Thus, chemicals with a Log Kow less than 1.3 are given a relative binding affinity (RBA) of less than 0.00001% (i.e, relative to estrogen, the natural ligand, set to 100%), or unmeasurable in our test system. While this rule appears to be generally applicable, there are more specific Log Kow ranges for binding activity that apply within many of the groups.

The fifth query identifies chemicals with a phenolic group as a hydrogen bond donating group which has the potential to bind at the A site in the ER (V in Figure 3; Table 2). Specific substructure searches are used to limit these chemicals to groups which have been tested. If the chemical is a phenol, the RBA can be estimated with a QSAR model for this group of chemicals.

The sixth query identifies chemicals with aniline, ester, or other polar group that can serve as a hydrogen bond acceptor at the B site in the receptor (VI in Figure 3; Table 2). Once again, specific substructure searches are used to limit the chemical group to those which have been tested. If the chemical falls within the domain of these tested groups of hydrogen bond acceptors, the RBA can be estimated with a QSAR model for this group of chemicals.

When the expert system was applied to the FI and AM inventories, the results in Table 2 demonstrated the value of using a screening methodology before any costly and time consuming screening and testing is conducted. The results show that only a few chemicals included in these inventories are likely to activate the ER pathway. Under the specific national authorities for which this study was conducted, initial screening and testing can be prioritized on a specific subset of chemicals, among several hundred within these inventories, that have the highest potential to interact with the ER.

As a final note, if the RBA for any chemical cannot be estimated, the chemical contains structural features which are not included in the chemicals that have been tested in the current training set. The approach, however, is based on a perspective that the domain of the expert system can be expanded to include additional chemical inventories with new and different structures by targeting strategic testing. This hypothesis based, strategic expansion of the expert system's domain will result in fewer and fewer chemicals that can pass through the queries and fall into a chemical group that has not been studied.

Chapter III. Accessibility of ER Screening Models

This section describes how the ER binding affinity model can be distributed to all OECD stakeholders either as a series of scientific papers or as one of the reproductive toxicity models in the (Q)SAR Application Toolbox. The advantages of distributing the ER binding affinity model as an expert system in the Toolbox are that the knowledge base can be preserved and expanded over time to encompass larger inventories while facilitating the identification of untested chemicals with a high probability of causing reproductive effects when they are tested and assessed.

Expert Systems for ER Binding Affinity

One of the more straight forward approaches to developing an expert system for a complex endpoint is to use decision trees with the queries based on simple "if, then else" logic. In modern chemical decision trees, the conditions placed on individual queries in order for them to be logically true can range from simple presence or absence of a general substructure to a complex parameter range derived from still other molecular models. As illustrated in this work, we have developed a decision tree model that incorporates simple chemical substructure queries, expert rules for when to invoke QSAR models for ER binding affinity, and queries involving 3D distances between interacting atom centers.

The application of decision trees is important to regulatory acceptance because it will inform the user about how well the decision tree model will cover the domain of the chemicals to be assessed (usefulness) and, at the same time, will provide a transparent explanation for the basis of the estimated endpoint. Explaining "why" each chemical produced the results it did in the decision tree is crucial to the subsequent discussions in a hazard assessment. For example, since binding to a receptor has many more specific constraints for which structures can even fit within the receptor "pocket(s)", a large number of chemical groups are expected to be "nonbinding". If representatives from these groups are tested and all found to be

negative, an expert rule-of-thumb can be written to explain the nonbinding estimate with invoked QSAR models.

To build a decision tree to estimate ER binding affinity, we incorporated a series of queries to identify nonbinding groups of chemicals and then added a series of more specific rules to group large categories of chemicals with a substantial range of binding affinities. Each of these categories was further classified, and quantitative models to estimate binding affinity for the entire category was included. Even more specific structural requirements were added to identify steroid-like chemicals of which many had large binding affinities similar to drugs and potent estrogenic chemicals.

Importance of the QSAR Application Toolbox

OECD contributions to making the use of (Q)SAR acceptable for regulatory applications has included a more systematic definition of terms, principles of validation for (Q)SAR together with guidance on how to apply the principles in a regulatory context, and the development of the categories approach for estimating missing values when more quantitative models are not available. One additional barrier to (Q)SAR, however, was the reality that the infrastructure for conducting (Q)SAR analysis in terms of skill-mix, equipment and software is substantial. Many member countries do not have the capacity to maintain additional infrastructure over and above risk assessment specialists for predictive toxicology. A significant contribution of OECD to the advancement of (Q)SAR methods was to develop the freely distributed QSAR Application Toolbox which has the potential to remove much of the infrastructure barrier and make the latest (Q)SAR methods available at no cost to all OECD stakeholders.

The intent of the QSAR Application Toolbox was to develop software that was capable of storing the data and models needed in hazard assessment, facilitating many of the tasks performed in the normal workflow of assessors, and reporting (Q)SAR estimates with acceptable transparency in the normal formats such as the SIDS data matrix for chemical categories. The "tasks" performed in filling data gaps involve the selection of (Q)SAR models that have appropriate domains for each chemical being assessed as well as the SIDS endpoint value that is missing for the target chemical.

The knowledge needed to select (Q)SAR models only for relevant endpoints and to use those models for specific chemicals falling within the reliable model domain is central to the question of enhancing regulatory acceptance of (Q)SAR itself by decision makers. That knowledge can be acquired by hiring experts, through experience with evaluating (Q)SAR methods, or from computerized archives of expert knowledge known as expert systems.

The (Q)SAR Application Toolbox was designed to accommodate as full array of expert system technology and to make the expert knowledge fully transparent along with complete explanations of how expert knowledge can be used to make an estimate for a specific endpoint and untested chemical.

In the simplest terms, the "expert system" encodes information that controls the use of any task performed by the Toolbox. The information can be prescribed by a consensus of hazard assessment expert or by experts in chemistry and toxicology in concert with the (Q)SAR Application Toolbox Management Group. Once encoded, an exhaustive description of the literature references and deliberations that led to the expert "rule" can be provided to the user each time the "rule" is invoked, even if the next application is generations from now.

The reason expert system technology is so important is that structure-activity relationships are only reliable for chemicals that interact through the same mechanisms. This underlying principle would mean that the user would have to be familiar with the multidimensional and arcane sciences involving chemical interactions with biological systems.

Obviously, this complex reality places an undue burden on users of the models. The solution is to encode the expert knowledge of chemical interactions and provide the user with a fully transparent description of what considerations were made, how the estimate was calculated from these considerations, and literature citation to underpin them.

Implementation in the QSAR Application Toolbox

The ER binding decision tree model represents an encapsulation of both undirected research in the literature from more than two decades as well as directed research over the past four years to tailor QSAR methods to specific regulatory needs. Although many regulatory decisions cannot wait for 3-4 years of testing, adapting this decision tree to each new chemical inventory and to other nuclear receptors will significantly decrease the numbers of new chemicals that have to be tested to achieve comparable results.

There are many software packages commercially available for the development of decision trees with chemicals. We have compared the decision tree requirements to the capabilities of the QSAR Application Toolbox which is freely distributed, and we find that there are no technical barriers that would prevent this ER binding affinity model to be distributed as part of the Toolbox. If agreeable to the appropriate OECD advisory groups, approximately six months would be needed to create and evaluate the decision tree as a module in the Toolbox.

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Please Note: The research being conducted on *in vitro* and QSAR methods was not sufficiently developed to be ready for use in the initial round of EPA EDSP priority setting. (see http://www.epa.gov/scipoly/oscpendo/prioritysetting/approach.htm), but may be used in subsequent rounds of EDSP priority setting. Mention of a substance in the meeting summary or EPA presentations concerning the development of such methods does not mean the Agency has or will make a determination that any use of the substance will pose a significant risk. Further, this research is insufficient to allow EPA to draw any conclusions as to whether these substances are "endocrine disruptors"; the substances listed are simply compounds that have been or may prove to be useful in developing ranking and prioritization methods.

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Figure 1. Relationship between ER binding (Log Relative Binding Affinity; RBA) and Log K_{ow} for low affinity binders interacting at Site A (e.g., p-alkylphenols) or Site B (e.g., p,n-alkylanilines).

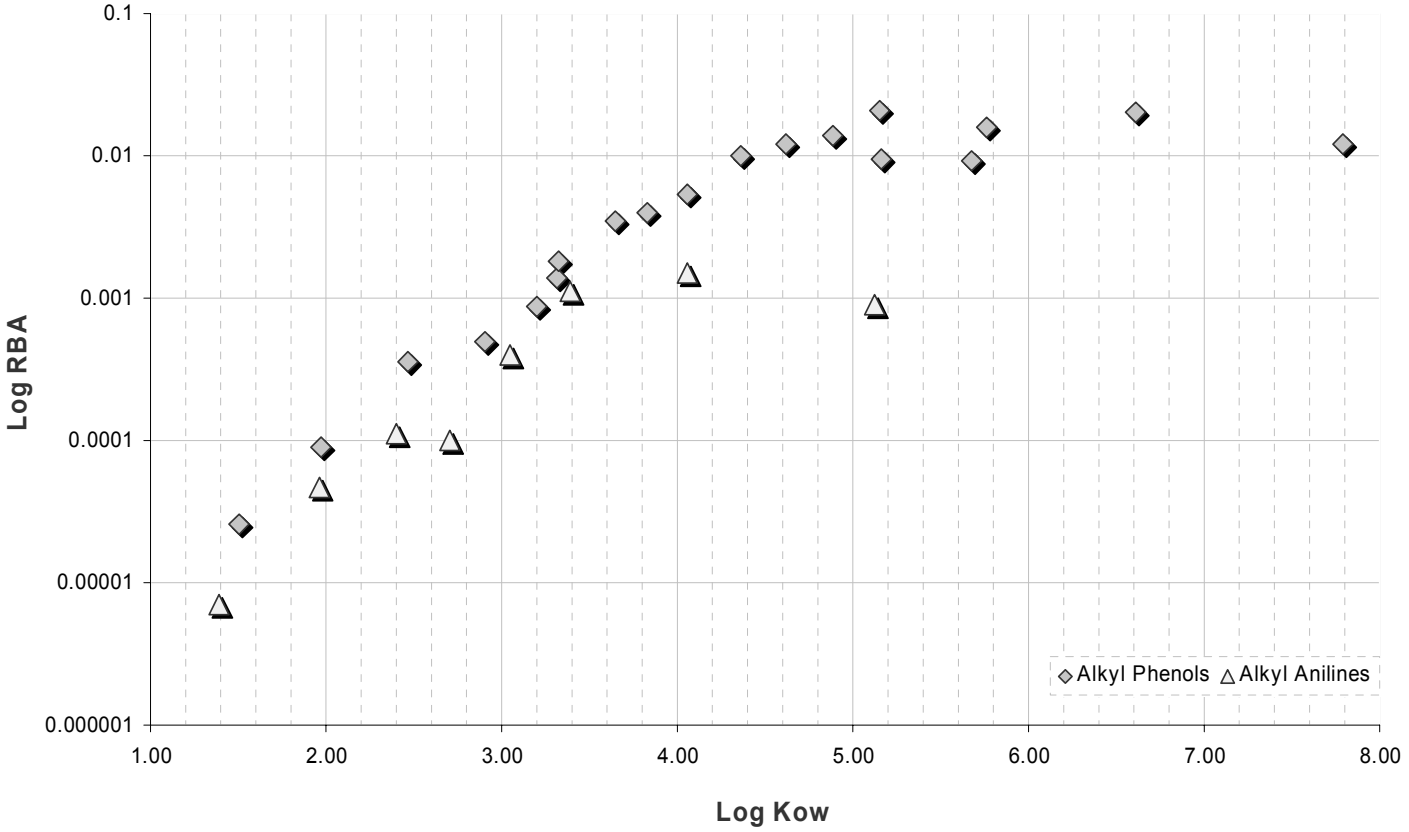

Figure 2. Demonstrating transparency at each step of the decision tree using the chemical group-wise approach where the existing chemical knowledge-base (e.g, training set chemicals) is compared to the inventory chemicals being assessed. The example shows the Sulfonic Acid Dyes structures tested and the representative chemicals in the inventory. Chemicals are presented in increasing order of Log Kow.

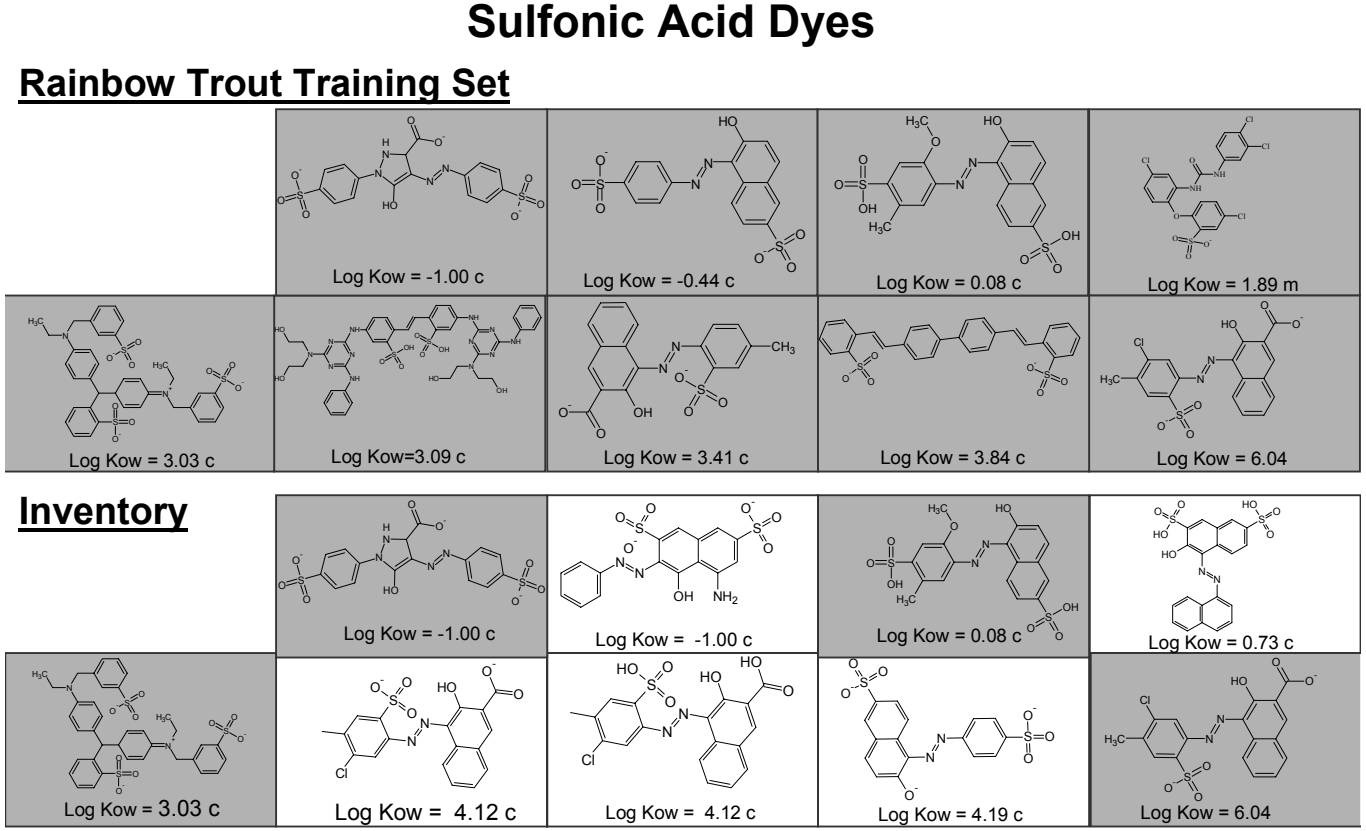

Figure 3. The Decision Tree.

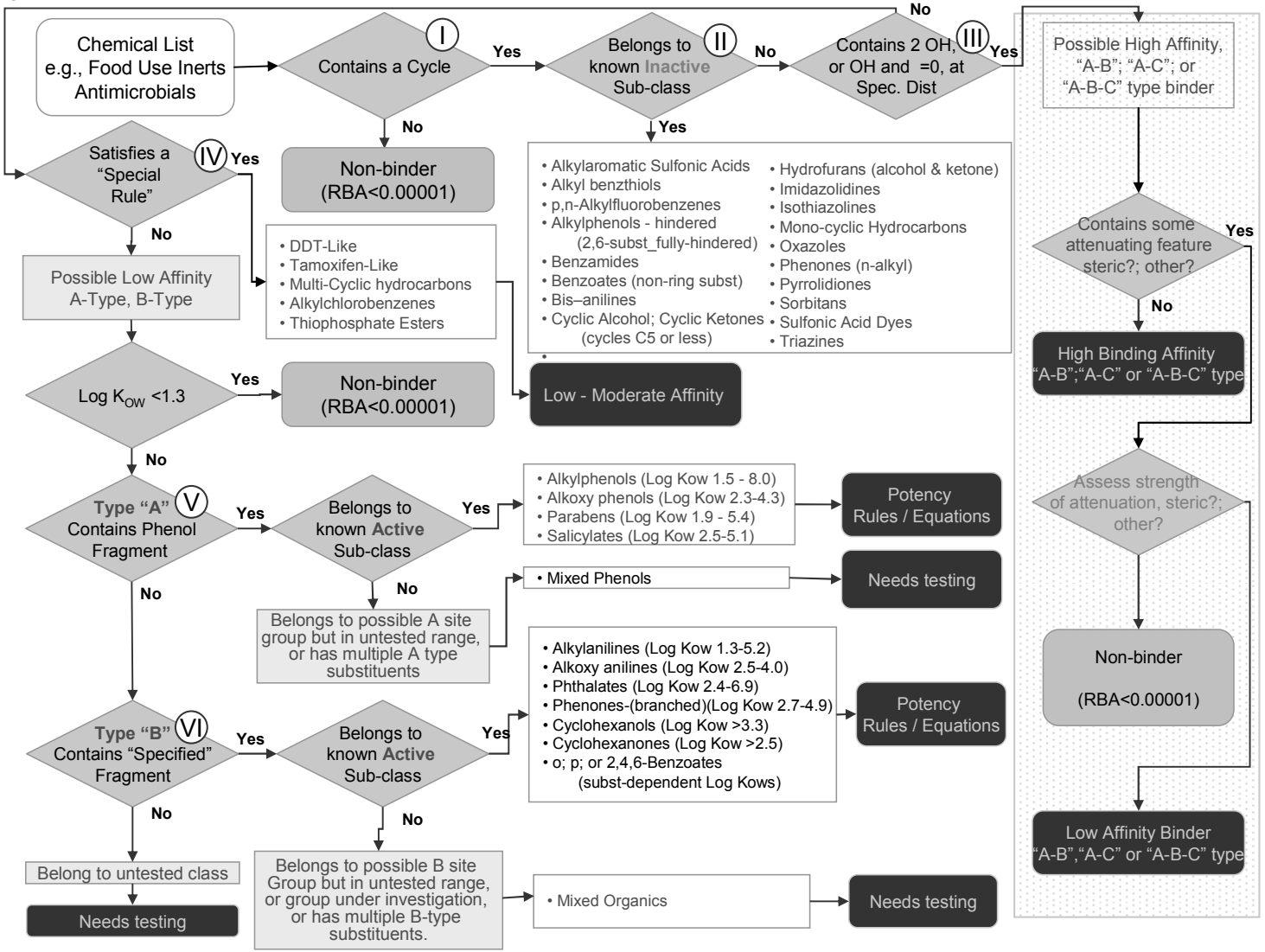

Table 1. Chemicals tested within groups for which no ER binding was found.

