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Fundamental And Guiding Principles For (Q)SAR Analysis Of Chemical Carcinogens with Mechanistic Considerations

**Series on Testing and Assessment
No. 229**

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OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 224

**REPORT OF THE WORKSHOP ON A FRAMEWORK FOR THE DEVELOPMENT AND USE
OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT**

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2015

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FOREWORD

In this document, prepared by US EPA and Health Canada, the importance of mechanistic consideration in (Q)SAR analysis, the critical role of mechanistic consideration in improving various (Q)SAR approaches and possible integrative approaches of combining chemoinformatics and bioinformatics are discussed, mainly using carcinogenicity as an illustrative toxicity endpoint. The principles and issues described in this document are general and may also be used for various types of chemical assessment. This document builds upon the recently published NAFTA (Q)SAR Guidance Document (NAFTA_TWG, 2012) for hazard/risk assessment of pesticides with expansion to cover industrial chemicals and added focus on mechanistic considerations. The intended target of readership is for global chemical hazard/risk assessors in regulatory agencies, industries, non-governmental organization and academia who require reliable and scientifically supportable (Q)SAR information and predictions in their assessments as well as for developers/researchers who endeavour to produce scientifically reliable (Q)SAR predictive models and tools. This document is not intended to be a full-scale, detailed guidance document. Its principal goal is to encourage users of (Q)SAR to keep mechanistic considerations in mind when conducting (Q)SAR analysis.

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

“QSAR is based on well-defined endpoints of intrinsic chemical activities and molecular descriptors, which can be mechanistically interpreted. Chemicals in a QSAR training set ought to have a common mechanism of interaction so that the context of structural requirements defining the domain can be articulated and tested. Finally the estimation of complex endpoints ought to be controlled by a QSAR-based expert system if the estimation of missing values or hazard screening in heterogeneous inventories is to avoid fueling the skepticism of QSAR” –

G. Veith (2004)

TABLE OF CONTENTS

FOREWORD.....	6
1. INTRODUCTION.....	10
.....	11
2. GENERAL PRINCIPLES AND KEY ELEMENTS OF MECHANISM-BASED (Q)SAR ANALYSIS OF CARCINOGENS.....	12
2.1 Toxicokinetics and toxicodynamics as the key components of mechanism-based (Q)SAR analysis..	13
2.1.1. Toxicokinetics considerations:	16
2.1.2. Toxicodynamics considerations:	17
3. IMPORTANCE OF MECHANISTIC CONSIDERATION IN (Q)SAR ANALYSIS AND HAZARD / RISK ASSESSMENT	21
3.1 Importance of mechanistic consideration in (Q)SAR analysis	21
3.2 Importance of mechanistic consideration in hazard/risk assessment communities and stakeholders..	22
4. OECD PRINCIPLES FOR VALIDATION OF (Q)SARS: A BRIEF REVISIT FROM THE MECHANISTIC POINT OF VIEW.....	24
5. ROLE AND IMPORTANCE OF MECHANISTIC CONSIDERATION / UNDERSTANDING IN THE DEVELOPMENT, ASSESSMENT, AND USE OF (Q)SAR APPROACHES.....	27
5.1 Chemical / structural class approach.....	27
5.2 Structural alerts approach	27
5.2.1 Overview of structural alerts:	27
5.2.2 Genotoxic structural alerts:	28
5.2.3 Nongenotoxic structural alerts:	28
5.3 Category / Grouping approach.....	30
5.4 Analog identification / comparison.....	31
5.5 Read across	33
5.6 QSAR equations.....	35
5.7 Combining multiple (Q)SAR models/approaches	36
6. SPECIFIC MECHANISTIC CONSIDERATIONS FOR (Q)SAR ANALYSIS OF DIFFERENT CLASSES OF CARCINOGENS	37
6.1 Direct-acting genotoxic carcinogens.....	37
6.2 Indirect-acting genotoxic carcinogens	38
6.3 Nongenotoxic carcinogens.....	39
7. INTEGRATING CHEMOINFORMATICS, BIOINFORMATICS AND SHORT-TERM PREDICTIVE ASSAYS IN MECHANISM-BASED (Q)SAR ANALYSIS OF CARCINOGENICITY	42
7.1 Short-term biological tests providing mechanistic information for (Q)SAR analysis of carcinogens.	42
7.1.1 Genotoxicity assays - Perspective on their use to pre-screen potential carcinogens	42
7.1.2 Type/Quality of DNA adduct data	44

7.1.3 Cell transformation assays (CTAs)	45
7.1.4 Toxicogenomics	46
7.2 Integrative approaches and tools.....	47
7.2.1 OncoLogic Functional Arm as an example of a conceptual integrative approach specific to carcinogenicity analysis	47
7.2.2 OECD QSAR Toolbox – a simple tool allowing complex analyses	48
7.2.3 ToxCast and ‘Hallmarks of Cancer’ approach	51
7.2.4 Mode of Action (MOA)/Human Relevance (HR) and Adverse Outcome Pathways (AOP)	51
7.2.5 Strategic testing using a combined in silico + in vitro approach.....	52
8. BRIEF OVERVIEW OF MECHANISTIC (Q)SAR ANALYSIS OF OTHER TOXICITY ENDPOINTS	56
8.1 Skin sensitization	56
8.2 Endocrine disruption – Estrogen Receptor (ER) Binding.....	57
8.3 Neurotoxicity	58
8.4 Developmental toxicity.....	59
9. CONCLUDING REMARKS AND FUTURE PERSPECTIVES	61
REFERENCES	63
APPENDIX	78

1. INTRODUCTION

Qualitative and quantitative structure activity relationship analysis—(Q)SAR---the study of the relationship between chemical structure and biological properties of substances---has long been used by researchers in academia and industries to develop new products or therapeutic agents with desirable properties. Since the passage of Toxic Substances Control Act (TSCA) in United States in 1976, (Q)SAR has been increasingly used in hazard/risk assessment, toxicity screening, and testing prioritization of new and existing industrial/environmental substances by the U.S. Environmental Protection Agency (US EPA) and by regulatory agencies throughout the world. Under the Government of Canada's Chemicals Management Plan, the commitment to address a large number of substances, many with limited data, has highlighted the importance of pursuing alternative hazard assessment methodologies including (Q)SAR that are able to accommodate substances with varying toxicological information. (Q)SAR is now also an indispensable tool for a variety of other activities such as drug development, design of safer substances, green chemistry, assessment of toxicity potentials of cosmetics products without animal testing, and the substance regulatory compliance under European Union's (EU) programs such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the CLP (Classification, Labelling and Packaging) or the BPR (Biocidal Product Regulation).