Acyclic Chemicals	**CAS**	**Chemical Name**	**LogKow**
Acyclic borates	121437	Trimethylborate	-1.00
Acyclic alcohol	77996	1,1,1-tris(hydroxymethyl)propane	-1.00
Acyclic alcohol	111273	1-hexanol	2.03
Acyclic alcohol	104767	2-ethyl-1-hexanol	3.01
Acyclic alcohol	112301	1-decanol	4.24
Acyclic alcohol/amine	637398	triethanolamine hydrochloride	-1.00
Acyclic amine salt	67633630	N-ethyl-N,N-dimethyl-3-[(1-oxoisooctadecyl)amino]-1-propanaminium, ethyl sulfate	6.43
Acyclic amine	112572	1,2-ethanediamine, n-(2-aminoethyl)-n'-2-(2-aminoethyl)aminoethyl	-1.00
Acyclic amine	78900	1,2-diaminopropane	-0.91
Acyclic amine	2783177	1,12-diaminedodecane	3.51
Acyclic aminocarboxylic acid	67436	Diethylenetriamine pentaacetic acid	-1.00
Acyclic aminocarboxylic salt	139899	Trisodium (2-hydroxyethyl)ethylenediaminetriacetic acid	-1.00
Acyclic carbamate	55406536	3-iodo-2-propynyl N-butylcarbamate	2.65
Acyclic mixed sulfonic salt	38916426	DL-Aspartic acid, N-(3-carboxy-1-oxo-3-sulfopropyl)-N-octadecyl-, tetrasodium salt	5.33
Acyclic ester	97632	Ethyl methacrylate	1.94
Acyclic ester	689894	Methylsorbate	1.96
Acyclic mixed salt	42808366	Octadecanoic acid, 9(or_10)-(sulfooxy)-, 1-butylester, sodium salt	6.76
Acyclic aldehyde	4313035	(2E,_4E)-2,4-heptadienal	1.86
Acyclic ketone	504201	2,6-dimethyl-2,5-heptadien-4-one	2.68
Acyclic phosphonate	4672382	propylphosphonic acid	0.28
Acyclic quat.amine salt	35141367	1-Propanaminium, N,N,N,-trimethyl-3(trimethoxysilyl)-, chloride; 1-propanaminium,	-1.00
Acyclic quat.amine salt	56375792	tributylmethylammonium chloride	0.24
Acyclic quat.amine salt	112005	N-dodecyltrimethylammonium chloride	1.22
Acyclic sulfate salt	126921	sodium 2-ethylhexylsulfate	2.75
Acyclic sulfate salt	142314	sodium octylsulfate	2.88
Other groups/subgroups			
Alkylaromatic Sulfonic Acids	657841	sodium_p-toluenesulfonate	-0.62
Alkylaromatic Sulfonic Acids	98691	4-ethylbenzenesulfonic_acid	0.38
Alkylaromatic Sulfonic Acids	532025	naphthalene-2-sulfonic_acid_sodium salt	0.63
Alkylaromatic Sulfonic Acids	130143	Naphthalene-1-sulfonic acid, sodium salt	0.92
Alkylaromatic Sulfonic Acids	6149037	4-Octylbenzenesulfonic acid, sodium salt	3.56
Alkylaromatic Sulfonic Acids	27176870	Dodecylbenzenesulfonic_acid_	5.67
Alkyl Benzthiols	106456	p-toluene_thiol	2.88
Alkyl Benzthiols	2396681	4-tert-butylthiophenol	4.19
p-Alkylfluorobenzene	462066	Fluorobenzene	2.27
p-Alkylfluorobenzene	403394	1-fluoro-4-isopropylbenzene	3.71
p-Alkylfluorobenzene	28593148	4-fluoropentylbenzene	4.90
Benzamide	10546700	N-propylbenzamide	1.72
Benzamide	--	2-methyl-n-propyl-benzamide	2.08
Benzoates - non-ring subst	93992	Phenylbenzoate	3.59
Benzoates - non-ring subst	136607	n-butylbenzoate	3.84

Benzoates - non-ring subst	6789884	Hexyl benzoate; Benzoic acid, n-hexyl ester	3.84
Benzoates - non-ring subst	94473	Phenethylbenzoate	4.01
Benzoates - non-ring subst	94462	Isoamylbenzoate	4.15
Bis-Anilines	101779	4,4'-diaminodiphenylmethane	1.59
Bis-Anilines	54628216	4,4'-diamino-2,2'-dimethylbibenyl	3.57
Bis-Anilines	4073987	4,4'-methylenebis(2,6-dimethylaniline)	4.01
Bis-Anilines	101688	1,1'-methylenebis 4-isocyanato benzene	4.08
Bis-Anilines	101611	4,4'-methylenebis[N,N-dimethyl]aniline	4.45
Bis-Anilines	2390592	Ethanaminium,N-[4-[bis[4-(diethylamino)phenyl]methylene]-2,5-cyclohexadien-1-ylidene]-N-ethyl-,chloride	5.78
Bis-Anilines	2716101	4,4'-[1,4-phenylenebis(1-methylethylidene)]bis-benzenamine	6.04
Bis-Anilines	13680358	4,4'-methylenebis(2,6-diethylanilne)	6.15
Cyclic Alcohols - other	96413	Cyclopentanol	0.71
Cyclic Ketones - other	120923	Cyclopentanone	0.38
Cyclic Ketones - other	78591	Isophorone	1.70
Cyclic Ketones - other	4694126	2,4,4-trimethylcyclopentanone	1.86
Cyclic Ketones - other	76222	(+/-)-Camphor	2.18
Furans- sugar	57501	Sucrose	-1.00
hydroFurans	97994	tetrahydrofurfuryl_alcohol	0.02
hydroFurans	105215	5-butyldihydrofuran-2(3H)-one	1.15
Imidazolidines	116256	dimethyl-1-hydroxymethylhydantoin	-0.86
Imidazolidines	16079882	1-bromo-3-chloro-5,5-dimethyl hydantoin	0.39
Imidazolidines	77485	1,3-dibromo-5,5-dimethyl-2,4-imidazolidinedione	0.54
Imidazolidines	118525	1,3-dichloro-5,5-dimethylhydantoin	0.55
Isothiazolines	2634335	1,2-benzisothiazol-3-one	0.61
Isothiazolines	26530201	2-octyl-3(2H)-isothiazolone	2.45
Mono-Cyclic Hydrocarbons	108883	Toluene	2.73
Mono-Cyclic Hydrocarbons	106423	p-Xylene (anhydrous)	3.15
Mono-Cyclic Hydrocarbons	98828	Cumene	3.66
Mono-Cyclic Hydrocarbons	5989275	(R)-(+)-Limonene	4.57
Oxazoles	6542376	1-aza-3,7-dioxabicyclo(3.3.0) octane-5-methanol	-1.00
Oxazoles	7747355	5-ethyl-1-aza-3,7-dioxabicyclo(3.3.0)-octane	0.26
Phenones - n-chain	98862	Acetophenone	1.63
Phenones - n-chain	942927	n-hexanophenone	3.64
Phenones - n-chain	1671756	Heptanophenone	4.13
Pyrrolidiones	872504	1-methyl-2-pyrrolidone	-0.38
Pyrrolidiones	2687947	N-octylpyrrolidone; 1-Octyl-2-pyrrolidone	3.31
Sorbitans	1338392	Sorbitan,_monododecanoate_(9CI)	3.15
Sorbitans	1338438	(z)-sorbitan,_mono-9-octadecenoate	5.89
Sulfonic Acid Dyes	1934210	Acid_Yellow_23	-1.00

Sulfonic Acid Dyes	2783940	C.I._Food_Yellow_3	-0.44
Sulfonic Acid Dyes	25956176	C.I. Food Red 17	0.08
Sulfonic Acid Dyes	3567257	Sulcofuron-sodium, monohydrate	1.89
Sulfonic Acid Dyes	3844459	C.I. Acid Blue 9, disodium salt	3.03
Sulfonic Acid Dyes	4404437	Fluorescent Brightener 28	3.09
Sulfonic Acid Dyes	5281049	Pigment Red 57-1	3.41
Sulfonic Acid Dyes	27344418	Disodium 4,4'-bis(2-sulfostyryl)biphenyl	3.84
Sulfonic Acid Dyes	7023612	2-Naphthalenecarboxylicacid,4-[(5-chloro-4-methyl-2-sulfophenyl)azo]-3-hydroxy-,calcium salt(1:1)	6.04
Triazines	7673098	N,N',N-trichloro-1,3,5-triazine-2,4,6-triamine	-0.38
Triazines	2893789	Dichloro-s-triazinetrione	1.28
Triazines	5915413	6-chloro-n(1,1-dimethylethyl)-n'-ethyl-1,3,5-triazine-2,4-diamine	3.06
Triazines	28159980	N'-tert-butyl-n-cyclopropyl-6-(methylthio)-1,3,5-triazine-2,4-diamine	3.38

Table 2. Number of Chemicals in the specific regulatory inventories under study that have either low potential for ER activation, or higher potential for ER activation based on the Decision Tree.

Chemicals in Groups with Low Potential for ER Activation (RBA<0.00001%)	**FI**	**AM**
Acyclics (all groups)	230	122
Alkylaromatic Sulfonic Acids	78	3
Sulfonic Acid Dyes	9	1
Sorbitans	7	0
Monocyclic Hydrocarbons	7	0
Cyclic caged Hydrocarbons	1	0
Pyrrolidiones	3	0
Hydrofurans	3	0
n-Alkyl Phenones	1	0
Oxazoles	0	3
Triazines	1	13
Isothiazolines	3	7
Imidazolidines	0	8
Cyclic Inorganics	0	3
2,6-subst Alkylphenol	1	0
Cyclic Pentanones/Others	2	0
Low ER Potential Chemicals in Higher Potential ER Activation Groups		
Cyclic Hexanones of Log Kow<1.5	1	0
Cyclic Hexanols of Log Kow<2.3	1	0
p-Chlorobenzenes of Log Kow<4	1	0
Low ER Potential Chemicals in Mixed Functional/Heteratom Groups		
Mixed Phenols	7	3
Mixed Organics	20	29
Organometallics	2	4
Total Chemicals with Low Potential for ER Activation	**378**	**196**

Chemicals in Groups with Higher Potential for ER Activation (RBA>0.00001%)	**FI**	**AM**
Alkyl Phenols	3	9
Alkoxy Phenols	1	0
Parabens	3	0
Salicylates	1	0
Phthalates	3	0
Thiophosphate Esters	1	0
Actives in Mixed Functional /Heteratom Classes		
Mixed Phenols	2	6
Mixed Organics	1	0
Total Chemicals with Higher Potential for ER Activation	**15**	**15**

ANNEX 4: PRESENTATION BY US-EPA

(Q)SARs OF ESTROGEN BINDING AFFINITY TO SUPPORT PRIORITIZATION OF HETEROGENOUS CHEMICALS WITHIN DEFINED INVENTORIES FOR SCREENING AND TESTING

Feb 17, 2009

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Chapter I. Identifying Hazards to ER-Mediated Pathways

OECD Endocrine Disruptor Testing and Assessment (EDTA)
"*Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals*"
Validation Management Group-Non-Animal (VMG-NA) objective:
identify non-animal assays for endocrine testing
develop tools necessary for:
Level 1 -Sorting and Prioritization with existing data and/or (Q)SAR systems)
Level 2 - *In vitro* assays providing mechanistic data

US EPA legislative mandate (Food Quality Protection Act):
Requires screening chemicals within specified inventories for effects similar to that of estrogen or other such endocrine effects
Need to prioritize *in vitro/in vivo* screening and testing options for classes of compounds where 'endocrine data' is lacking:
-Inert ingredients used in formulations of pesticides used on crops
-Antimicrobial active ingredient pesticides

Chapter I. Identifying Hazards to ER-Mediated Pathways

Risk Management Context

Establish a risk-based means to focus endocrine screening and testing program efforts

-Most chemicals needing evaluation do not have existing toxicity data

-In combination with an existing exposure evaluation, need a hazard-based method to prioritize chemicals for *in vitro/in vivo* screening

Goal: Develop a (Q)SAR-based hazard component to prioritizing chemicals for subsequent *in vitro* and/or *in vivo* screening

Regulatory Context

Development and use of a QSAR in regulatory risk assessment requires clear problem definition

- The purpose of the QSAR application must be well-defined (e.g., priority setting for testing, and chemical-specific risk assessment are two very different purposes with different acceptance criteria)
- The chemicals of regulatory concern must be defined to establish an appropriate training set for QSAR development and/or to assess appropriateness of QSAR application

A QSAR can only be as good as the underlying toxicological understanding and data it is based upon

- Toxicological activity is assessed based on a well-defined endpoint in a well-defined assay

OECD Principles for QSAR Validation

- **Transparent**
  - How the QSAR estimate can be explained mechanistically
  - How reasonable is the estimate compared with data for similar chemicals
- **Defined Assay**
  - Well-defined biological endpoint –
    - Informing SIDS endpoint
    - Toxicity pathway – plausible linkage of MIE to higher level measure
  - Well-defined chemistry
    - Does assay allow testing of type of chemicals on regulatory lists?
    - Are chemicals present in assay in form and concentration assumed?
- **Defined Applicability Domain**
  - Well-defined application – risk context
    - Does the QSAR domain cover the regulatory question?
- **Unambiguous algorithm**
  - Expert Systems – logic tree, rules, local models
- **Mechanistic interpretation**
  - Can estimate be explained mechanistically- chemistry & biology ?
  - Toxicity Pathway to Adverse Outcome
  - Relationship of chemical parameters to activity

OECD QSAR Principles were developed to guide member countries as they validate QSAR approaches for their national regulatory needs

Characteristics of QSARs approaches that will enhance regulatory acceptance of using estimated values for priority setting:

1) Alignment of a QSAR application with a specific regulatory purpose
   -Does it inform important SIDS endpoint?
   -Does it provide comparable estimates for all chemicals in a specified inventory?

2) Public and scientific community understanding/acceptance
   -Is the (Q)SAR method adequately described?
   - Can estimates be explained mechanistically?
   - Are estimates reasonable based on data for comparable chemicals?

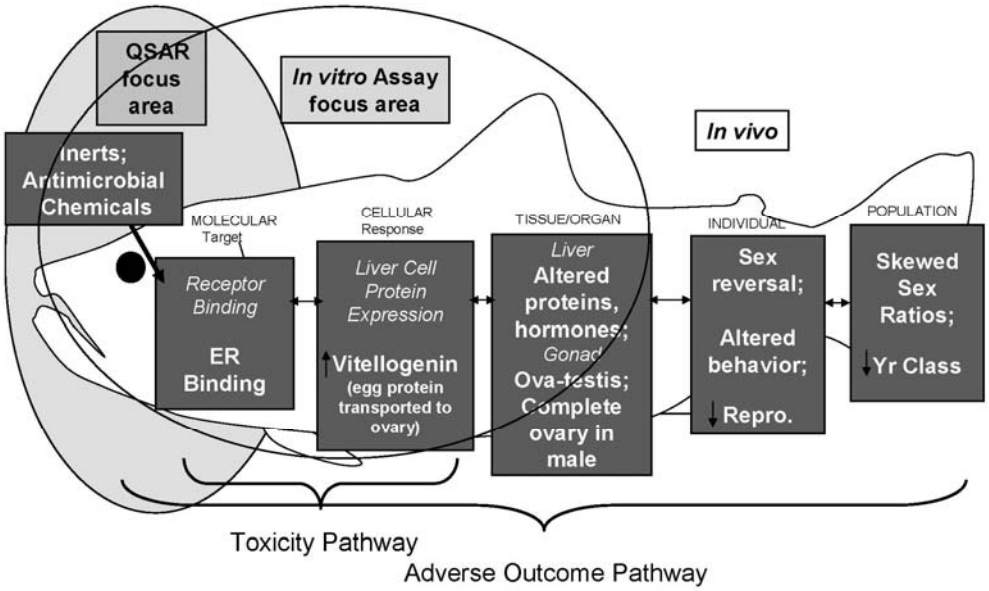
Does the (Q)SAR expert system for this OECD consultation inform an important SIDS endpoint?