There are basically two approaches to (Q)SAR analysis—statistical and mechanistic. Statistical approach mainly focuses on statistical association between toxicity and structural fragments or physicochemical properties whereas mechanistic approach mainly uses mechanism of action and supporting short-term and/or medium-term predictive assays as a guide to develop (Q)SAR analysis and discover molecular features that may contribute to toxicity. Most experienced (Q)SAR researchers use both approaches interchangeably depending on their experience and available data/knowledge. Ideally, (Q)SAR studies should attempt to achieve statistical association with mechanistic backing but the ideal is subject to availability of relevant data/knowledge and supportive information for analyzing targeted chemicals in question. In the past decade, the chemistry- and statistically-based methods have made significant progress as a result of greater availability of computational and predictive technologies. However, the ongoing reduction in the number of cancer bioassays and other toxicity studies, due to economical and ethical reasons, will lead to a shrinking supply of robust animal toxicity data needed for statistical analysis. On the other hand, there has been a tremendous increase in mechanistic studies to investigate the molecular initiating event (MIE), mode of action (MOA), and adverse outcome pathway (AOP) of various chemically induced toxicity endpoints in recent years. Knowledge of mechanism can greatly improve (Q)SAR studies by proper classification/grouping of substances, selection of most appropriate molecular descriptors, increasing reliability/confidence in (Q)SAR study and helping to design goal-oriented confirmatory studies. At the same time, the development of various predictive assays, toxicogenomics, high throughput screening (HTS) assays, Tox21 and ToxCast projects can provide a wealth of bioinformatics inputs that can complement chemoinformatics-based (Q)SAR studies for ideal integrative assessment.

In this document, the importance of mechanistic consideration in (Q)SAR analysis, the critical role of mechanistic consideration in improving various (Q)SAR approaches and possible integrative approaches of combining chemoinformatics and bioinformatics are discussed, mainly using carcinogenicity as an illustrative toxicity endpoint. The principles and issues described in this document are general and

may also be used for various types of chemical assessment. This document builds upon the recently published NAFTA (Q)SAR Guidance Document (NAFTA_TWG, 2012) for hazard/risk assessment of pesticides with expansion to cover industrial substances and added focus on mechanistic considerations. The intended target of readership is for global chemical hazard/risk assessors---in regulatory agencies, industries, non-governmental organization and academia---who require reliable and scientifically supportable (Q)SAR information and predictions in their assessments as well as for developers/researchers who endeavor to produce scientifically reliable (Q)SAR predictive models and tools. This document is not intended to be a full-scale, detailed guidance document; its principal goal is to encourage users of (Q)SAR to keep mechanistic considerations in mind when conducting (Q)SAR analysis.

2. GENERAL PRINCIPLES AND KEY ELEMENTS OF MECHANISM-BASED (Q)SAR ANALYSIS OF CARCINOGENS

The ability to predict or assess the toxicity potential of substances is greatly dependent on the mechanistic complexity of the toxicity endpoints. In general, endpoints with relatively simpler key molecular initiating mechanisms (e.g., genotoxicity via DNA binding, sensitization via protein binding) can be predicted/assessed more easily than those with complex mechanisms (e.g., carcinogenicity, neurotoxicity, teratogenicity). In this guidance document, carcinogenicity will be used to illustrate this point. It is well documented that chemical carcinogenesis is a multi-stage, multi-factorial process, which conceptually consists of three operational stages---initiation, promotion, and progression, each with diverse underlying mechanisms. Initiation involves a mutational event that may include gene mutation or chromosome aberration/instability. Promotion involves clonal expansion of initiated cells by a variety of means such as cell proliferation, inhibition of programmed cell death, persistent chronic inflammation, inhibition of terminal differentiation, and loss of growth control. Progression may involve a second mutational event, the loss of tumor suppressor gene, impairment of immune surveillance, acquisition of increased blood supply via angiogenesis and ability to metastasize. As a result of the significantly differing mechanisms of these three stages, the key molecular descriptors suitable for use in (Q)SAR prediction of potential activities in these three stages differ accordingly (Table I). To be a complete carcinogen, a substance must be able to trigger, either directly or indirectly, activity in all three stages of the process. The relative importance of the substance's contribution to each of these three stages differs from substance to substance. Generally, the genotoxicity and carcinogenicity based (Q)SAR models are built using toxicological data obtained from studies that are not specifically designed for the purpose of model development. Moreover, the choice of descriptors in statistical model algorithms is often based more on achieving best statistical performance than on evidence of direct correlation with specific mechanisms of action for the various stages of carcinogenicity as described in Table I.

Similar to the interpretation of experimental results, predictions from a (Q)SAR model on a mammalian toxicological endpoint should be interpreted in relation to whether the underlying pathways are likely to be relevant to humans. Moreover, as far as possible, and again similarly to the interpretation of experimental results, differences in metabolic alterations and/or key events in the pathways that lead to the final adverse outcome, should be taken into account. If enough information exists from experience in humans, one strength of (Q)SAR models employing human training set data over the animal "model"/test data is that such (Q)SAR models make predictions directly of the human hazard endpoint (Adverse Outcome). Frequently, the model builders fail to consider among other factors, the human relevance of pathways underlying mammalian toxicological findings, explicitly accounting for metabolic alterations and/or key events in the pathways that lead to the final adverse outcome as well as kinetics. Model algorithms are designed to identify correlation of molecular structure of a substance and/or its physicochemical parameters (relating to absorption / bioavailability / reactivity) with the adverse outcome and tend to ignore the role of concentration/dose or the various steps leading to the effect. Based on the *predominant* mechanism of action, carcinogens may be loosely classified as genotoxic and epigenetic / nongenotoxic. Genotoxic carcinogens, also known as DNA-reactive carcinogens, generally are substances that directly interact with DNA either as parent substances or as reactive metabolites to form DNA adducts which, if unrepaired, may initiate carcinogenesis. Epigenetic / nongenotoxic carcinogens are agents that mainly act on the promotion and progression stage and cause indirect DNA damage without binding to DNA. The (Q)SAR approaches of genotoxic and nongenotoxic carcinogens should therefore be quite different.

In essence, mechanism-based (Q)SAR studies use known or likely general or class specific or receptor-specific mechanism of action as a guide to determine and develop the best molecular descriptors and structural features to include in the model development exercise. If a hypothesized mechanism is used, then the hypothesis should be stated. To the extent possible, available biological/functional information and short-term predictive tests should also be used to support or complement the structural input. One significant advantage of mechanism-based (Q)SAR studies is that they can be done with relatively few substances without any statistical analysis if a clear mechanism can be reasonably well defined.

Table I: Three stages of carcinogenesis and their mechanistic consideration and descriptors

	Initiation	Promotion	Progression
Main event(s)	Direct DNA binding Indirect DNA damage	Clonal expansion via: ↑ cell proliferation ↓ apoptosis ↓ differentiation ↑↓ homeostasis ↑↓ gene expression	Overcoming suppression & enabling immortality (e.g., immunosuppression, <i>p53</i> , angiogenesis, etc.)
Key mechanistic considerations	Electrophile, resonance stabilization, DNA adduct, reactive oxygen species, etc.	Receptor, cytotoxicity, gene expression, etc.	Free radical, receptor, gene suppression, chromosome instability, immunosuppression, etc.
		Signal transduction, homeostasis, etc.	
(Q)SAR molecular descriptors	Electrophilicity, HOMO/LUMO, delocalization, ...	Molecular size/shape, docking, biopersistence, DNA methylation, hormonal antagonists/agonists/precursors,...	Reduction potential, DNA methylation, ...

2.1 Toxicokinetics and toxicodynamics as the key components of mechanism-based (Q)SAR analysis

Conceptually, there are two key components that must be carefully considered in mechanism-based (Q)SAR: toxicokinetics / pharmacokinetics and toxicodynamics / pharmacodynamics (Figure 1). The toxicokinetics step basically determines the likelihood that the substance and/or its ultimate toxicant(s) can reach the target organ(s) where the toxicodynamics step takes place to carry out the molecular initiating event(s) of the toxic action.

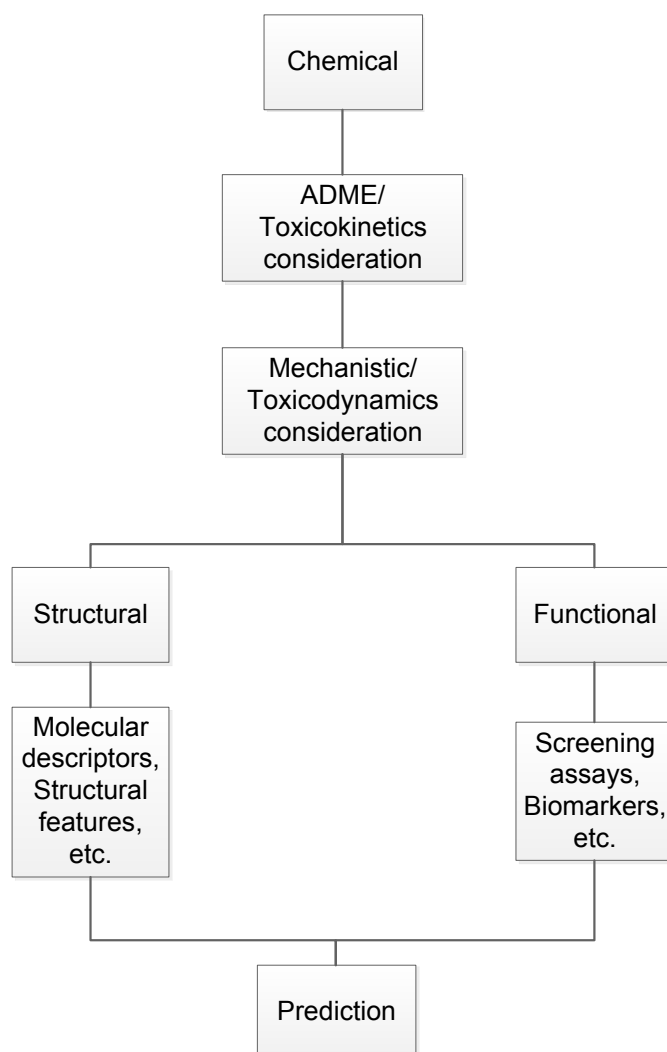
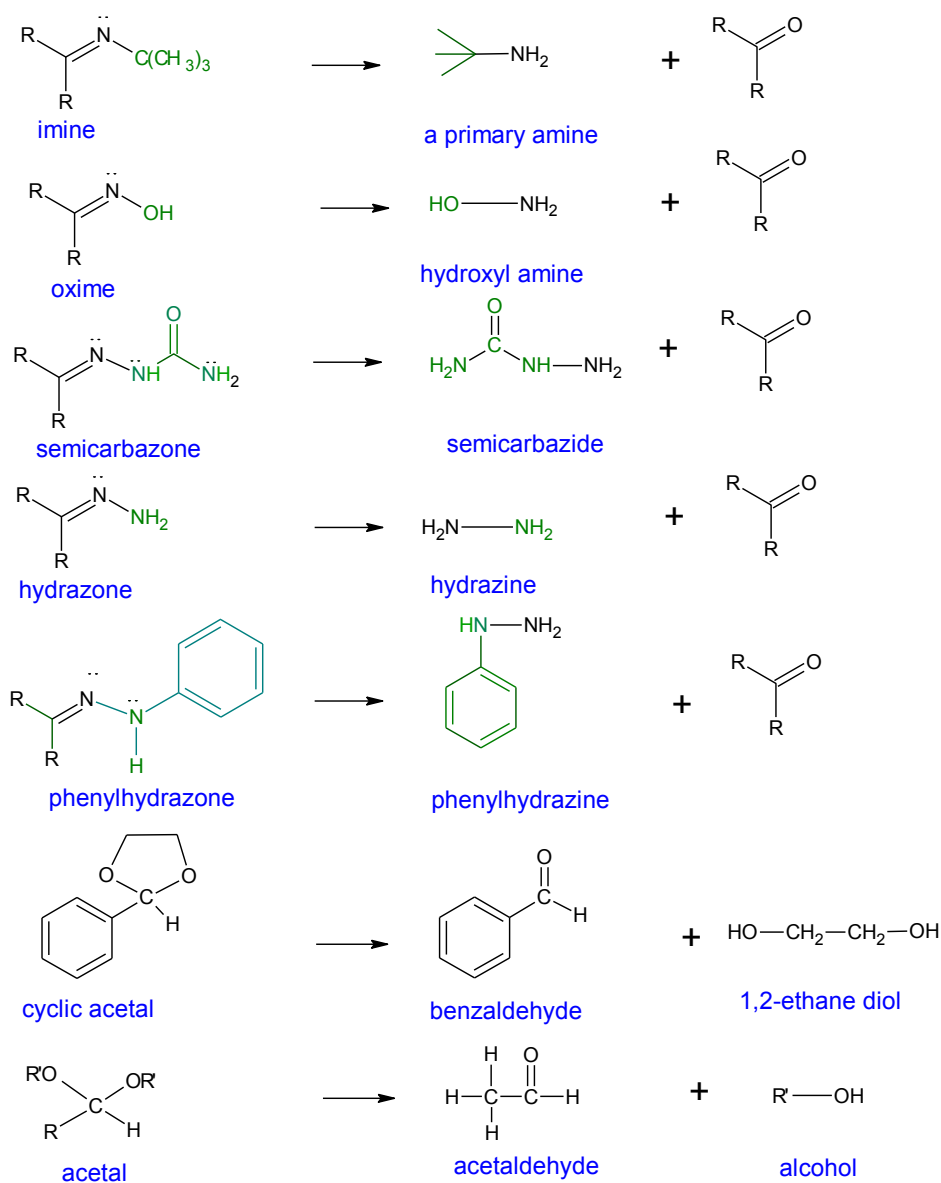
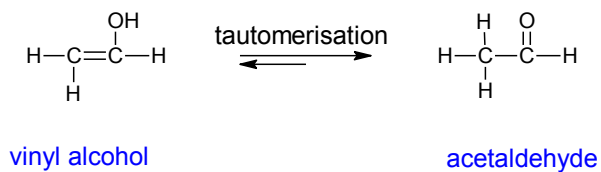