Mechanistic linkage between SIDS endpoint (impaired reproduction) and hazard identification endpoint for QSAR modeling (ER binding)

ER-mediated adverse outcome pathway ending in reproductive impairment
- ER binding is the molecular initiating event
- The expert system identifies chemicals that can initiate the pathway
  - -Provides decision-making rationale for regulatory community
  - -Provides conceptual model useful for generating testable hypotheses

ER Pathway:
- -Initial focus consistent with related legislative directive
- -Chemical binding to ER known to have potential to cause adverse effects
- -Existing evidence that diverse chemical structures bind ER
- -Bioassays for ER binding available from drug-design, although extant methods were focused on potent anti-estrogens
- -Drug-design provides mechanistic insights on multiple types of interaction with ER binding site

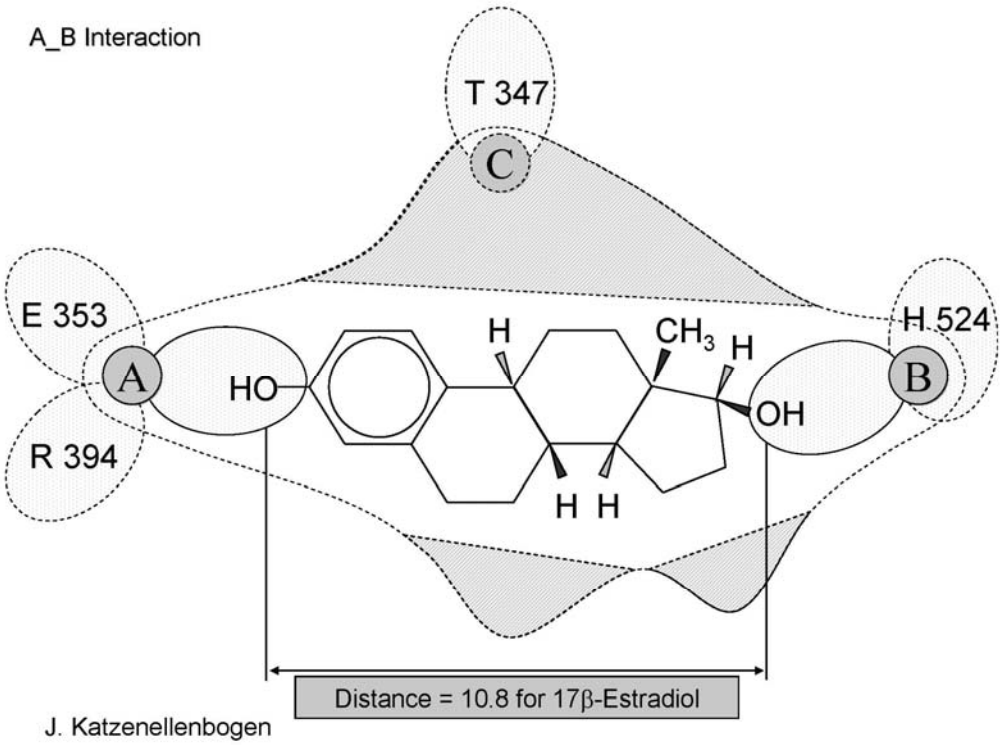
Adverse Outcome Pathway ER-mediated Reproductive Impairment



Can the (Q)SAR estimates by explained mechanistically? Search for Mechanistic Insights based on Known ER Ligand Interactions

Energy and steric constraints dictated by the ER itself shapes the domain of the chemical structures that can bind to the receptor

Evidence, from drug-design, crystal structure, docking studies indicates:
- multiple ER sub-pockets, with varying H-bonding
  (e.g., Katzenellenbogen et al)
- steroids, high potency estrogens and anti-estrogens interact with at least two sub-pockets (H-bonding at specified distances)

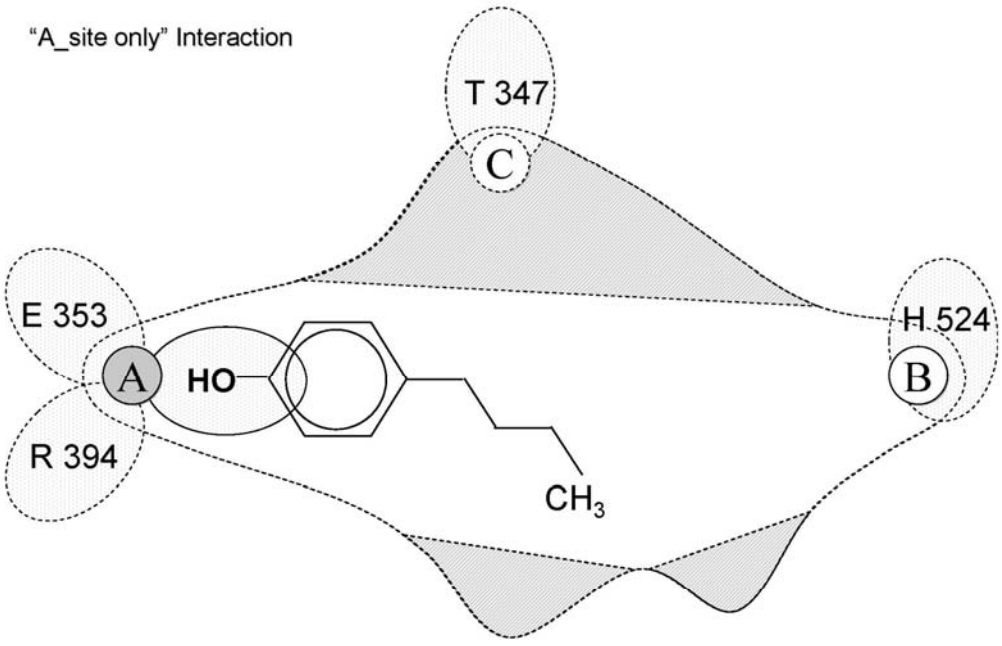


Can the (Q)SAR estimates by explained mechanistically? Hypothesize ER interactions of Inventory Chemicals

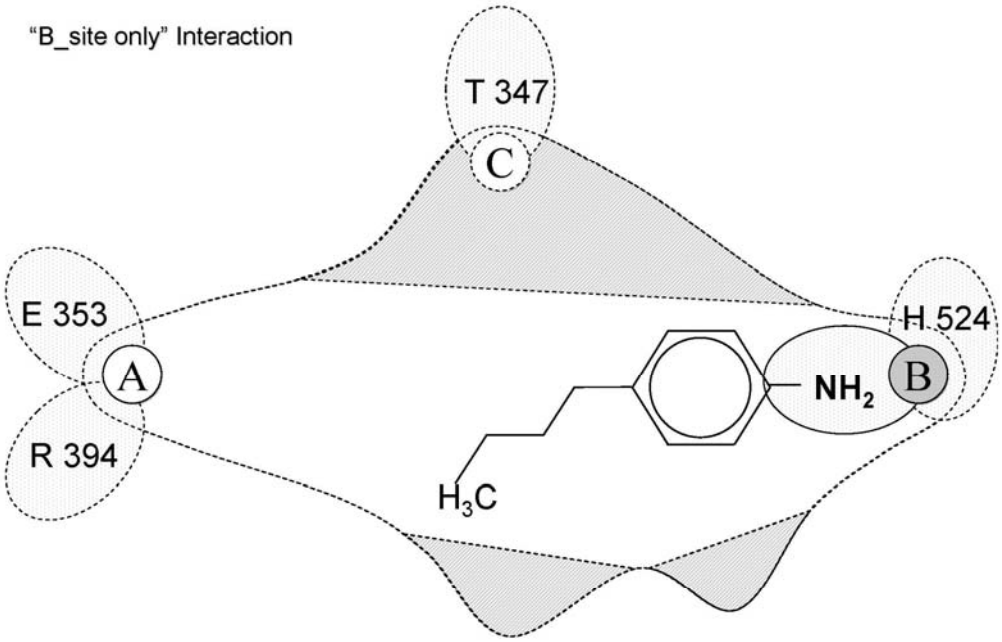
Inert ingredients and antimicrobial pesticides are non-steroidal and do not contain multiple H-bonding groups at distance needed for steroid-like interactions

Hypotheses:

- Any inerts, antimicrobials that bind will be through a low affinity process
- A small % of these chemicals will bind to the ER
- A chemical grouping approach will provide a useful regulatory application framework
- Chemical can be grouped based on common ER sub-pocket binding site (e.g., based on common binding mechanisms)



J. Katzenellenbogen



J. Katzenellenbogen

Does the (Q)SAR expert system for this OECD consultation provide coverage for all chemicals in a specified chemical inventory(ies)?

OECD Principles describe (Q)SAR model domain in terms of the chemical structures used to create the model
- Usefulness evaluated by comparing domain of the (Q)SAR model to the domain of a specific regulatory inventory

Most (Q)SAR models do not use a specific regulatory inventory to develop the model domain

The ER expert system that is the focus of the OECD consultation provides (Q)SAR estimates of estrogenic activity derived from a knowledge-base domain based on specific chemical inventories of regulatory interest

QSAR Model Domain vs Regulatory Domain

Common approach:

- Test chemicals (or use data found in literature)
- Define model domain
- Check if an untested chemical is in domain, if so apply model

Current approach for inert ingredients and antimicrobial pesticides

- Acquire specific inventories requiring screening under US mandates
- Characterize structures in the inventories
- Compare these structures to structures in existing ER binding datasets
- Strategically expand knowledge-base of ER binding domain
  - to cover regulatory lists

Food use Inert Ingredients

Inert chemicals in pesticides used on food crops
The 2004 List included:

893 entries = 393 discrete chemicals + 500 non-discrete substances
(44% discrete : 56% non-discrete)

393 discrete chemicals include:

- **366 organic chemicals (93%)**
- **24 inorganic chemicals (6%)**
- **3 organometallic compounds (1%)**

500 non-discrete substances include:

- **147 polymers of mixed chain length**
- **170 mixtures**
- **183 undefined substances**

Antimicrobial Pesticides

Antimicrobials and sanitizers list included:

299 = 211 discrete chemicals + 88 non-discrete substances
(71% discrete : 29% non-discrete)

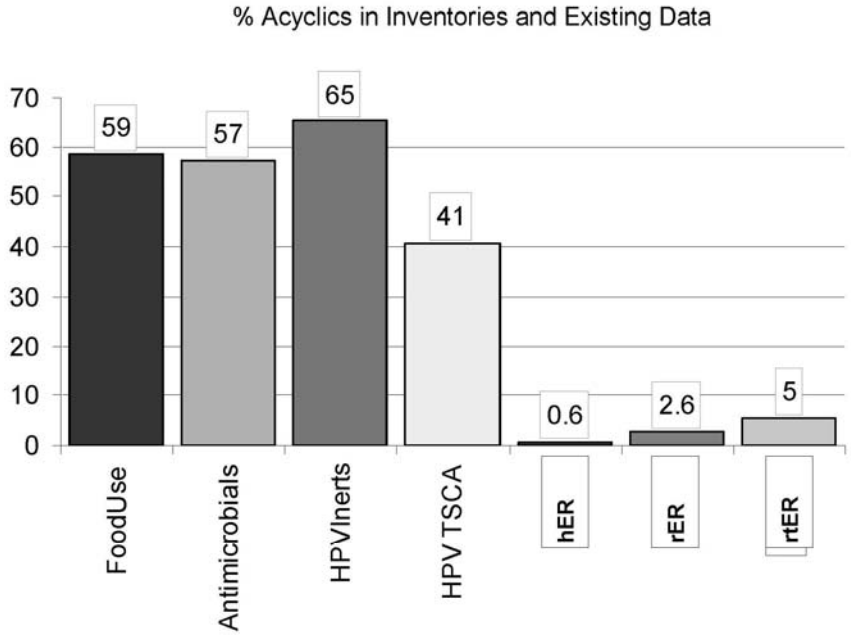
211 discrete chemicals include:
153 organic chemicals (72%)
52 inorganic chemicals (25%)
6 acyclic organometallic compounds (3%)

88 non-discrete substances include:
25 polymers of mixed chain length
35 mixtures
28 undefined substances

Original ER Binding Training Sets

- ER binding data sets from 1990s – 2004
  (hER – human; rER-rat; rtER- rainbow trout):
  - Steroids, anti-estrogens *(high potency binders)*
  - Organochlorine pesticides (OCPs)
  - Alkylphenols
  - Minimal overlap with chemicals in regulatory inventories of interest

	hER	rER	rtER	Food Use Inerts	Anti-microbial Pesticide	HPV Inerts	HPV Industrial Chemicals
Steroid, Anti-E2, OCPs	150 (30 %)	91 (40%)	37	2 (<1%)	2 (1%)	6 (1%)	178 (3%)
Alkyl-phenols	35 (7%)	13 (6%)	22	3 (1%)	7 (3%)	6 (1%)	71 (1%)
Overlap with regulatory inventories as % of total				2%	4%	2%	4%



Chapter II. Aligning ER Binding Domain to Regulatory Inventories

Establishing a knowledge-base to cover regulatory inventories using a well-defined endpoint

1) Databases developed for drug design are often inadequate to develop screening models for environmental contaminants

2) (Q)SAR models must encompass the domains of regulatory inventories to ensure "good and useful science"

3) Knowledge-base of the ER binding model can be strategically expanded to encompass the inert ingredient and antimicrobial inventories

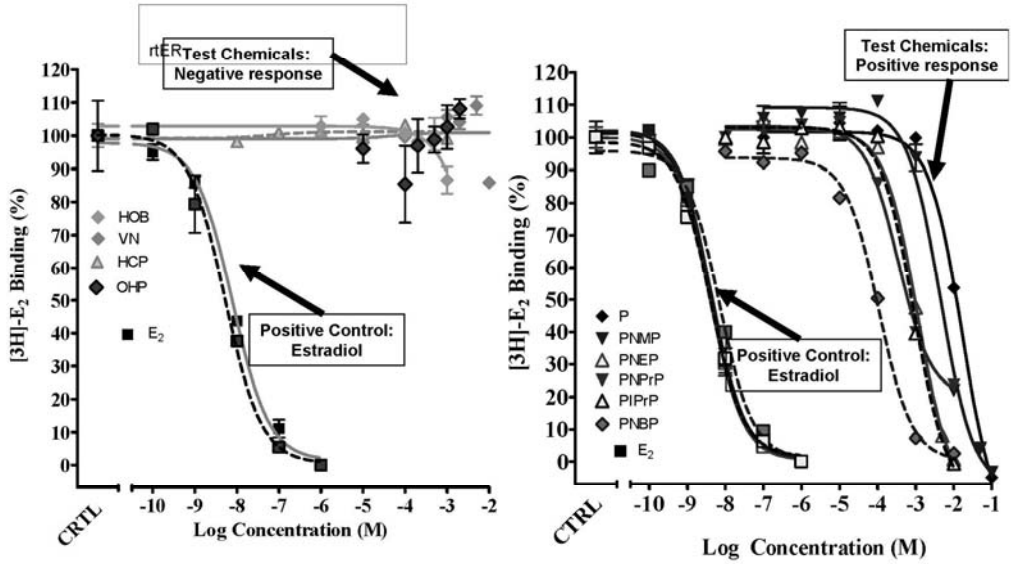
Well-Defined Endpoint: Biology

Focus on molecular initiating event within context of ER-mediated adverse outcome pathway for ER binding

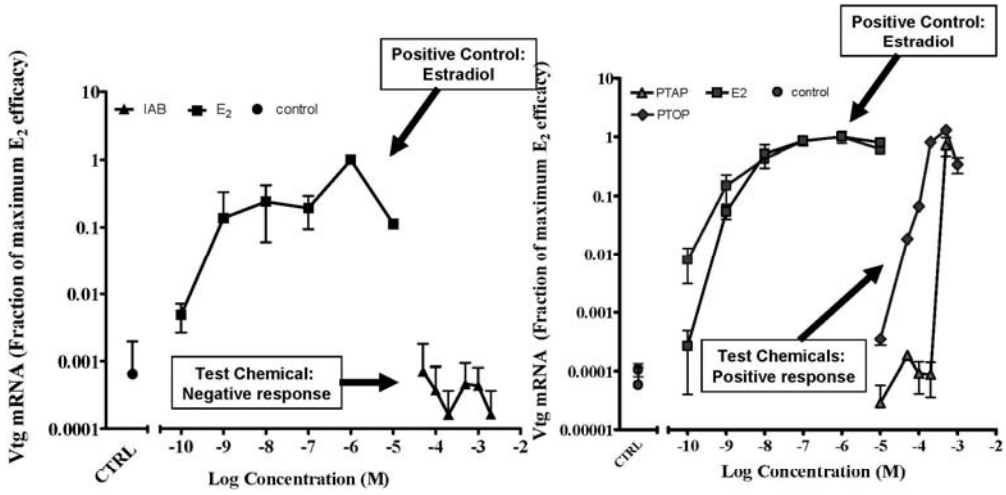
Optimize bioassay methods for chemicals with low affinity

1) rtER binding is assessed using a standard competitive binding assay optimized for inerts and anitmicrobials;
   - chemicals are tested to compound solubility in the assay media to determine any potential to bind ER
2) equivocal binding curves are interpreted by looking for gene expression (ER-mediated vitellogenin mRNA production in metabolically competent trout liver slices);
3) additional experiments to verify competitive binding (Ki) are done as needed

Data Example - primary *In vitro* assay used :
Estrogen Receptor Binding Displacement Assay



Data example – Confirmatory *in vitro* Assay:
Gene Activation



Well-Defined Endpoint: Chemistry

(What do you know and what are you assuming?)

- Chemical purity
- 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?
- Bioavailability of the test chemical in the assay
  - Rate of chemical 'disappearance' within the system (e.g. hydrolysis rates; partitioning to surfaces other solutes)
  - Chemical solubility
    - Freely dissolved vs. bound and unavailable

Expanding the Knowledge-base to Cover Regulatory Inventories

Process:

-subdivide chemical on lists into groups

-group by chemical attributes mechanistically related to the hazard endpoint - "testable rules" (e.g., binding types)

-during development, iteratively evaluate against inventory chemicals to ensure sufficient data and "rules" to covers all chemicals

-if: any inventory chemical is not defined by a subgroups (e.g., outside the domain)

then: expand data and rules until entire regulatory domain is covered

Therefore, chemicals were selected for testing to achieve two goals:

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

As Knowledge-Base Expands, Hypotheses can be Tested and Rules Developed

Given the large representation of Acyclic compounds in the inventory, is there sufficient evidence to say all acyclics are non-binders?

Given that hydrophobic sub-pockets have been described, is there evidence for a relationship of binding and Log P?

Are there general rules for covering chemical groups that do not bind?

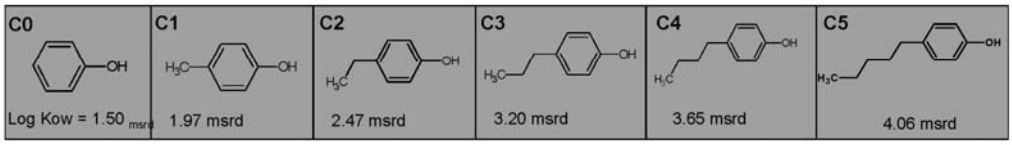
As Knowledge-Base Expands, Hypotheses can be Tested and Rules Developed

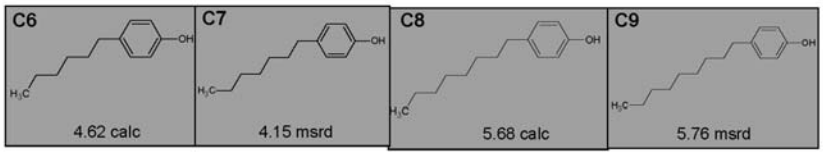
Given the large representation of Acyclic compounds in the inventory, is there sufficient evidence to say all acyclics are non-binders?

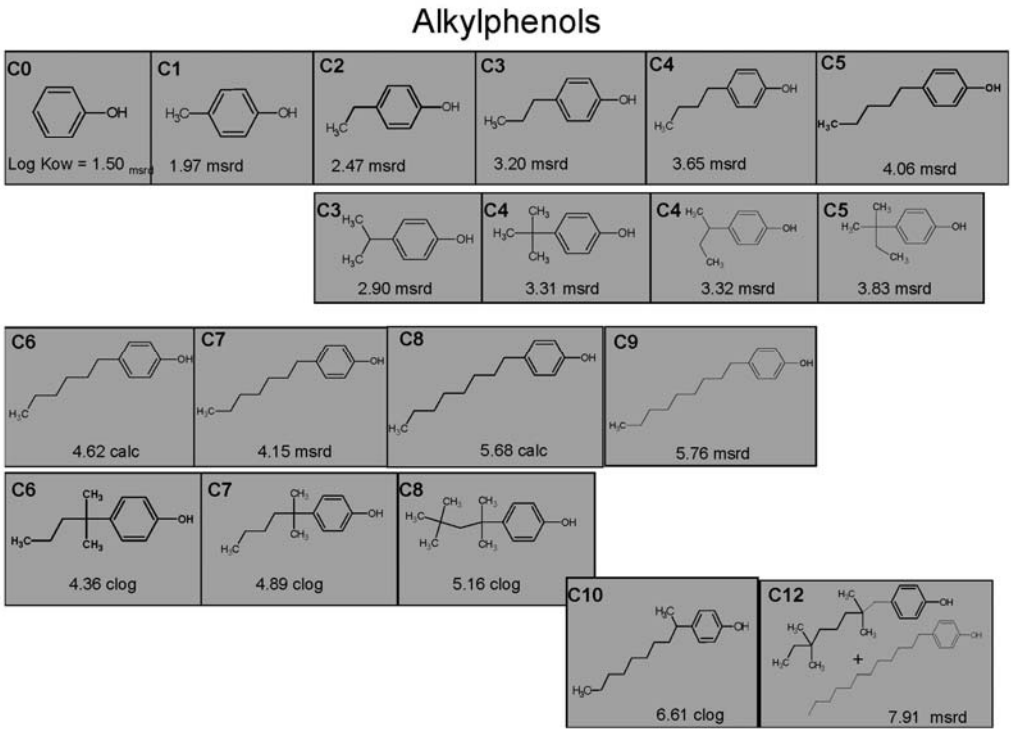
Given that hydrophobic sub-pockets have been described, is there evidence for a relationship of binding and Log P?

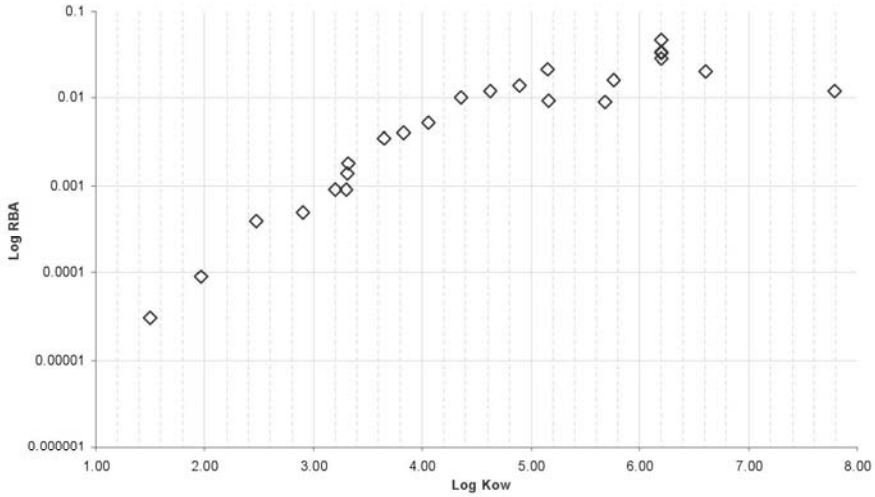
Are there general rules for covering chemical groups that do not bind?

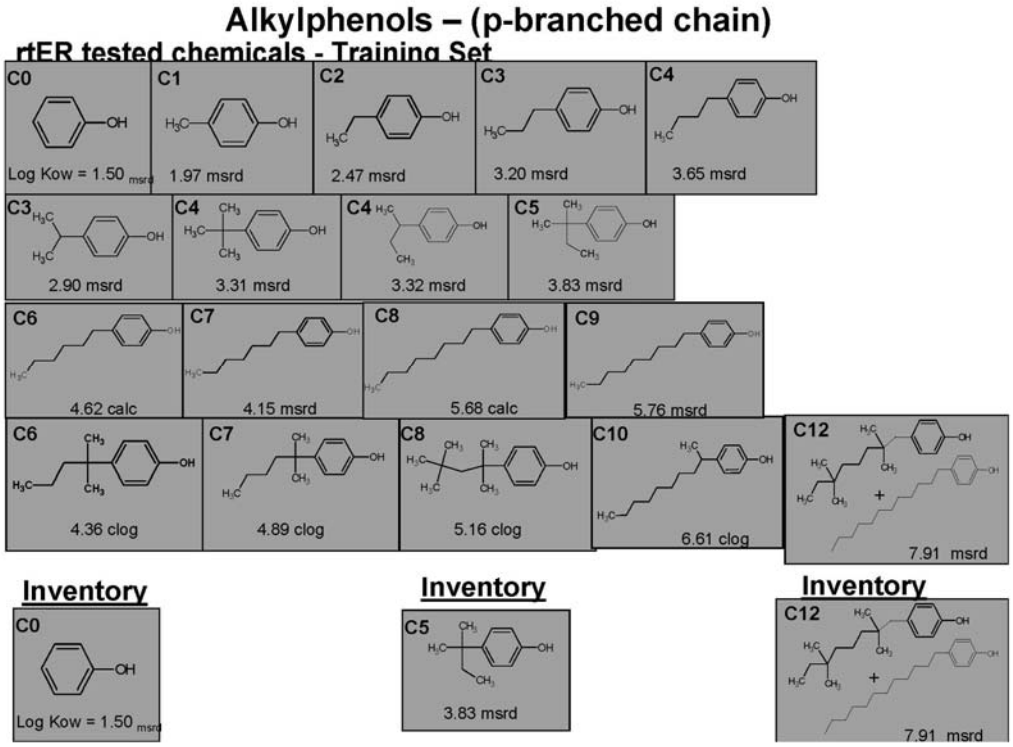
Homologous Series
Alkylphenols

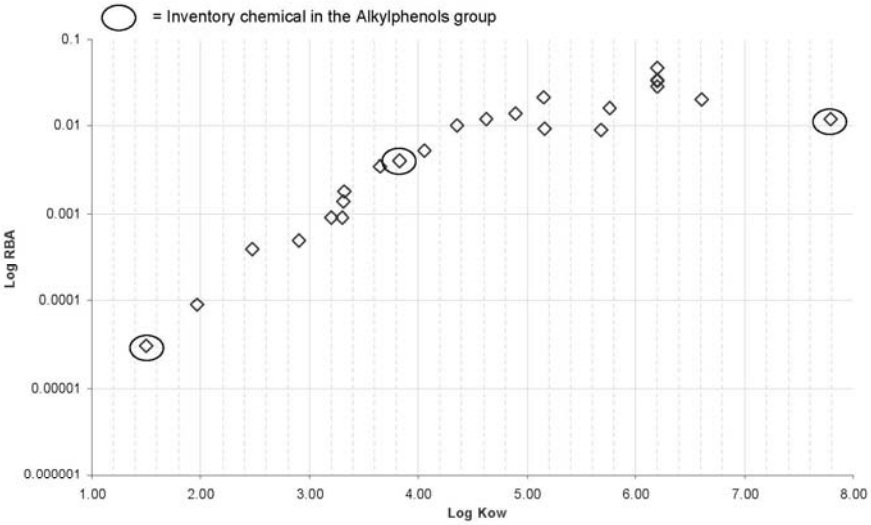





Alkylphenols
C0
Log Kow = 1.50 msrd
C1
1.97 msrd
C2
2.47 msrd
C3
3.20 msrd
C4
3.65 msrd
C5
4.06 msrd
C3
2.90 msrd
C4
3.31 msrd
C4
3.32 msrd
C5
3.83 msrd
C6
4.62 calc
C7
4.15 msrd
C8
5.68 calc
C9
5.76 msrd
C6
4.36 clog
C7
4.89 clog
C8
5.16 clog
C10
6.61 clog
C12
7.91 msrd

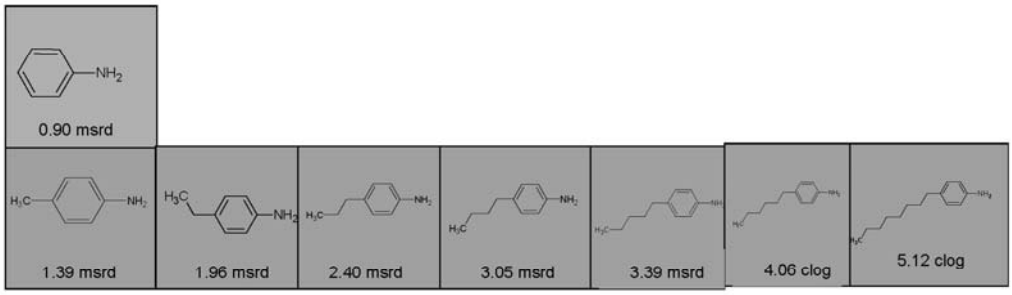

Log RBA
0.1
0.01
0.001
0.0001
0.00001
0.000001
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Log Kow


Alkylphenols – (p-branched chain)
rtER tested chemicals - Training Set
C0
Log Kow = 1.50 msrd
C1
1.97 msrd
C2
2.47 msrd
C3
3.20 msrd
C4
3.65 msrd
C3
2.90 msrd
C4
3.31 msrd
C4
3.32 msrd
C5
3.83 msrd
C6
4.62 calc
C7
4.15 msrd
C8
5.68 calc
C9
5.76 msrd
C6
4.36 clog
C7
4.89 clog
C8
5.16 clog
C10
6.61 clog
C12
7.91 msrd
Inventory
C0
Log Kow = 1.50 msrd
Inventory
C5
3.83 msrd
Inventory
C12
7.91 msrd

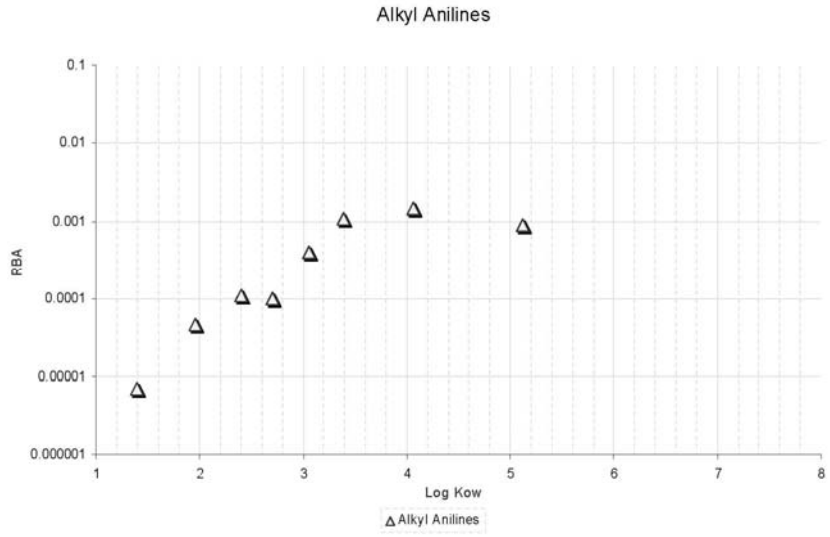

= Inventory chemical in the Alkylphenols group
Log RBA
0.1
0.01
0.001
0.0001
0.00001
0.000001
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Log Kow

Alkylanilines – (p-n chain)

rtER tested chemicals - Training Set

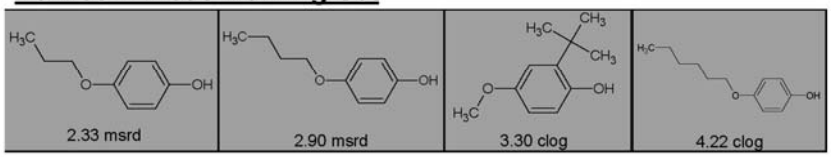


Relationship between Log Kow and RBA for alkylanilines.

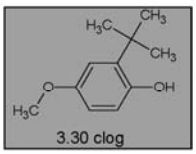


Alkylphenols – (alkoxy)

Rainbow Trout Training Set

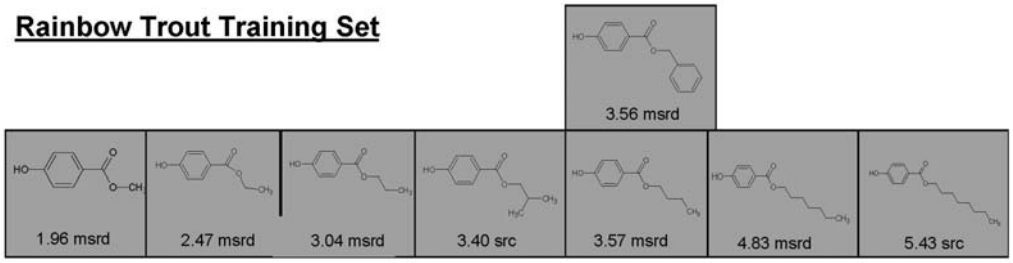


Inventory



Parabens – (mono-hydroxy)

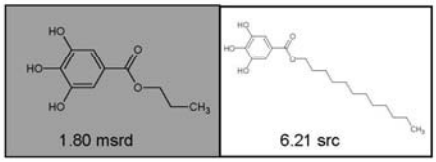
Rainbow Trout Training Set



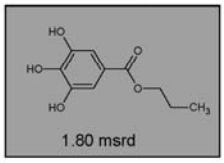
Inventory

Parabens – (tri-hydroxy)