Figure 1: Key components of mechanism-based (Q)SAR

The toxicokinetics component can be assessed/hypothesized by examining the substance's physicochemical properties (e.g., MW, solubility, pKa, logP, vapor pressure, particle size, stability/reactivity), assessing the role of route of expected/potential exposure on reactive chemicals to reach target organs (see Sections 2.1.1 and 6), identifying structural features that may be conducive to hydrolysis (see Figure 2(A)) or tautomerization (see Figure 2(B)); see also OECD QSAR Toolbox) to yield reactive chemicals, and potential metabolic activation/detoxification (see Section 6.2; see also Parkinson and Ogilvie, 2008; Shen, 2008; Klaunig, 2013). Comparison with structurally and mechanistically related analogs can often provide important (Q)SAR clues; minor changes at or around reactive sites or detoxification sites can have major effects on activation/detoxification (see Section 6.2). If available, software developed for assessing absorption, distribution, metabolism, excretion (ADME) or models for physiologically based pharmacokinetics (PBPK) or toxicokinetics (PBTk) should be used to support available data.



(A)



(B)

Figure 2: Examples of structural features that may generate reactive intermediates via (A) acid hydrolysis or (B) tautomerization.

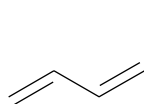
2.1.1. Toxicokinetics considerations:

One of the simplest and most effective toxicokinetics assessments of a substance is its “bioavailability”. Substances that have very high MW, logP, and/ or molecular size and which have very low water solubility or solubility in relevant human liquids (e.g. stomach or lung fluid) and/or have large particle size which may not be inhaled into deeper lung tissues are often considered “not significantly bioavailable/ systemically available” for a particular relevant route of administration. However, it should be cautioned that under some scenarios (e.g., lung overload with inhaled high MW polymer, ingestion of polysulfated polymers), some of these “not bioavailable” substances may still be of cancer concern.

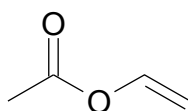
The route of exposure/administration is also particularly important for direct-acting reactive chemical carcinogens with short half-life in the body because they can have far greater toxic effects at the portal of entry (e.g., nasal cavity and respiratory system via inhalation, skin and subcutaneous via skin painting or injection, stomach via gavage) than distal organs due to rapid hydrolysis and/or detoxification by cellular protective nucleophiles such as GSH. For example, bis-(chloromethyl) ether, one of the most potent human respiratory carcinogens, is not expected to be carcinogenic by the oral route because it hydrolyzes in water within seconds (Section 5.2). For highly unstable direct-acting substances, inhalation and injection may be the only possible routes of administration that can demonstrate their potential carcinogenic activity.

Another frequently used toxicokinetics consideration is to determine the trend, range of activity or “cut-off point” of toxicity of a homologous series of substances with the same mechanism of action. To the extent possible, toxicokinetics should be always used together with structural alerts to reach scientifically supportable (Q)SAR predictions. In fact, indiscriminate use of structural alert without prudent toxicokinetics consideration is one of the major causes of “false positives” in (Q)SAR studies. (Q)SAR algorithms that account for mitigating factors as well as bioavailability (e.g. Leadscope Model Applier, Multicase CaseUltra) tend to produce fewer false positives relative to Structural Alert (SA) based algorithms (e.g. Toxtree).

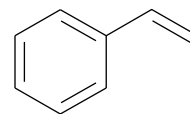
One of the most difficult tasks for considering the toxicokinetics of data-poor substances is to project the most likely ultimate carcinogenic intermediate(s) and potential target organ(s) / tissue(s). Some of the (Q)SAR tools such as OECD QSAR Toolbox, Oasis TIMES and Lhasa METEOR are designed to predict potential metabolites of a chemical and can serve to identify the ultimate carcinogenic species. Very often, assumptions are needed in (Q)SAR analysis, particularly for screening / prioritization purposes. Depending on the specific purpose of the (Q)SAR analysis, users should be aware of the limitations associated with the assumptions. For example, unhindered terminal double bonds are generally assumed to generate an epoxide group as the likely ultimate genotoxic carcinogen. This is in fact the case for substances like 1,3-butadiene (Sangaraju et al., 2012). However, for the structurally related vinyl acetate, the resulting epoxide is not expected to be the ultimate carcinogen due to instability caused by the adjacent oxygen; instead, intracellular hydrolysis to cytotoxic acetic acid and release of vinyl alcohol which tautomerizes to reactive acetaldehyde are believed to contribute to the nasal carcinogenic action of the chemical in rodents (Bogdanffy and Valentine, 2003). There is also evidence that the epoxide of styrene may not be the ultimate carcinogenic intermediate in mouse lung carcinogenesis (Cruzan et al., 2013). There is also some evidence that humans may have lower capacity to metabolically activate styrene to styrene oxide than rodents and that the ring hydroxylated styrene metabolites, not styrene oxide, may play a more important role in styrene-induced mouse lung tumors (Cruzan et al., 2012, 2013).