Rainbow Trout Training Set

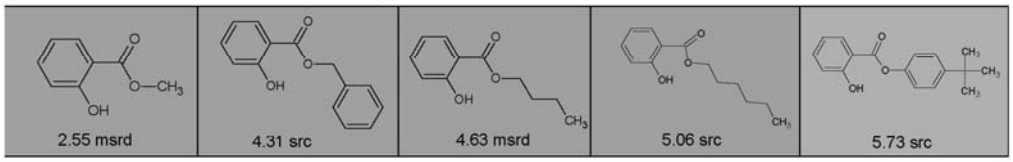


Inventory

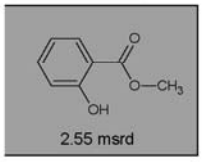


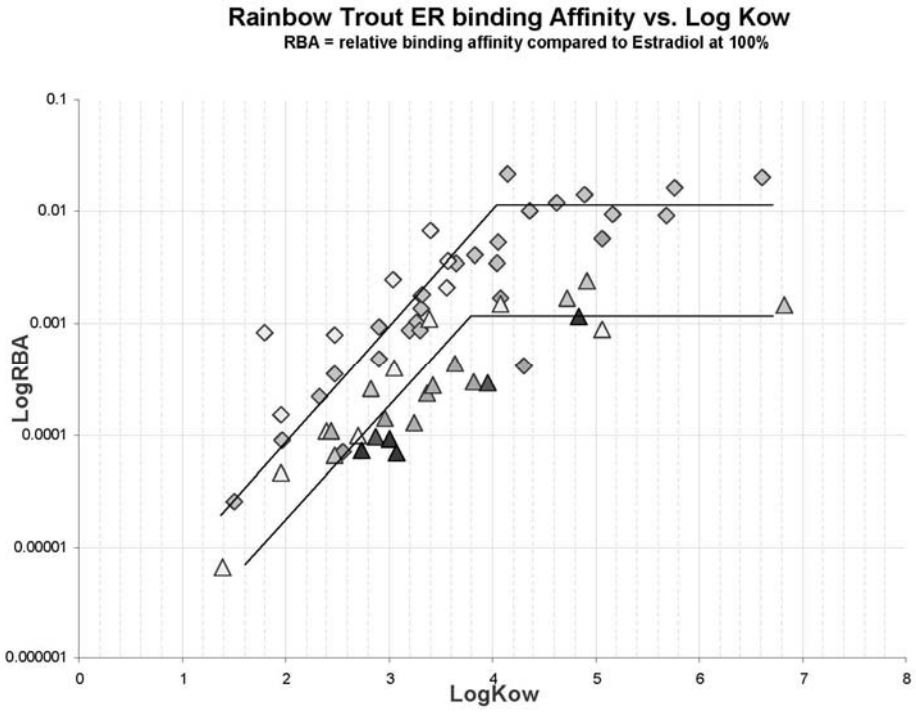
Salicylates

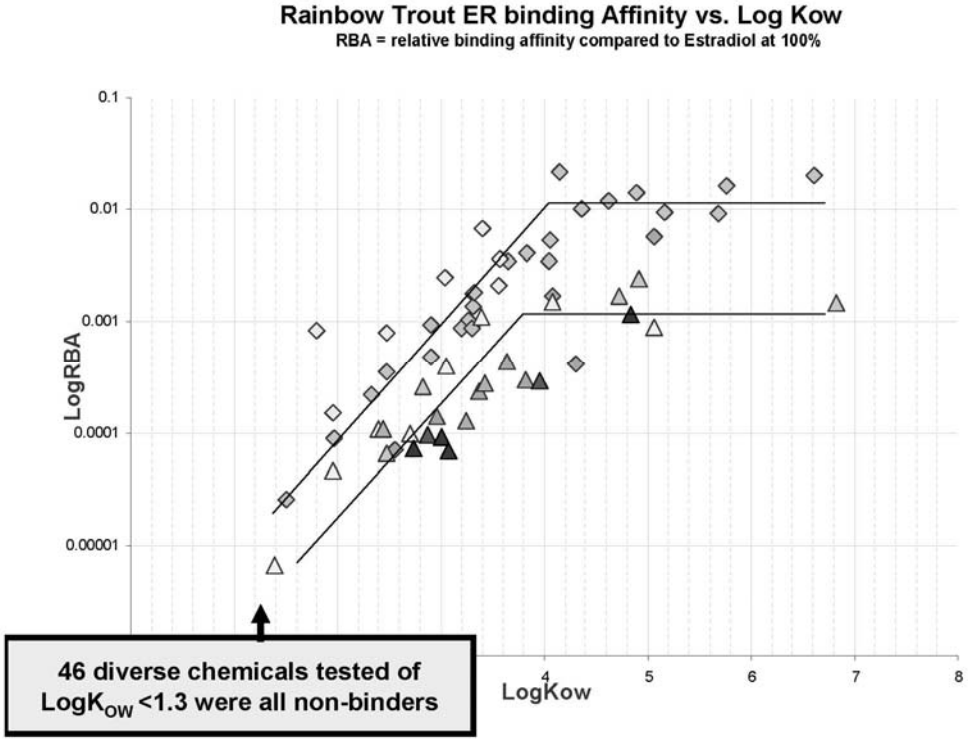
Rainbow Trout Training Set

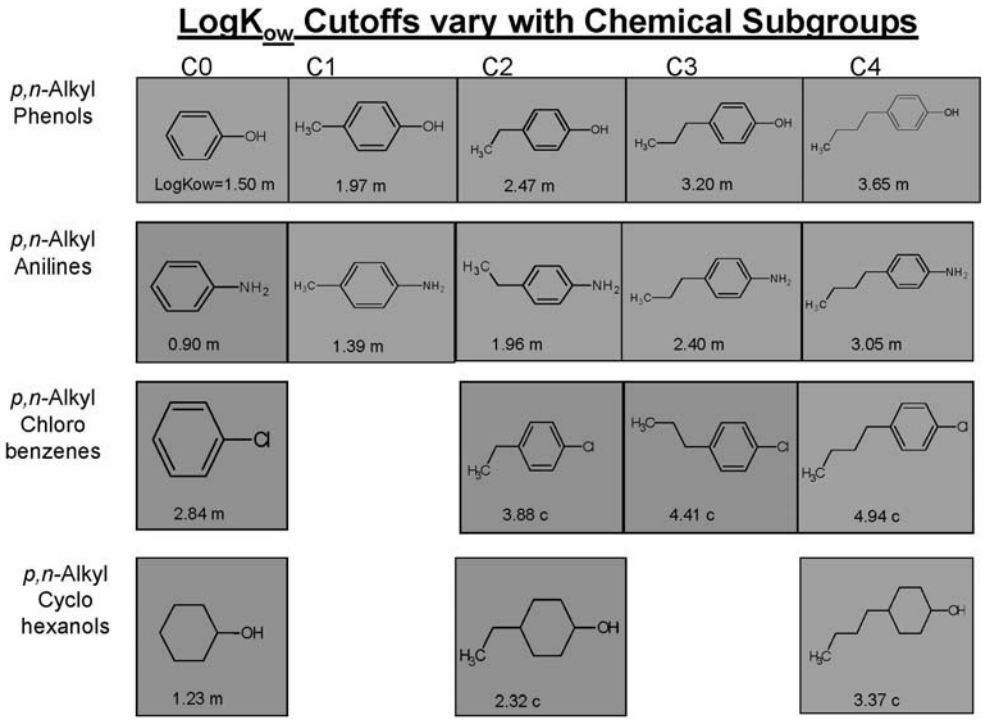


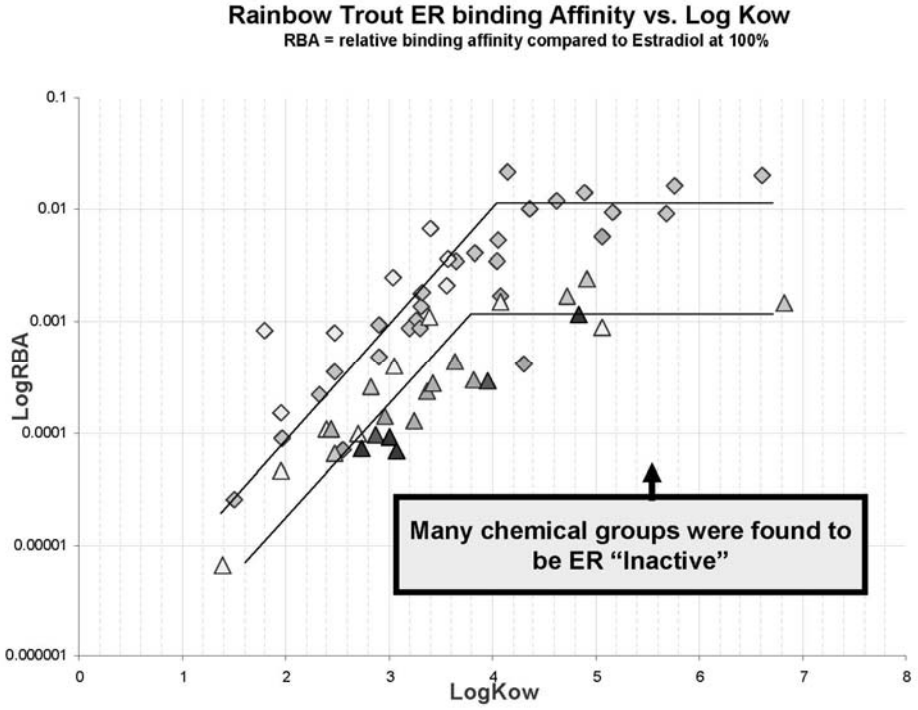
Inventory




Rainbow Trout ER binding Affinity vs. Log Kow
RBA = relative binding affinity compared to Estradiol at 100%
LogRBA
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
1
2
3
4
5
6
7
8
LogKow


Rainbow Trout ER binding Affinity vs. Log Kow
RBA = relative binding affinity compared to Estradiol at 100%
LogRBA
0.1
0.01
0.001
0.0001
0.00001
4
5
6
7
8
LogKow
46 diverse chemicals tested of
LogKOW <1.3 were all non-binders


LogK$_{ow}$ Cutoffs vary with Chemical Subgroups
C0
C1
C2
C3
C4
p,n-Alkyl Phenols
LogKow=1.50 m
1.97 m
2.47 m
3.20 m
3.65 m
p,n-Alkyl Anilines
0.90 m
1.39 m
1.96 m
2.40 m
3.05 m
p,n-Alkyl Chloro benzenes
2.84 m
3.88 c
4.41 c
4.94 c
p,n-Alkyl Cyclo hexanols
1.23 m
2.32 c
3.37 c

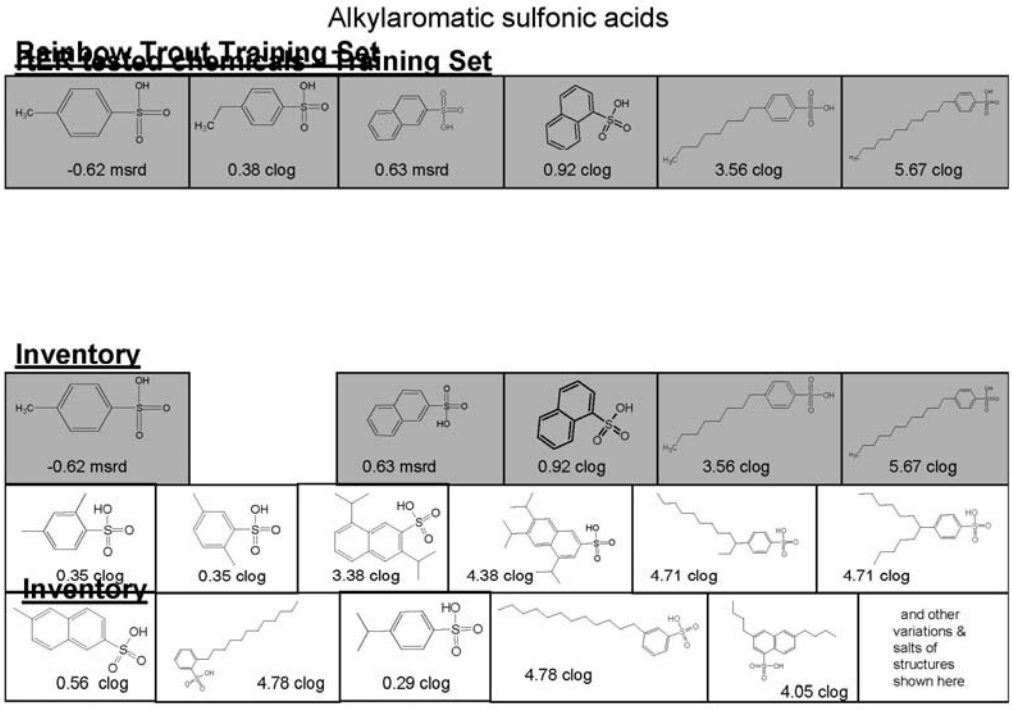

Rainbow Trout ER binding Affinity vs. Log Kow
RBA = relative binding affinity compared to Estradiol at 100%
LogRBA
LogKow
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
1
2
3
4
5
6
7
8
Many chemical groups were found to be ER "Inactive"

As Knowledge-Base Expands, Hypotheses can be Tested and Rules Developed

Given the large representation of Acyclic compounds in the inventory, is there sufficient evidence to say all acyclics are non-binders?

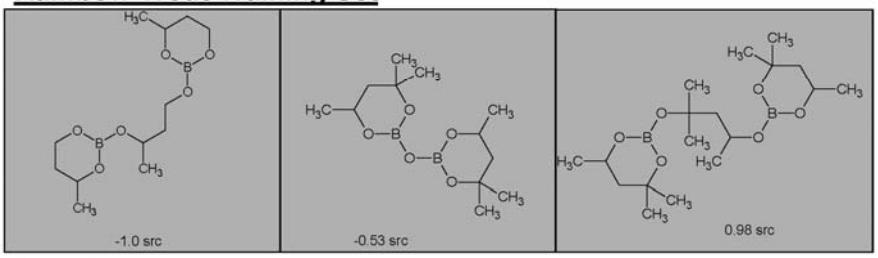
Given that hydrophobic sub-pockets have been described, is there evidence for a relationship of binding and Log P?

Are there general rules for covering chemical groups that do not bind?

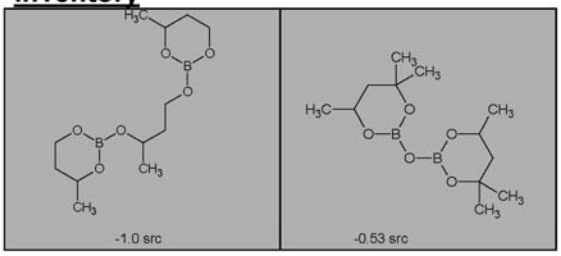


Mixed Organics – (diboranes)

Rainbow Trout Training Set

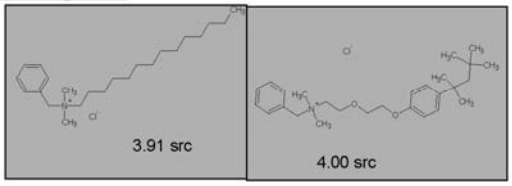


Inventory



Mixed Organics – (quat. Amines)

Rainbow Trout Training Set



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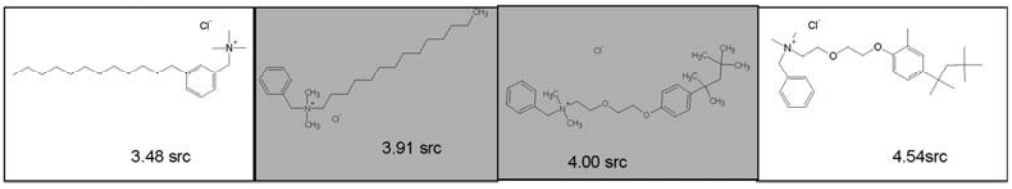
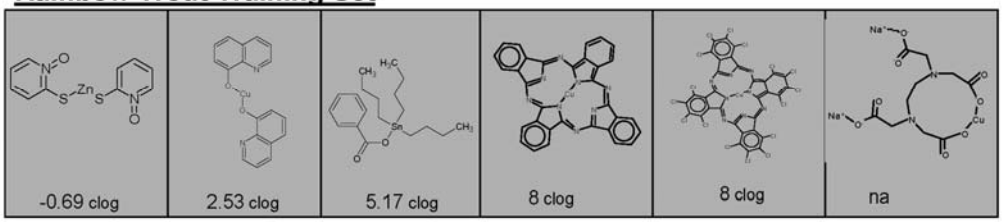
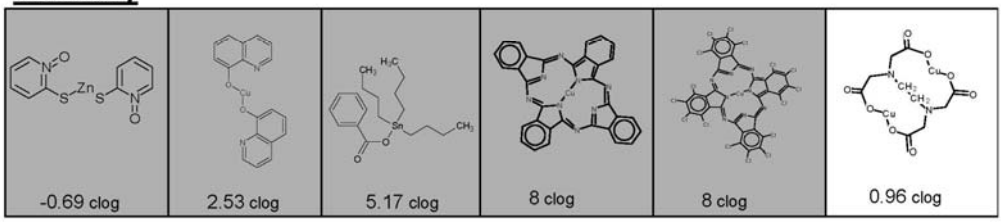


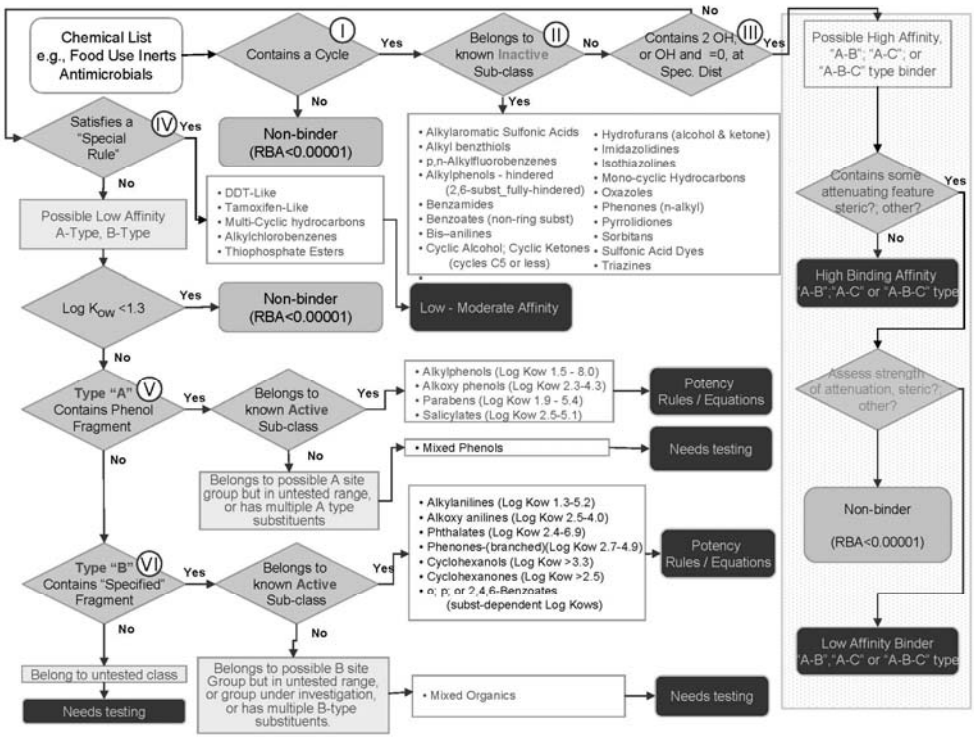
Organometallic

Rainbow Trout Training Set



Inventory





Results:

Chemical has low potential for ER activity if:

-Belongs to a group where testing showed no evidence of ER interaction (RBA < 0.00001);
-Log Kow <1.3, or meets other group-specific Log Kow cutoffs

General characteristics of these chemicals:

-Acyclic compounds (e.g., no benzene rings)
-Cyclic compounds that donot contain a likely H-bonding group;

RBA = relative binding affinity; (a ratio of measured chemical affinity for the ER relative to 17-beta-Estradiol = 100%)
Log Kow = log of octanol/water partition coefficient (also known as Log P); is an indicator of lipophilicity

Results:

Chemical has Higher Potential for ER Activity if:

-Belongs to chemical group with evidence of ER interaction, (RBA > 0.00001), and:
-Log Kow > 1.3, and
- Log Kow < than a chemical group-specific high Log Kow cutoff

General characteristics of these chemicals:

-Contains at least one cycle (e.g., benzene ring);
-Contains a possible H-bonding group;

Food Use Inert Ingredients and Antimicrobial Pesticides

Food Use Inerts		Antimicrobials
393	*Total Chemicals*	211
378 (96%)	*Low Potential*	196 (93%)
15 (4%)	*Higher Potential*	15 (7%)

Summary

ER Expert System is transparent and useful:

- A priority setting tool was developed to focus further testing efforts on the 4 to 7% of chemicals within two defined inventories that have plausible toxicological potential for an important adverse outcome.
- All chemicals in the regulatory inventories were covered.
- Data to build expert system is grounded in ER-mediated Adverse Outcome pathway, informing SIDS endpoint
  - reproductive effects have already been established for some low affinity chemicals
  - more testing will establish lowest ER affinity resulting in relevant adverse effect
  - data and references can be updated as new knowledge is obtained

ER Expert System facilitates identification of untested chemicals with a high probability of causing reproductive effects

- (Q)SAR Expert System can be transparently evaluated against other chemical inventories of regulatory interest and expanded where needed if chemical classes are outside the existing model domain

Please Note: The research being conducted on *in vitro* and QSAR methods was not sufficiently developed to be ready for use in the initial round of EPA EDSP priority setting (see http://www.epa.gov/scipoly/oscpendo/prioritysetting/approach.htm), but may be used in subsequent rounds of EDSP priority setting. Mention of a substance in the meeting summary or EPA presentations concerning the development of such methods does not mean the Agency has or will make a determination that any use of the substance will pose a significant risk. Further, this research is insufficient to allow EPA to draw any conclusions as to whether these substances are "endocrine disruptors"; the substances listed are simply compounds that have been or may prove to be useful in developing ranking and prioritization methods.

ANNEX 5: QUESTIONS ADDRESSED BY THE REVIEWERS

1) The estrogen receptor (ER) binding affinity expert system is based on *in vitro* data derived from methods for measuring ER binding affinity that are intended to be reflective of the chemicals in the regulatory domain of interest. Is this premise met? Please also comment on the applicability of the ER binding affinity methodology to determine a wide range of potential binding affinity for diverse chemicals with varying water solubility, Log Kow, etc.

2) In this ER expert system the effort was made to show the biological relevance of the binding data derived from the in vitro assays (and which provides the knowledge base for the ER expert system) in the context of a toxicity or adverse outcome pathway where ER binding is linked through a series of intermediate effects leading to reproductive impairment. Do you think that this expert system can be used to group chemicals into toxicologically meaningful groups and that within these groups it is possible to fill data gaps on reproductive toxicity by read-across or trend analysis?

3) It is intended that the ER expert system use a chemical hierarchy based on different binding mechanisms, which permits each estimated value to be accompanied by an explanation of the basis for the estimate as well as of how it compares to measured data for other members of the same subgroup. Do the expert system outputs clearly and effectively provide this explanatory information? Please make suggestions to improve the explanatory information and how to present it. Please comment on the strengths and limitations of this approach for enhancing transparency and documenting the overlap of model and regulatory domains.

4) The ER expert system provides predictions for each chemical, with each individual prediction traceable to chemical subgroups with individual binding mechanism and endpoint databases. Please comment on the clarity and transparency by which this information is provided in the expert system outputs. Please make suggestions for improvement.

5) The OECD validation principles include: a defined endpoint; an unambiguous algorithm; a mechanistic interpretation, a defined domain of model applicability; and appropriate measures of goodness of fit, robustness, and predictivity (see *http://www.oecd.org/dataoecd/33/37/37849783.pdf* and *http://www.oecd.org/dataoecd/55/35/38130292.pdf*). The previous questions have addressed specific scientific issues associated with these principles. Please comment here on the documentation of the ER expert system with respect to the OECD validation principles. Please provide suggestions, as appropriate, to enhance the clarity or transparency of the expert system documentation with regard to the principles.

6) The QSAR Model Reporting Format (QMRF) developed by the European Commission (see *http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/qrf/QMRF_version_1.2.pdf*) is envisioned to apply on many forms of statistical models and expert systems. Please comment on any critical elements of the proposed ER binding affinity expert system that cannot be adequately summarized by this reporting format.

ANNEX 6: REVIEW REPORT BY DR ONO

The review comments for the ER binding affinity expert system for the OECD (Q)SAR Toolbox

Atsushi Ono Ph.D.
Division of Risk Assessment,
National Institute of Health Sciences, Japan.

This report describes the review comments on the ER binding affinity expert system concerning the issues suggested to be addressed at the Expert Consultation.

1. Applicability domain of the estrogen receptor (ER) binding affinity expert system.

The EPA has concentrated to the 393 chemicals of inert ingredients in pesticides and the 211 of antimicrobial active ingredients as the target chemical domain for model development. Each structural query (rule) of the expert system was developed based on experimental data (binding assay) of representative chemicals from the each structural subgroup which was defined narrowly. Therefore, the developed query seems to be reliable within these class (sub-structure), though the detailed validation is necessary.

Although all structure which used for the model development is not shown, It seems that structures of almost chemicals which are included in the list are relatively simple according to structural classification in table 2. If risk assessors intend to apply this model to other chemical lists including diverse chemicals, the covered structure of the current expert system may be (too) restrictive.

As described in the document, the expert model can be expanded for the other sub-structures in same manner. However, it is not easy to classify the compound with a complex structure and build a reliable rule for each subgroup.

2. The biological relevance and utility in the grouping compounds.

The disrupting effect on the ER downstream gene transcription by the compound is one of the important causes of reproductive toxicity. It was shown that ER binding affinity is well correlated with transcription activity in many reports and also our analysis. Therefore, the ER binding affinity is useful information to fill data gaps on reproductive toxicity within structural subclasses.

Moreover, the endocrine system is strongly associated with the nervous system and the immune system in the homeostatic feedback system. Therefore, exogenous estrogens have potential to induce unexpected adverse effects through these feedback systems and some of which have been missed by traditional toxicological testing.

This expert system is useful for grouping chemicals not only on reproductive toxicity but also on such unexpected toxicological potentials.

3. Strengths and limitations of the expert system.

The decision tree is possible to provide clear explanatory information in prediction results for each subgroup and each compound. This is the strength of this approach compared to the other statistical approaches many of which include “black-box” in some parts of prediction for users.

In addition, the one of expectable features of this expert system is to define the criteria of negative compounds such as queries1, 2 and also Log Kow<1.3. Because this system intends to be used for regulatory purpose, the negative error must be prevented as much as possible. Thus, these negative selection queries must be validated more carefully. For example, acyclic chemicals are defined as non-binders in Query 1, however, it was reported that disulfiram (CAS No, 97-77-8) which does not contain a cyclic structure has affinity to ER of logRBA=-1.34 (*Toxicol In Vitro.* 2008 ;22(1):225-231).

It seems to be a limitation of this approach that it is not easy to build reliable rules to cover wide variety of structures.

4. The clarity and transparency of the expert system.

To keep the clarity and transparency, it is desirable that the system can automatically profile the subclass of chemicals even without the expertise to use. The output should be including full description of discriminate rules applied on the compound.

It is easy to understand that the prediction results shown in color figures of correlation with LogKow and comparison with the experimental data as well as existing toxicological knowledge of compounds including the same subclass, similar to the DNA Binding and Protein Binding of the (Q) SAR Toolbox.

5. The documentation of the ER expert system with respect to the OECD validation principles.

The consultation document sufficiently described the endpoint and algorithm. For mechanistic interpretation, the method and background data to distinguish A type and B type binders are not shown sufficiently. Moreover, the cited Katzenellenbogen's publication seems not to be appropriate to explain the A site and the B site of the ligand binding pocket of ER.

From the fact that the decision rules were defined for each structural subclass, the applicable substructure domain is clear. The primary target of this expert system was the EPA's list of pesticide inert ingredients; consequently this system is able to apply to most of structural subclass of compounds included in that list. However, the range of application on other chemical lists with regulatory interest (HPV etc.,) is unclear.

I could not find the description about the goodness of fit, robustness and predictivity of this system in this document. To confirm these, the validation test using the dataset independent from the dataset used for model development should be performed. I could not find the information about the external validation.

6. The critical elements of the ER expert system that cannot be adequately summarized by the QSAR Model Reporting Format (QMRF).

The QMRF required contents almost accord with the OECD validation principles. About the QC values (goodness of fit, robustness, and predictivity etc.,) of like this system based on multiple decision rules, the evaluation on the individual rule as well as on the whole model is useful like other statistical models. Thus, specific reporting format is expected for the expert system.

7. Other comments

In para.21, it is described that a single statistical (Q)SAR model including chemicals of different binding type is likely to give spurious results. However, the model based on interaction energy with the target receptor (i.e. 3D docking model) can treat chemicals with different interaction types in a single model.

QSARs are a developing technology, and many reliable training data are necessary to build the reliable model, recursive improvement and validation using new data is important for the future.

The review comments for the ER binding affinity expert system for the OECD (Q)SAR Toolbox

Atsushi Ono Ph.D.
Division of Risk Assessment,
National Institute of Health Sciences, Japan.

Expert consultation meeting of the ER expert system
17, Feb , 2009
OECD Headquarters, Paris

Issue 1. Applicability domain

Test structures for model development

Target chemicals

Total 604 discrete chemical structures
393 of the inert ingredients in pesticides(FI)
211 of antimicrobial active ingredients(AM)

Results from decision tree (Table 2)

30 (/604) chemicals were estimated to have ER binding activity.

2

Issue 1. Applicability domain

Test structures for model development

How many compounds were tested ER binding assay ?
How many compounds shown binding affinity to ER ?
Availability of the binding data (positive chemicals)?

Target chemicals

Total 604 discrete chemical structures
393 of the inert ingredients in pesticides(FI)
211 of antimicrobial active ingredients(AM)

Results from decision tree (Table 2)

30 (/604) chemicals were estimated to have ER binding activity.
Was the validation test (binding assay) done?
Positive/Negative error ?

3

Issue 1. Applicability domain

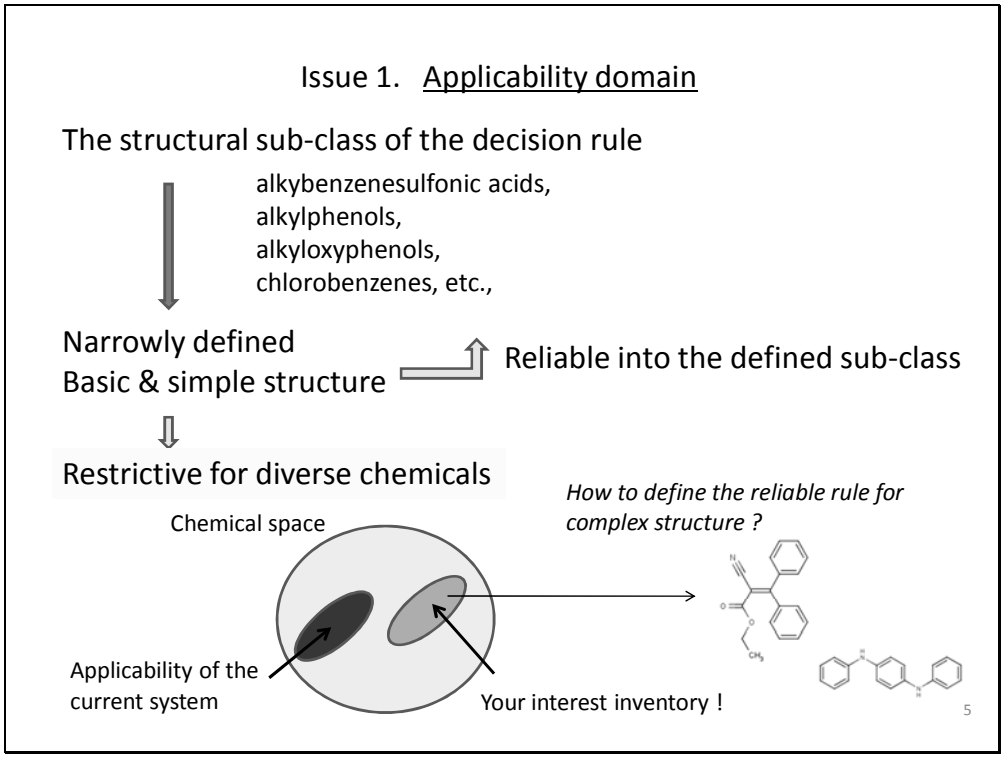
The structural sub-class of the decision rule

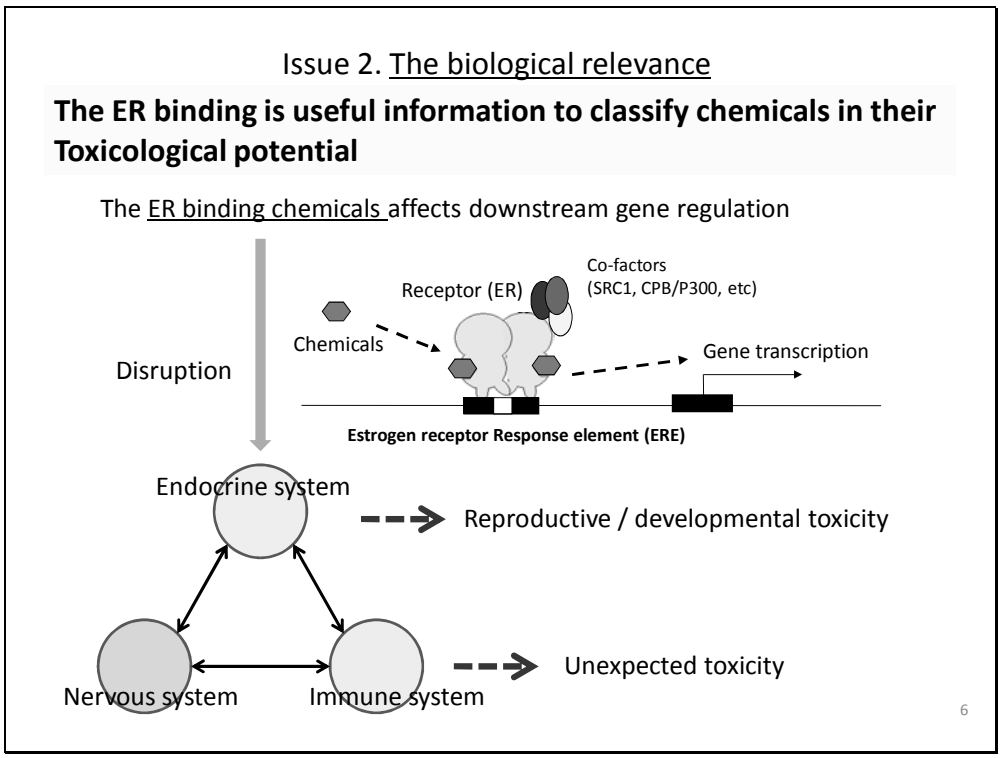
alkybenzenesulfonic acids,
alkylphenols,
alkyloxyphenols,
chlorobenzenes, etc.,

Narrowly defined
Basic & simple structure

Reliable into the defined sub-class

4


Issue 1. Applicability domain
The structural sub-class of the decision rule
alkybenzenesulfonic acids,
alkylphenols,
alkyloxyphenols,
chlorobenzenes, etc.,
Narrowly defined
Basic & simple structure
Reliable into the defined sub-class
Restrictive for diverse chemicals
How to define the reliable rule for complex structure ?
Chemical space
Applicability of the current system
Your interest inventory !
5

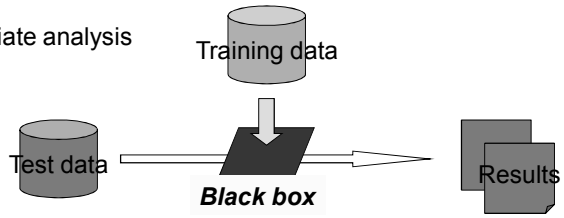

Issue 2. The biological relevance
The ER binding is useful information to classify chemicals in their Toxicological potential
The ER binding chemicals affects downstream gene regulation
Disruption
Receptor (ER)
Co-factors
(SRC1, CPB/P300, etc)
Chemicals
Gene transcription
Estrogen receptor Response element (ERE)
Endocrine system
Reproductive / developmental toxicity
Nervous system
Immune system
Unexpected toxicity
6

Issue 3. **Strengths** and limitations

The rules of decision tree based on sub-structure are descriptive.

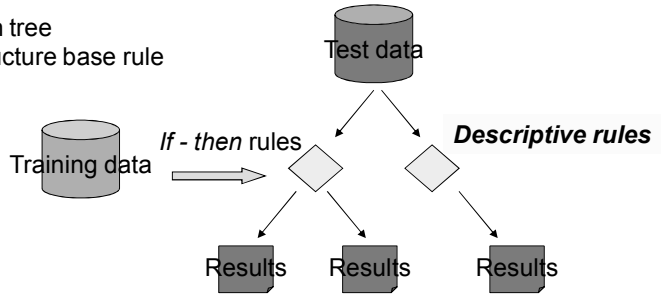
Statistical method

Descriptors base multivariate analysis
Pharmacophore
3D-docking model
etc.,



Expert system

Decision tree
Sub-structure base rule



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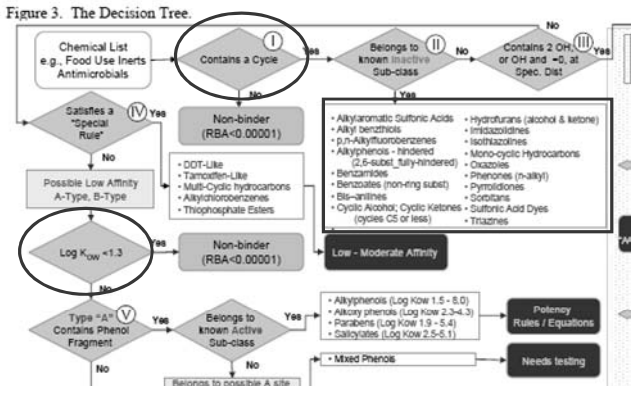
Issue 3. **Strengths** and limitations

The regulatory screening

Clear definition of negative compounds



Important and useful

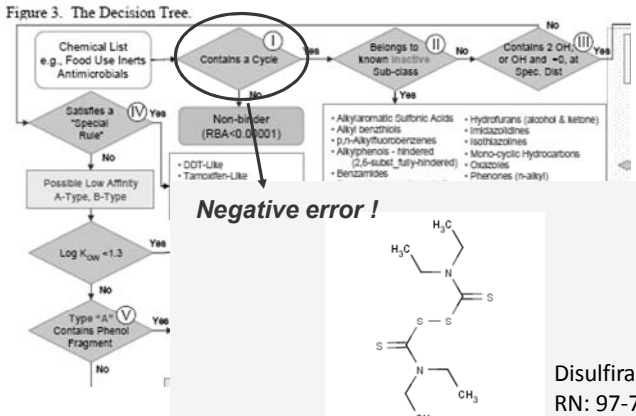
Careful validation is necessary

8

Issue 3. Strengths and **limitations**

The regulatory screening

Negative error is not allowable



Negative error !

Disulfiram
RN: 97-77-8

ER binding activity (Antagonized in uterotropic assay)
(*Toxicol In Vitro*. 2008 ;22(1):225-231).