1,3-butadiene



vinyl acetate



styrene

Toxicokinetic variations in target organs/tissues (e.g., presence of β -lyase in kidney or β -glucuronidase in urinary bladder to re-activate detoxified phase II conjugated metabolites of toxic substances), qualitative or quantitative species or target organ differences in activating / detoxifying enzymes, and *in vivo* vs. *in vitro* systems are also complicating factors that may require mode of action studies to help in (Q)SAR studies.

2.1.2. Toxicodynamics considerations:

The toxicodynamics step can be assessed / hypothesized by examining the substance's structural moieties that are known to be associated with specific toxicity (commonly known as "structural alert (SA)" (Section 5.2)) as well as the toxicity profile of the substance and its structurally or functionally related substances. Toxicity endpoint-specific as well as mechanism-specific SAs have to be developed and continuously improved. Many of these have been captured in the OECD QSAR Toolbox (Diderich, 2010). Most of them either center on the electrophilic reactivity or receptor-mediated activity.

By far the most frequently used SAs are those for electrophiles. Many toxicants are either electrophilic or can be activated to electrophilic chemical intermediate(s) to react covalently with nucleophilic sites in macromolecules to carry out molecular initiating events (MIE). A number of factors can affect the toxicity endpoint, toxic potential, and target organ/tissue of any given electrophile. The key factors include:

Softness or hardness of the electrophile: Figure 3 lists a number of electrophiles and nucleophilic sites in macromolecules in the relative order of hardness/softness. In general, electrophiles prefer to react with nucleophiles of similar degree of hardness/softness. Most genotoxic carcinogens have hard electrophilic ultimate carcinogens that covalently bind to the hard nucleophilic sites in DNA. Chemicals with soft electrophiles *tend* to bind to the nucleophilic sites in protein preferentially to initiate nongenotoxic activities such as sensitization, neurotoxicity, hematotoxicity, hepatotoxicity, and nongenotoxic carcinogenicity. Some soft electrophiles are capable of binding to DNA but usually only at high doses or at portal of entry.

Examples of Hard and Soft Electrophiles

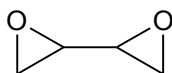
Soft electrophiles	Aldehydes, polarized double bonds
	Epoxides, strained-ring lactones, alkyl sulfates, alkyl halides
	Arylcarbonium ions
	Benzylic carbonium ions, nitrenium ions
Hard electrophiles	Alkylcarbonium ions

Nucleophilic Sites in Macromolecules

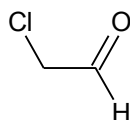
Soft nucleophiles	Thiol groups of cysteinyl residues in protein and glutathione
	Sulfur atoms of methionyl residues in protein
	Primary amino groups in protein (arginine and lysine)
	Amino groups of purine bases in RNA and DNA
	Oxygen atoms of purines and pyrimidines
Hard nucleophiles	Phosphate oxygen (P=O) of RNA and DNA

Figure 3: Examples of hard and soft electrophiles and nucleophilic sites

Monofunctional vs. bifunctional vs. polyfunctional and intergroup distance: Substances that contain more than one electrophilic group tend to have a higher potential for carcinogenicity than those with only one electrophilic functional group because of the possibility for crosslinking. The groups can be identical (e.g., 1,3-butadiene diepoxide) or different (e.g., chloroacetaldehyde). The ideal distance between groups appears to be in the range of 1 to 6 carbon atoms to allow the formation of DNA cyclic adducts which tend to be more persistent.

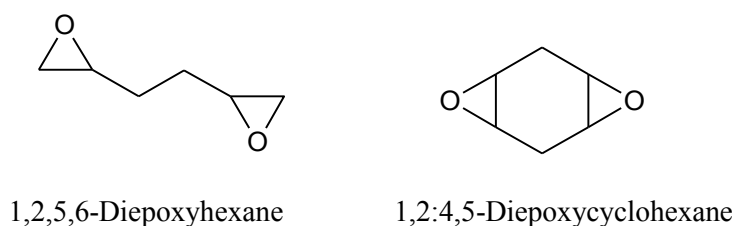


1,3-butadiene diepoxide



chloroacetaldehyde

Molecular flexibility / rigidity: Molecular flexibility can be an important factor. For example, a diepoxide connected by an aliphatic chain is carcinogenic but a diepoxide on a rigid cyclohexane ring is not.



Balance between reactivity and stability: Reactive intermediates which are too reactive have very short half-lives (e.g., free radicals) and therefore can only react with cellular proteins or lipids at the immediate vicinity of the site of their generation. In order for reactive electrophiles to bind covalently to DNA, they may resort to resonance stabilization for the transient stability needed to reach DNA. Some possible examples of resonance stabilization playing a role to allow carcinogens to reach DNA are in Figure 4.

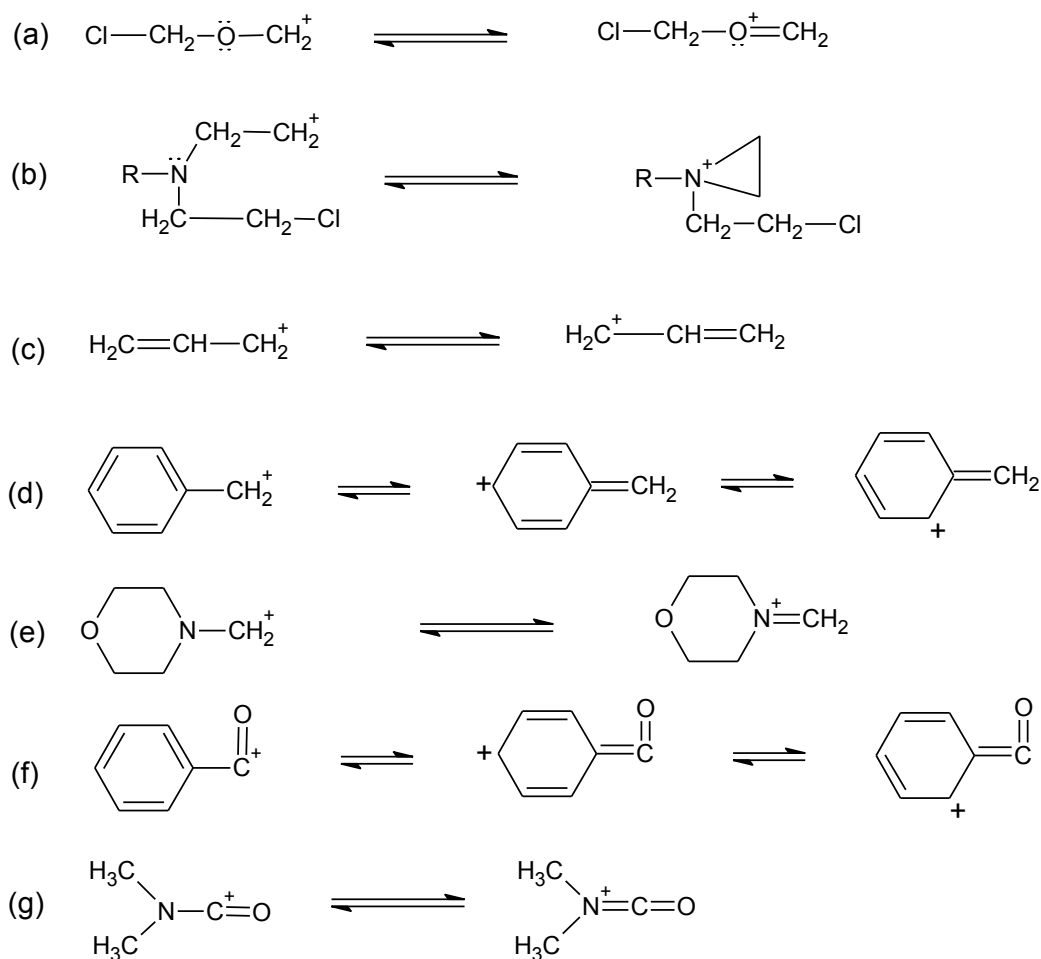
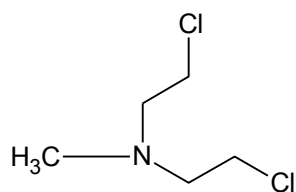
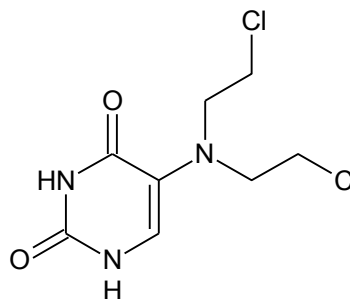


Figure 4: Resonance stabilization of reactive intermediates from (a) bis-(chloromethyl)-ether, (b) nitrogen mustard, (c) allyl chloride, (d) benzyl chloride, (e) bis-(morpholino) methane, (f) benzoyl chloride, and (g) dimethylcarbamyl chloride

Role of molecule of attachment: The molecule to which a SA is attached can have substantial modifying effects on the toxicity potential of the substance. Aside from the usual toxicokinetic/ADME effects (see Section 6.1), some combinations can have synergistic effects. For example, attaching a nitrogen mustard moiety to uracil leads to a substance (uracil mustard) that is substantially more carcinogenic than mustard itself possibly because uracil mustard combines two mechanistic potentials — electrophilicity and misincorporation of nucleoside analog—for carcinogenesis (Shimkin et al. 1966). Most of the potent carcinogenic polycyclic aromatic hydrocarbons are both good DNA intercalators as well as being electrophilic through activation to diol-epoxides.



Nitrogen mustard



Uracil mustard

In contrast to the unifying concept for genotoxic carcinogenic mechanisms, nongenotoxic carcinogens act by a variety of mechanisms (see Sections 5.2.3 and 6.3) with initial molecular targets usually distributed throughout the cell (e.g., nuclear receptors, cell membrane, cytoplasmic protein, organelles, etc.). (Q)SAR analysis of nongenotoxic carcinogens is dependent on availability of knowledge on the specific mechanism(s) involved. By far, the most extensively studied mechanism is receptor-mediated cell proliferation. In contrast to covalent binding of electrophiles to DNA by genotoxic carcinogens, receptor binding by nongenotoxic carcinogens is noncovalent and therefore reversible. The two key components for (Q)SAR analysis of receptor-mediated carcinogens are (a) ability of the substance to bind to the receptor, and (b) biological half-life in body to allow continuous and sustained binding (Sections 5.2.3 and 6.3).

Most (Q)SAR studies mainly focus on the molecular initiating event (MIE) -- such as DNA adduction, protein binding, receptor binding-- as the sole toxicodynamic component. In reality, from MIE to the final outcomes/manifestations of carcinogenic or toxic effects, a series of key events must occur. Adverse Outcome Pathways (AOP) and Mode of Action (MOA) frameworks have been recently developed to study these linkages and key events (Section 7.2.4). Most of the substances subjected to (Q)SAR studies are unlikely to contain sufficient data/information for substance specific AOP or MOA consideration. However, if a substance can be somehow related, structurally or functionally, to a certain substance or substance group with AOP/MOA information, then useful mechanistic information may be extracted to support the case. AOP/MOA based grouping is currently being considered for incorporation into the OECD (Q)SAR Toolbox.

3. IMPORTANCE OF MECHANISTIC CONSIDERATION IN (Q)SAR ANALYSIS AND HAZARD / RISK ASSESSMENT

The majority of existing automated (Q)SAR models designed to predict carcinogenicity of chemicals use statistically based algorithms employing molecular fragments and/or molecular descriptors relating to charge/reactivity, distance and bioavailability and do not directly reflect known or likely intermediate steps in the complex carcinogenicity pathway nor consider metabolism. Therefore, such models may often provide little mechanistic insight into the predicted carcinogenic activity of unknown chemicals. To the extent possible, mechanistic and toxicokinetic consideration should be conducted to help identify/postulate possible metabolite(s) that may contribute to molecular initiating event and other key events of carcinogenicity and determine data gaps needed to formulate AOP.

3.1 Importance of mechanistic consideration in (Q)SAR analysis

Mechanistic consideration is a crucial element for developing scientifically sound (Q)SAR analysis. The importance can be illustrated in the following points:

Selection of toxicity endpoint: The degree of difficulty of (Q)SAR analysis is generally proportional to the mechanistic complexity of the toxicity endpoint selected for the study. Endpoints with relatively well defined mechanism (e.g., skin sensitization, mutagenicity) tend to be easier to predict than those with multiple mechanisms (e.g., carcinogenicity or reproductive/developmental toxicity). Effect levels that involve multiple toxicity endpoints (e.g., No Observed Adverse Effect Level and Lowest Observed Adverse Effect Level) may be even problematic to qualify as a well-defined endpoint from the mechanistic point of view (see Section 4).

Selection of appropriate molecular descriptors: As discussed in Section 2, different mechanisms require different molecular descriptors for the most effective and scientifically sound (Q)SAR analysis. Without mechanism-related descriptors, (Q)SAR analysis may not adequately address the mechanistic association.