9

Issue 4. The clarity and transparency

Suggestion;

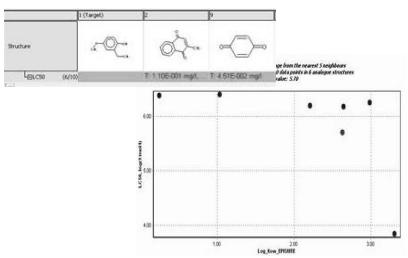
Automated chemical classification

allow same results for everybody

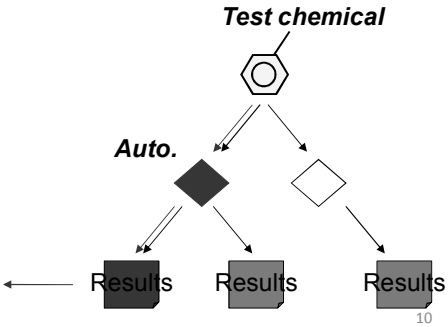
Full description of prediction

Comparative results with existing knowledge

check the reliability of result



Descriptive results



10

Issue 5. To the OECD validation principles.

Good !

Defined endpoint
- ER binding affinity

Unambiguous algorithm
- Expert system based on decision rules (tree)

Defined domain of model applicability
- Clearly defined applicable substructure

Require !

Mechanistic interpretation
- Lack of mechanistic information for distinguish between A-, B- type binder

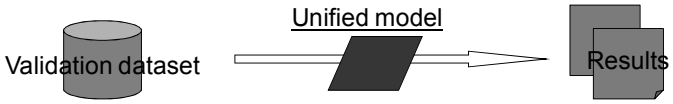
Appropriate measures of goodness of fit, robustness, and predictivity
- Lack of information of validation using external chemicals

11

Issue 6. To the EU – QMRF reporting.

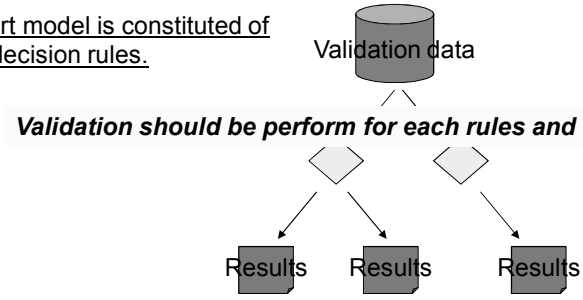
How to report (validate) the performance of the expert model

The models based on statistical multivariate analysis have single unified rule.



Validation should be perform for whole model and the goodness of fit, robustness, and predictivity are single results.

The expert model is constituted of multiple decision rules.

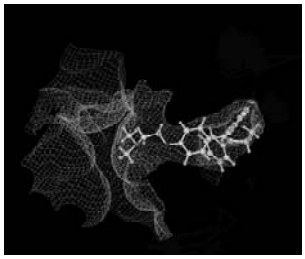


Validation should be perform for each rules and also whole model .

12

Other comment

3D docking model based on 3D structures of both ER ligand binding cavity and compound can be estimate variety of compounds in one model.



Ligand binding cavity of ER alpha

QSARs are developing (challenging) technology, and many reliable training data are necessary to build the reliable model, recursive improvement and validation using new data is important for the future.

The combination of different type of QSAR approaches in OECD Toolbox with the ER binding expert system would be useful for cluster chemicals into toxicological meaningful group.

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ANNEX 7: REVIEW REPORT BY DR COATS

A (Q)SAR Approach for Estimating Estrogen Receptor Binding Affinity

Reviewer:

Joel R. Coats, Ph.D.
Professor of Entomology & Toxicology
Iowa State University
Ames, Iowa, USA

Perspective of Reviewer

Research and Graduate Teaching

1. environmental toxicology & chemistry

2. insect toxicology: mode of action, QSAR

Primary focus: pesticides

Endocrine Disruption

1. Growing awareness – over the past 15 years

2. Many faces of "endocrine disruption"

3. Many approaches:
   a. observational
   b. experimental

Endocrine Disruption

4. Major regulatory challenges
   a. examples
   b. effects
   c. mechanisms
   d. expense of testing

5. Standardization needed
   a. testing methods
   b. QSAR-based approaches

(Q)SAR-Based Expert System

Specific Issues 1

Comment on the applicability of the ER binding affinity methodology to determine a wide range of potential binding affinity for diverse chemicals with varying water solubility, LogKow etc.

The expert system is applicable to many types of organic compounds, with wide variation in physicochemical properties. Constraints include some properties mentioned, e.g. limits of soubility in water, or others pertinent to the ER binding such as volatility, polymerization potential.

Specific Issues 2

Can this expert system be used to group chemicals into toxicologically meaningful groups?

It has the potential to prioritize chemicals for testing, by identifying those chemicals most likely to interact with the endocrine receptor (ER).

Specific Issues 2 (cont'd)

Is it possible to fill data gaps on reproductive toxicity by read-across or trend analysis?

Yes, data gaps can be filled with predicted capacity of compounds to interact with the ER and subsequently impact reproduction. It can render that missing information with a high probability of correctness (although not 100%).

Specific Issues 3

Do the expert system outputs clearly and effectively provide (mechanistic) explanatory information?

The decision tree provides considerable information on the logic/steps followed that produces a prediction. The mechanistic perspective is a strength of the system.

Specific Issues 3 (cont'd)

Please make suggestions to improve the explanatory in formation and how to present it.

More detail would be helpful at several steps in the decision tree, e.g. "contains 2OH or OH and =O at specific distance" or "contains some attenuating feature steric? other?"

Specific Issues 3 (cont'd)

Comment on strengths and weaknesses of the approach for enhancing transparency and documenting the overlap of model and regulatory domains.

Specific Issues 3 (cont'd)

Strengths:

1. **straightforward decision tree allows clarity of steps and outcome**
2. **model can be expanded to encompass more subgroups, to facilitate more overlap with a given regulatory domain.**

Specific Issues 3 (cont'd)

Limitations:

1. **Would be helpful to include more background on the definition of relative binding affinity and rationale for specific values of it that are deemed safe or not.**

2. **It is difficult to comprehend the number and types of chemicals in all possible regulatory domains, therefore degree of overlap is difficult to ascertain.**

Specific Issues 4

Comment on clarity and transparency by which this information is provided in the expert system outputs and make suggestions for improvement.

1. **If the output could include access to background references or summarized conclusions from those relevant references (liver slice paper, etc.), more value and perhaps more understanding of the decision tree could be realized.**

Specific Issues 4 (cont'd)

2. If metabolism concept (activation or detoxification) could be included in relation to the in vitro testing, it would provide an additional dimension to predictive capability.

Specific Issues 5

Please comment here on the documentation of the ER expert system with respect to the OECD validation principles and provide suggestions to enhance the clarity or transparency of the expert system documentation with regard to the principles.

Specific Issues 5 (cont'd)

1. **The ER binding endpoint is clearly explained. It is a simple assay and much data is available.**

2. **The algorithm is the decision tree, and it is unambiguous for the data set utilized. Predictions definitely are reproducible. The model should provide ample background data and/or references to support the categories and decisions in the expert system.**

Specific Issues 5 (cont'd)

3. **The applicability domain is clearly defined for the set of chemicals presented in the system. When a larger set of compounds is developed, with appropriate physicochemical properties, the domain will be expanded considerably. The mechanistic aspect should remain constant.**

Specific Issues 5 (cont'd)

4. The statistical measures, such as goodness of fit, are less important than predictivity for this (Q)SAR model. The decision tree should be highly predictive for ER binding, although this endpoint will not always precisely extrapolate to reproductive toxicity.

Specific Issues 6

Comment on any critical elements of the proposed ER binding affinity expert system that cannot be adequately summarized by this reporting format.

<u>Principle 2</u>

The algorithm is essentially the decision tree, for this expert system that is based on a (Q)SAR. Uncertain if only the ER binding algorithm would be appropriate to report (?)

Specific Issues 6 (cont'd)

Principal 3

The broader domain of applicability would be important in final model.

Principal 4

Predictivity can be evaluated through the use of an external validation set of chemicals. Goodness-of-fit can be measured by analysis of the ratios of false positives and false negatives. The validation of the model would be valuable, but expression of goodness-of-fit statistics may not be feasible.

Conclusions

1. **Very strong experimental bases for model:**
    a. **measured concentrations**
    b. **time frames**
    c. **activation**
    d. **detoxification**

Conclusions

2. Very strong mechanistic bases for model:
    a. Well understood binding assay
    b. Hydrogen bonding sites
    c. Vitellogenin mRNA

Conclusions

3. Expert system can be expanded for a broader domain of chemicals.

Conclusions

4. Limitation:

ER binding will not translate to reproductive toxicity with 100% predictability

Conclusions

5. Major Advantages:

Prioritizes chemicals for testing

- **saves time**
- **saves money**
- **saves animals**

Conclusions

6. Major Utility:

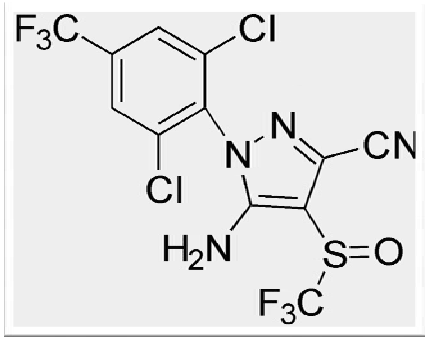
Serve probabilistic risk assessment by contributing rapid, inexpensive information to a weight-of-evidence approach.

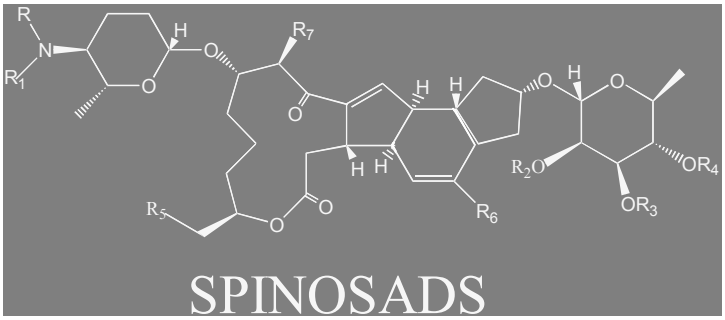
New Insecticides

Equal

New Challenges

Fipronil





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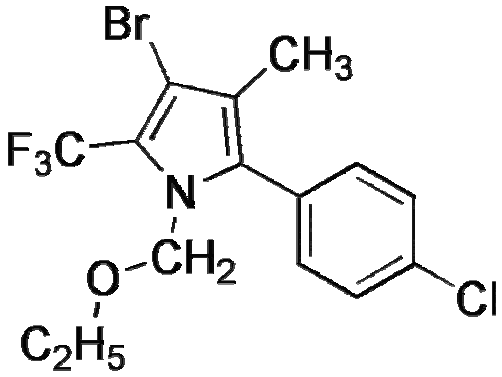
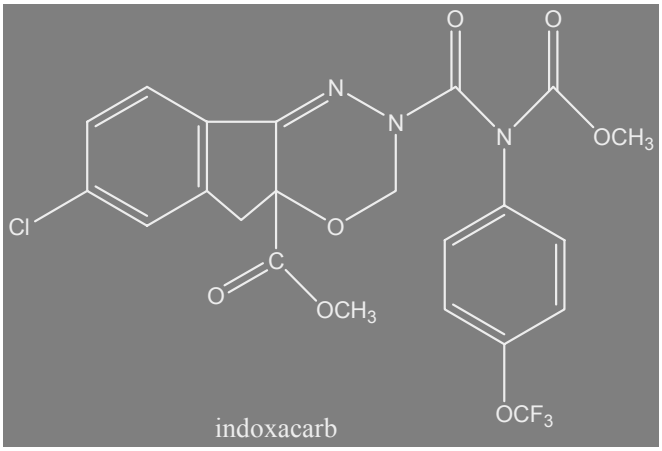
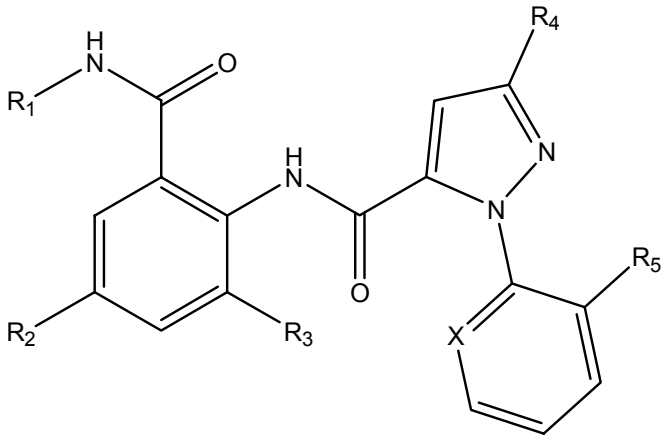
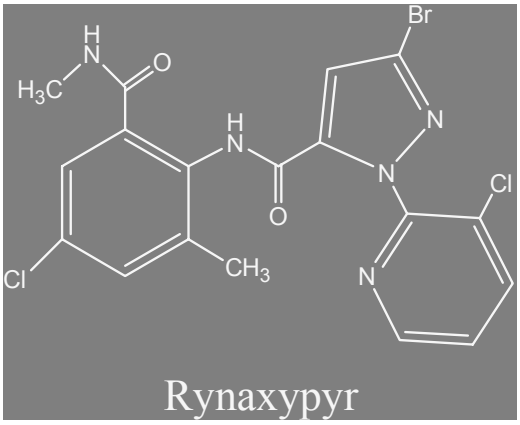


Indoxacarb

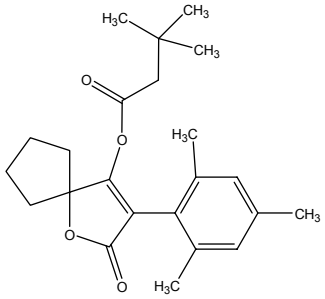




Anthranilic Diamides

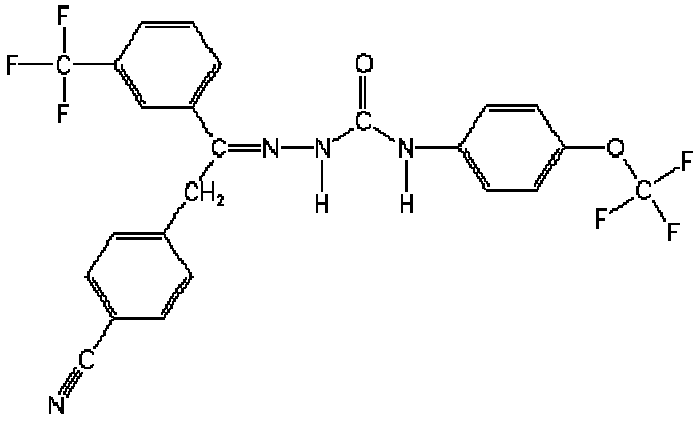


Rynaxypyr



Spiromesifen
a tetronic acid

Metaflumizone



ANNEX 8: REVIEW REPORT BY DR BONNELL

REVIEW OF THE PROPOSED OECD EXPERT SYSTEM FOR ESTROGEN BINDING AFFINITY

OECD ER Binding Workshop
February 17th 2009

Mark Bonnell, Michel Lortie, Kate Crump –
Ecological Assessment Division, Environment Canada
Tim Singer – New Substances Division, Health Canada

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Overview

- General impressions
- Answers to specific OECD questions 1-6
- Demonstration of decision tree using 5 suspected ER binders notified as new chemicals in Canada
- Additional comments

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Some Points From Regulatory Scientists

- ER binding as a “biomarker” or more subtle expression of the potential for adverse effects has not traditionally been used in hazard assessment (PBT) for priority setting or risk assessment
- Few regulatory scientists even understand the ER binding concept, especially on the ecological side
- Currently no regulatory actions have been taken in Canada solely based on endocrine disruption (although the weight of evidence for BPA pointed in that direction), traditional effects endpoints (e.g., NOEC) have been used for priority setting and risk assessment
- ER binding has been used by Environment Canada and Health Canada to request further ED screening tests (radio-labelled ER binding ligand test)

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Some Points From Regulatory Scientists

- Most likely would be used with other ED indicators or modes of action to prioritize chemicals or for risk assessment in a weight of evidence
- Foresee 2 main applications of the expert system:
  - Chemical grouping of inventories (e.g., DSL medium priorities)
  - Tier 1 information for determining endocrine disrupting potential (e.g., leads to request for further testing) on a chemical by chemical basis

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General Impressions

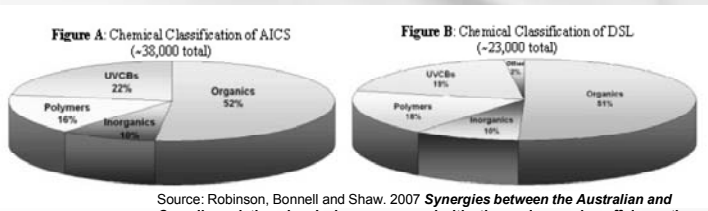
- Agree with the rule-based approach and logic tree proposed as opposed to the previous statistical approaches to ER binding based on stearic properties of a chemical alone
  - Environment Canada never felt confident that a result from previous ER binding models suggested endocrine disruption potential (tested on chemical based on model results which turned out negative)
- It seems that the proposed approach attempts to do the same as older ER binding models (e.g., specific distances of –OH groups, cyclic considerations) but provides more transparency and domain coverage to regulatory inventories

General Impressions

- Logk_{ow}s of acid dyes in training set (figure 2) seem to be too high (usually <1)
- The decision tree was relatively easy to flow and well laid out – will it be included in the in silico version ?
  - Suggest highlighting the decision pathway for transparency
- The use of a binary decision rule (i.e., RBA <0.00001 and RBA >0.00001) for outcomes is probably wise rather than specific bins of RBA results, given the inherent variability and extrapolation to hormonal effects in vivo
- Not sure how potency values are used to score RBA in the end

Question 1: Inventory Relevance

Q: Are the model's chemicals in the regulatory domain of interest and can they consider the diversity of chemical properties encountered in inventories ?



Source: Robinson, Bonnell and Shaw. 2007 ***Synergies between the Australian and Canadian existing chemicals programs: prioritisation and screening off domestic existing chemical inventories.*** SETAC World Congress, Sydney Australia.

A: Yes and No.