Accounting for metabolism: QSAR model algorithms often do not directly reflect or integrate metabolic transformation (bioactivation / detoxification) by explicitly predicting the likelihood of possible metabolites contributing to the various steps in the Adverse Outcome Pathway for carcinogenicity. As a result, when such a rodent carcinogenicity (Q)SAR model predicts positive outcome for a target chemical it fails to inform the user if the carcinogenic effect was actually caused by one or more particular metabolites just similar to that the rodent bioassay itself (without targeted special additional studies) and it also fails to provide information about the roles of metabolites in many earlier key events involved in the carcinogenicity process. Model algorithms that account for metabolism provide information on both activating and deactivating pathways that help to better interpret a prediction in mechanistic terms. For instance, the algorithm of Oasis TIMES, a hybrid expert system, consists of a mammalian metabolism simulator that would generate potential metabolites, provide information on different metabolic pathways and flag the active species (parent and/or metabolite(s)) (Mekenyan et al. 2012, Mekenyan et al. 2004, Serafimova et al. 2007).

Predictive domain of training set or knowledge base: Chemical structure has traditionally been used as a basis for assessing the predictive domain/coverage of training set/knowledge base for the (Q)SAR prediction of substances of interest. With increasing mechanistic studies and high throughput screening assays, toxicants can now be grouped by the mechanism of action as well for example by way of specific structural alerts. Mechanistic consideration should be used as an additional criterion for assessing predictive domain/coverage.

Database stratification for mechanistic homogeneity: It has been amply demonstrated that (Q)SAR analysis using structurally homogeneous or homologous series of substances (i.e., “local” models) tend to be more accurate than that from heterogeneous database (i.e., “global” models). This is because structurally closely related substances are more likely to have a common mechanism of action. Likewise, (Q)SAR studies of mechanistically homogeneous substances (e.g., peroxisome proliferator carcinogens) may be fruitful in uncovering structural features (see Section 6.3)

Interpretation of outliers: In QSAR, *outliers* are data points that clearly and unexpectedly do not fit the QSAR model developed from the overwhelming majority of other data points in the study. These are valuable in defining the limitations under which compounds act by a common molecular mechanism modeled by one or more descriptors, and/or experimental limitations of the biological test data (Verma and Hansch, 2005). Some researchers often remove outliers to improve statistical associations, sometimes without adequate justification. Such a practice should be exercised with caution because outliers are potential fertile ground for discovering new mechanistic insights. To the extent possible, mechanistic consideration should be included as part of the approaches to justify exclusion of outliers.

Guidance to hypothesis testing: One of the key roles of (Q)SAR analysis is also to find data gaps and generate hypothesis testing to improve predictive capability and knowledge base. Mechanistic consideration can give guidance for selection of proper goal-oriented tests.

Nature of modelled output: Most (Q)SAR models (typically statistical) are designed to provide a qualitative dichotomous yes / no or positive / negative prediction on a substance’s carcinogenic potential without mechanistic basis. Such predictions may be helpful for screening purposes but tend to be of limited use for in depth hazard assessments if not used in battery approaches, where many different models are used e.g. on the same carcinogenicity related endpoint and same training sets using different modeling approaches and/ or on different but carcinogenicity related endpoints using same trainings sets. For a better interpretation, it is valuable to consider or evaluate such predictions through weight of evidence approaches, and in light of mechanistic data obtained from other methods and/or (Q)SAR models for mechanistic endpoints. It is furthermore relevant to consider whether structural alerts and/ or other molecular descriptors of such models can be shown to be meaningful in relation to the carcinogenicity (e.g. related to reactivity and hence related to possible genotoxic mode of action of the carcinogenicity outcome).

Increasing confidence/reliability of (Q)SAR study: The confidence/reliability of a (Q)SAR study is dependent on a variety of factors such as the complexity of the endpoint, the experience/knowledge of the researcher/user, the availability of time and resources, the robustness of training database/knowledge base, scientific validity and predictive performance of the model, supporting documentation and predictive methodology. To the extent possible, achieving statistical association with mechanistic backing should be an ideal goal of (Q)SAR.

3.2 Importance of mechanistic consideration in hazard/risk assessment communities and stakeholders

(Q)SAR has now increasingly become an indispensable tool for hazard / risk assessment of industrial, pharmaceutical and environmental substances by academia, industries, governmental regulators, health and environmental advocates and stakeholders. Some of the examples of importance of mechanistic consideration in these endeavors are shown below.

Hazard assessment, screening and prioritization: Mechanism-based (Q)SAR is ideal for hazard assessment of data-poor substances. It has been routinely used to assess the toxic potential of new substances by the US EPA, Health Canada and other jurisdictions. Substances with a probable mechanism

can be compared to its analogs and a reasonable assessment can be made if the mechanisms are reasonably similar and comparative toxicokinetics information is available. In the absence of analogs, a possible mechanism may be postulated and a goal-oriented tiered testing plan can be suggested to the submitter. An example of a prioritization study was a detailed mechanism-based SAR analysis of the carcinogenic potential of over 290 drinking water disinfection byproducts (DBP) (Woo et al., 2002). Twenty higher concern substances were identified. They were prioritized for national drinking water monitoring and provided input to the US National Toxicology Program for selection of DBP for cancer bioassay.

Input to quantitative risk assessment: Mechanistic consideration is a crucial factor in quantitative risk assessment. Regulatory agencies generally consider genotoxic carcinogens to have no threshold and *proven* nongenotoxic carcinogens to have threshold. Inadequately tested carcinogens are, by default, considered to be genotoxic unless proven otherwise. The models used for genotoxic (e.g., q_1^* or linear extrapolation to origin from benchmark dose BMD_{10}) and nongenotoxic (e.g., margin of exposure, margin of safety) can give substantially different quantitative risk assessments with a major impact on regulatory compliance. There are however, some proposals for alternative approaches to harmonize risk assessment of genotoxic and nongenotoxic carcinogens.

Human significance of animal carcinogens: The concern that some animal carcinogens may not have human significance started with the observation that some target organs (e.g., forestomach) in rodents have no human counterpart. The mechanistic finding that the induction of male rat kidney tumors by some nongenotoxic substances was due to binding to $\alpha_{2\mu}$ globulin, led to global acceptance that such carcinogens are not of human relevance because humans do not have a significant amount of $\alpha_{2\mu}$ globulin. Since then, the induction of cancer in certain target organs by a growing list of mostly nongenotoxic chemical carcinogens is not considered to be of human significance. The importance of mechanism has led IARC to adopt the use of mechanistic consideration as one of the major criteria for grouping chemical carcinogens as Known Human Carcinogens (Group 1). A MOA/Human Relevance (HR) framework has been developed to provide transparency in MOAs and their qualitative and quantitative relevance to humans and implications for dose response (e.g., Seed et al., 2005; Meek et al., 2013). MOA/HR studies are now being carried out by the industry to provide supportive evidence for the safety of their products for humans despite marginally positive animal data.