- For discrete existing chemicals there is greater coverage, but for >50% of new chemicals notified in Canada (and many existing chemicals as well) many are not discrete substances are of high molecular weight, low $\log k_{ow}$ and very low WS
- We expect the chemical and chemical property domain of the ER binding expert system to be fairly restricted when our regulatory inventory is considered
  - 23,000 existing substances
  - 15,000 new substances

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Question 2: Chemical Categories

Q: Can the expert system be used to create meaningful toxicological categories that can be used to fill data gaps for other like substances ?

A: Yes we agree that this is a useful approach to chemical grouping which could provide a defendable rationale for read-across purposes. The category created could also provide the basis for requesting further testing based on the "weight of evidence" and transparent rationale provided by the expert system

Note: ER Binding categories would also likely be sub-categorized by structure for regulatory purposes (structure + mechanism)

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Question 3: Expert System Output

Q: Does the expert system clearly and effectively provide needed explanatory information ?

A: In general yes, but it was difficult to speak in detail about the clarity and transparency of the outputs based upon the information provided. In theory, the explanation of the basis for the estimate and a comparison with measured data would be essential for regulatory purposes.

Suggestions:

- Incorporate Figure 3 into the in silico version
- Highlight the decision pathway in Figure 3 for a queried chemical
- Have text explanation of why a particular pathway was chosen
- Explain why a chemical ends up in a particular bin (in addition to Figure 3)
- Explain what RBA >0.00001 or RBA <0.00001 means from an endocrine disruption point of view as well as the qualitative confidence associated with the result
- Incorporate much of the ErB consultation document as a helpfile for the in silico version
- Can the USEPA OPP set of chemicals with ER binding results be provided in the in silico version ? (sometimes we like to use independent read-across lookup)
- Provide domain coverage (quantitative or qualitative domain coverage results)
- Provide the chemical domains for each mechanistic group
- Add Figure 1 from Schmeider et al. in helpfile for in silico version to show linkage to "observed effects"

Question 4: Clarity and Transparency

Q: Comment on the clarity and transparency of the expert system output

A: This question seemed to be redundant with Q3. Following Figure 3, the clarity and transparency of results is very good except the provision of endpoint databases. It was not easy to see how this is linked to the RBA result or to the decision tree.

Suggestions:

- Provide the specific chemical domains (i.e., CAS#s) for each subcategory from I-VI

Question 5: OECD Validation Principles

Q1: Is the endpoint well defined ?

A1: The endpoint is well defined (i.e., RBA) but mechanisms and causation are probably not as well understood by most regulators. So there is regulatory uncertainty as to how positive results from this endpoint are correlated to observed endocrine disrupting effects (i.e., is RBA really a big factor for endocrine disruption or are other hormonal disturbances more important)

- Also what is the level of false positives (variability)

Question 5: OECD Validation Principles

Q2: Is the algorithm unambiguous ?

A2: Figure 3 clearly explains the expert decision tree (algorithm) and grouping method, but it is not clear how the threshold of RBA 0.00001 was selected and why or what is means ?

- Suggest that a clear well written explanation of RBA and its thresholds be included in the in silico work up

A2: The same can be said of the RBA Types (e.g., A-B, A-C)

- Same suggestion as above

A2: Add animated decision flow to figure 3 for in silico workup

Question 5: OECD Validation Principles

Q3: Is the domain of applicability well defined ?

A3: While the domain of applicability is well defined for each category as the decision tree is followed, the overall DOA based on the training chemicals should be easily found by users.

Suggest:

- adding all chemicals from USEPA OPP as a training set with relevant physical and chemical properties (e.g., K_{ow}, WS, MW, maximum cross-sectional diameter)
- Add CAS# to subgroup domains
- Inform users of overall domain based on training set and relevant global parameters
- Give domain scores as with other models for:
  - Global parameters
  - Mechanistic
  - Structural
  - Metabolism ?

Question 5: OECD Validation Principles

Q4: Are appropriate measures of goodness-of–fit, robustness and predictivity included ?

A4: This could not easily be determined from the available information. However, it seemed that this type of statistical approach is not as relevant for the expert system which is largely based on observed results from tests for chemical classes and expert rules. In fact, this “weight of evidence approach” is preferable compared with a statistical design (statistical relevance vs. biological relevance)

Suggest:

- basing predicitivity on whether a substance is correctly categorized, not on whether RBA >0.00001 = an EDC (this is too uncertain and maybe the result of more than one hormonal mechanism)
- Summary statistics may be needed for validation using external training sets

Question 5: OECD Validation Principles

Q5: Does the expert system provide a mechanistic interpretation ?

A4: Yes, although this could not easily be determined from the available information and depends on what is to be included in the in silico workup. However, it is essential that it be included in the expert system as a helpfile for each subcategory because of the potential need by regulators to understand and relay the rationale for groupings or for using the RBA for requesting further testing

Suggest:

- Clearly explaining RBA types and basis ER Binding mechanism

Question 6: QSAR Model Reporting Format (QMRF)

Q: Comment on critical QMRF components that cannot be addressed by the expert system

A: Some QMRF items that may be difficult to address include:

- Some goodness-of fit and robustness measures (e.g., 6.6, 6.7, 6.10, 6.11, etc)
- Some predicitivity measures (availability of an external training set ?)

Otherwise the QMRF can be applied to the expert system as if a QSAR

TEST CASES

5 New Substances Notified under the Canadian New Substance Notification Regulations (NSNR)

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Substance A Description

- MW = 288
- WS = 28 mg/L (pH 3.4)
- Logkow = 3.4 (pH 2.0)
- Functional Groups: Benzyl, Carboxylic acid, Methylene, Phenol

- Current OECD Toolbox RBA Result: very strong binder, OH
- LMC RBA model ca. 2006 result: very strong binder
- LMC RBA model ca. 2009 result: unsure

- EC/HC requested testing of this compound using reporter gene assay: no agonist or antagonist effects

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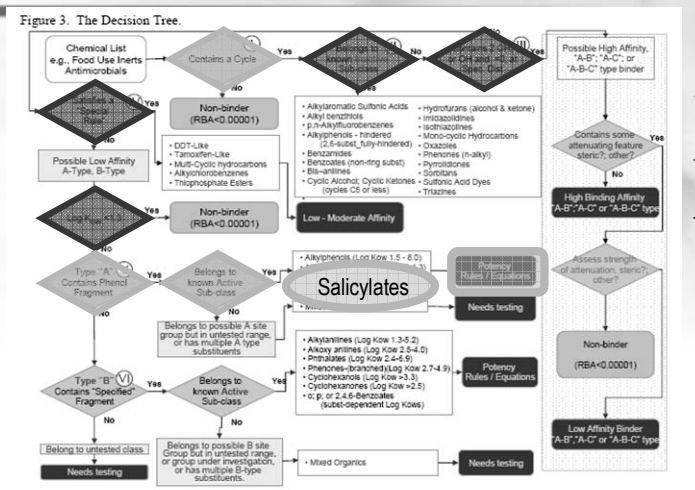
Substance A

Categorization: Phenols
Functional Groups: Benzyl, Carboxylic acid, Methylene, Phenol



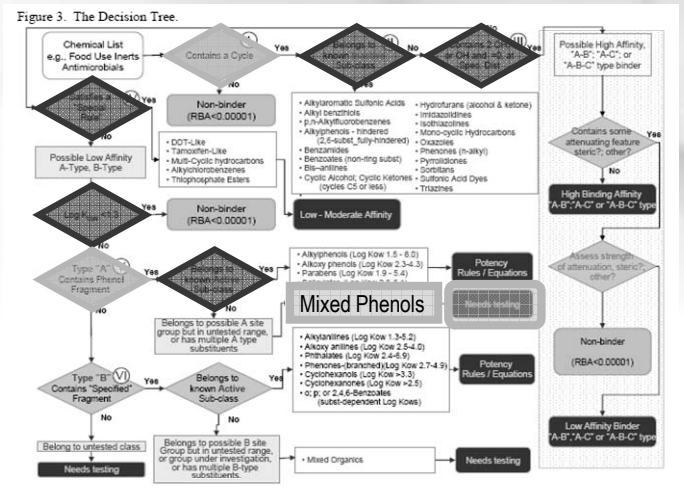
***In vitro* testing** – no ER agonist or antagonist activity

Substance B Description

- MW = 485
- WS = <0.0352 mg/L
- Logkow >4.2 (5.7 by KOWWIN)
- Functional Groups: Alcohol, Arene, Ether, Ketone, Methylene, Phenol
- Current OECD Toolbox RBA Result: very strong binder, OH
- LMC RBA model ca. 2009 result: RBA >10% (strong)

Substance B

Categorization: Phenols
Functional Groups: Alcohol, Arene, Ether, Ketone, Methylene, Phenol



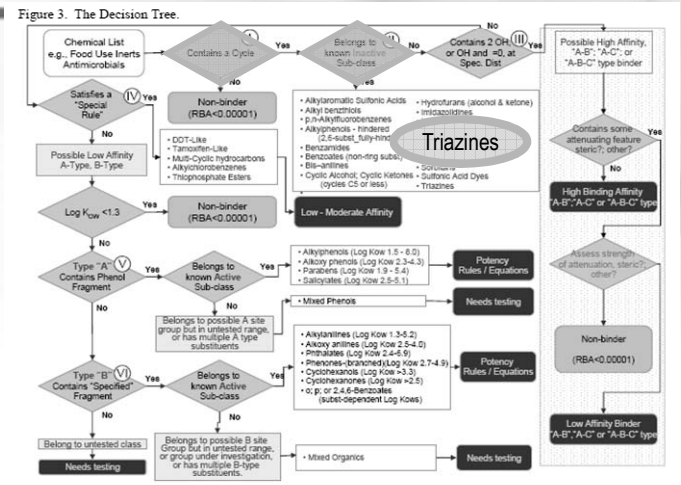
Substance C Description

- MW = 630
- WS = <0.046 mg/L
- Logkow >4.6 (KOWWIN = 7.6)
- Functional Groups: Arene, Ether, Methyl, Methylene, Phenol
- Current OECD Toolbox RBA Result: non-binder, MW > 500
- LMC RBA model ca. 2009 result: RBA >10% (strong)

Substance C Option1:

Categorization: Phenols, Triazines
Functional Groups: Arene, Ether, Methyl, Methylene, Phenol

Figure 3. The Decision Tree.



Mixed Functionality ?

Option 1: Triazine >> Phenols

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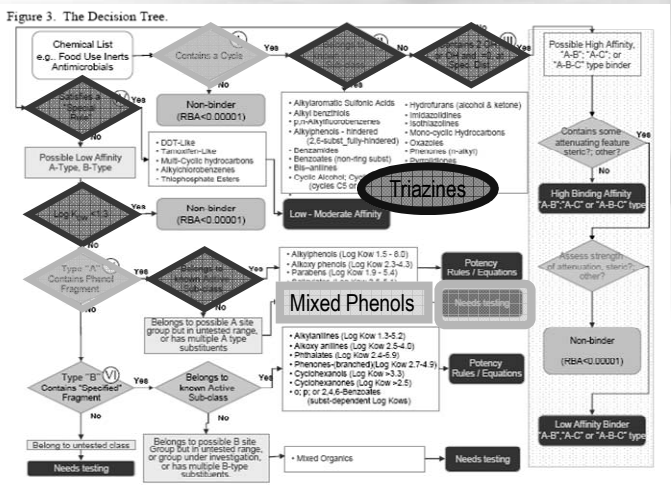
Substance C: Option 2

Categorization: Phenols, Triazines
Functional Groups: Arene, Ether, Methyl, Methylene, Phenol

Figure 3. The Decision Tree.



Option 2: Phenols >> Triazine

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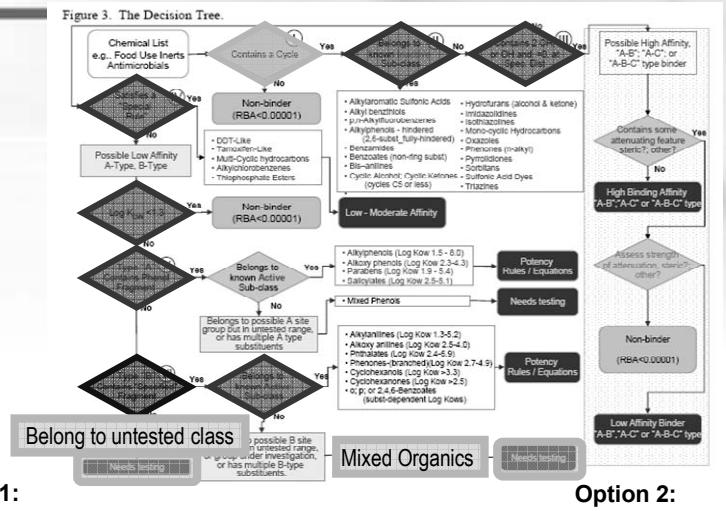
Substance D Description

- MW = 601
- WS = 0.03 mg/L
- Logkow = 6.0
- Functional Groups: Arene, Epoxide, Ether, Methyl, Methylene
- Current OECD Toolbox RBA Result: non-binder, MW > 500
- LMC RBA model ca. 2009 result: 0.1<RBA<10% (moderate)

Substance D

Categorization: Epoxides, Neutral Organics
Functional Groups: Arene, Epoxide, Ether, Methyl, Methylene



Option 1:
Ether ≠ "Specified" Fragment

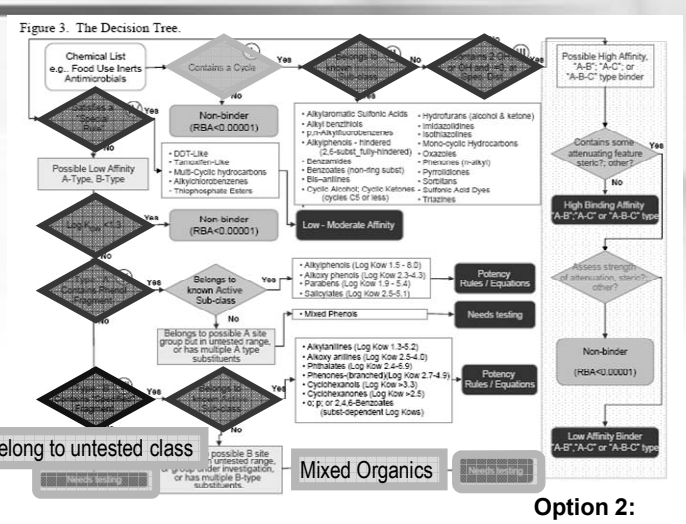
Option 2:
Ether = "Specified" Fragment

Substance E Description

- MW = 370
- WS = “negligible” (MSDS); 48 mg/L (WSKOWIN)
- Logkow = 2.9 (KOWWIN)
- Functional Groups: Alcohol, Arene, Epoxide, Ether, Methyl, Methylene
- Current OECD Toolbox RBA Result: non-binder, no OH or NH2 group
- LMC RBA model ca. 2009 result: 0.1<RBA<10% (moderate)

Substance E

Categorization: Epoxides
Functional Groups: Alcohol, Arene, Epoxide, Ether, Methyl, Methylene



Option 1:
Ether ≠ “Specified” Fragment

Option 2:
Ether = “Specified” Fragment

Summary of Test Case Results

- 5 substances which were predicted to have estrogen receptor binding affinity using other approaches - 2 could be clearly categorized based on the new expert system
- For 3 of the 5 test cases it was not clear as to where in the decision tree they would fall.
- Substance C has a triazine functionality, but also has phenols. Based on the training set, four triazine compounds were tested, but none are combined with single phenol moiety. It is unclear at this point with only the ER Consultation Document if substance C should fall in the triazine – not active category or the mixed phenols needs testing category.
- The expert system is not clear on the extent of the specified fragments with regards to ethers (Substance D and E). If they are part of the specified fragments, it would fall under mixed organics – needs testing, if not, then it would fall under untested class – needs testing. In this case, both result in the same recommendation, but the expert system does not appear clear (again based on the ER Consultation Document only).

Other Points

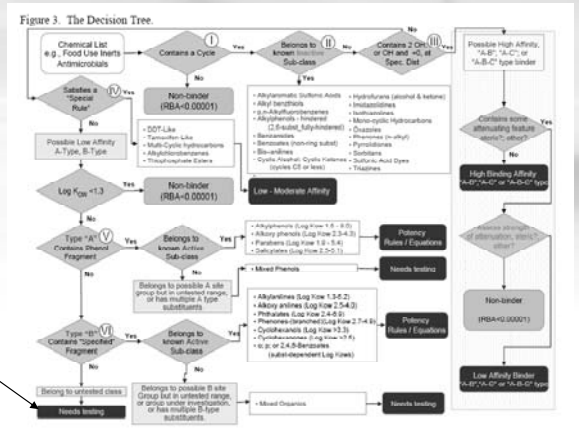
18. "*A more complex question, i.e., whether all ER binding chemicals regardless of measured potency, produce adverse effects in whole organisms or populations, is an important risk assessment question but is not a goal of the prioritization efforts*"

- However, this is a very important point to EC/HC as we are now conducting risk assessments, but still have the prioritization of 2600 "medium priorities" to accomplish
- So begs the question: What is the goal of the expert system ?

20. …"*Research in progress is evaluating the extent to which chemicals with very low apparent ER binding affinities (e.g., five to six orders of magnitude less than that of estradiol) can cause adverse effects in fish in vivo…".*

- This has been a major question for EC/HC risk assessors – so the scale up to in vivo becomes very important for the domain coverage

Other Points



COULD THIS LEAD TO TOO MANY CHEMCIALS SUGGESTED FOR TESTING ?

(I.E., MANY CYCLIC NEUTRAL ORGANICS, NO ALERT GROUPS WITH LOGKOW >1.3 ?)

Final Thoughts

- We see the application of the expert system for prioritization using categories as well as single chemical risk assessment
- The grouping of chemicals using the proposed expert system looks promising and we feel is the most transparent system to date with the best mechanistic explanation
- The subcategories provide a “reference chemical approach” that could provide regulators with the necessary “weight” to request further testing on a chemical by chemical basis
- The only way to truly assess the ability of this model to predict ER binding is to conduct a 'validation' study, where chemicals from across the diverse regulatory "chemical space" are selected randomly and, in parallel, tested experimentally and run through the decision tree
- Good quality training set – can it be expanded ?

Acknowledgements

Nils Sundin of the Ecological Assessment Division re-ran the LMC RBA model predictions for this presentation