Pollution prevention and molecular design of safer and greener substances: The quest to substitute toxic existing substances with safer alternatives and design safer and greener new substances are the ultimate goals of US EPA's pollution prevention and design for the environment programs in collaboration with industries and stakeholders. In both cases, mechanism-based (Q)SAR is the ideal tool for such tasks. A recent book on designing safer substances is a good source of information on various principles and approaches (Boethling and Vouchkova, 2012) and the application to specific endpoints such as carcinogenicity (Lai and Woo, 2012).

4. OECD PRINCIPLES FOR VALIDATION OF (Q)SARS: A BRIEF REVISIT FROM THE MECHANISTIC POINT OF VIEW

The reliability / confidence / certainty of (Q)SAR analysis in predicting the toxicity potential of untested substances has always been a subject of debate, skepticism and scrutiny. As may be seen from the previous sections, the reliability of (Q)SAR analysis is subject to limitations in (Q)SAR knowledge/expertise, empirical data available for modelling, predictive technologies, mechanistic understanding, time and resources, which can be highly variable and dependent upon the specific purposes/needs of the users and stakeholders. In 2002, an international effort to define the principles for validation of (Q)SAR was initiated. This work has widely been credited for increasing regulatory acceptance in the use of (Q)SARs. Originally called the Setubal Principles, the principles are now called OECD Principles for Validation of (Q)SARs (OECD, 2007). Readers are directed to the latest OECD guidance material for details which can be found at the following link: <http://www.oecd.org/chemicalsafety/risk-assessment/guidancedocumentsandreportsrelatedtoqsars.htm>.

The intent of this section is to briefly revisit these principles mainly from the mechanistic point of view with suggestions for possible future updates or reconsideration.

Principle 1: “defined endpoint”

The intent of this principle is to ensure that the endpoint should be well defined but the interpretation may be broadened to meet some regulatory needs. For example, a NOAEL (No Observed Adverse Effect Level) or a LOAEL (Lowest Observed Adverse Effect Level) “might be considered to be a defined endpoint in the sense that it is a defined information requirement of a given regulatory guideline, but cannot be regarded as a defined endpoint in the scientific sense”. While this is a necessary pragmatic approach, guidance should be given regarding possible compromise in the scientific soundness in such cases. The users and developers of (Q)SAR models should be made aware of the fact that the ability of (Q)SAR to make predictions with robust scientific support is affected by the mechanistic complexity of the toxicity endpoint in question. The determination of NOAEL/LOAEL requires examining all available contributing toxicity endpoints (e.g., cardiotoxicity, hepatotoxicity, reproductive toxicity, etc.) and manually selecting the most reasonable overall no effect or lowest effect level. Each of these contributing endpoints may have entirely different mechanisms of action. Therefore, (Q)SAR analysis of NOAEL/LOAEL tends to focus on the toxicokinetics parameters rather than both toxicokinetics and toxicodynamics. The reliability is even more questionable for global models using heterogeneous classes of substances because they are expected to have different toxicokinetics as well as different toxicodynamic parameters.

Principle 2: “unambiguous algorithm”

The intent of this principle is to ensure transparency in the model algorithm that generates predictions of an endpoint from information on chemical structure and/or physicochemical properties. To the extent possible, mechanistic consideration should be added to enhance robustness. Developers of commercial models with proprietary algorithm should be encouraged to provide, at least, information on the strengths and limitations of their models. In the absence of such information, users interested in evaluating a substance of interest should “test drive” the model using related substances with known information to see whether the substance is covered by the model.

Principle 3: “defined domain of applicability”

Defined domain of applicability is important because reliable predictions are generally limited to target substances structurally similar to the training database used to build the model. In addition to the structural domain, mechanistic domain must also be a consideration. The increasing successes of using structural alerts (SA) to predict sensitization, genotoxicity and genotoxic carcinogenicity potential of substances show the possibility of (Q)SAR analysis across different structural classes. The latest addition to the OECD QSAR Toolbox is SA for nongenotoxic carcinogens (see Section 6.3) with SAs for pulmonary sensitization and phototoxicity under development. More effort should be spent to explore the possible expansion to other toxicity endpoints as well. When using SA to predict toxicity potential, it is also important to consider the toxicokinetics factors of the chemical of interest relative to the related analogs used to define the domain of applicability. The potential route of exposure to the chemical of interest may also be a factor for highly reactive chemicals that may cause portal of entry effects.

Principle 4: “appropriate measures of goodness-of-fit, robustness and predictivity”

The “appropriate measures of goodness-of-fit, robustness” and “predictivity” correspond to the “internal validation” and “external validation” of the original Setubal principles 5 and 6, respectively. A number of studies (e.g., Gramatica, 2007; Kulkarni and Barton-Maclaren, 2014) indicated internally validated predictive models often may not be quite as satisfactory as those with external validation. Users should also be aware that each batch of substances to be analyzed is a different challenge; good predictivity in one batch of substances does not necessarily apply to the next batch if the structural and mechanistic characterizations are different.

Principle 5: “mechanistic interpretation, if possible”

It is inevitable that some (Q)SAR results may lack mechanistic interpretation. The current guidance stated that the intent of this principle is “not to reject models that have no apparent mechanistic basis, but to ensure that some consideration is given to the possibility of a mechanistic association....and to ensure that the association is documented.”

In view of the increasing importance of mechanistic understanding in determining hazard/risk assessment and human relevance of toxicity, mechanistic consideration should play an important role in (Q)SAR analysis of toxicity endpoints, especially those with complex mechanism(s). The language in OECD principle 5 (defined mechanism of action, *if possible*) may not seem to be strong enough. If a defined mechanism of action is not available, the (Q)SAR analysis should be interpreted with caution with data gaps identified. On the other hand, the confidence/reliability of the (Q)SAR analysis may be enhanced if mechanistic support is available. The advent of Tox21 and ToxCast may provide more mechanistic insights to the (Q)SAR field.

(Q)SAR model reporting format (QMRF) is the OECD test guideline for documenting model related information whereas (Q)SAR prediction reporting format (QPRF) is the test report for a specific substance tested with the test guideline. QPRFs should follow the same outline as QMRFs except that the interpretation is now on a specific substance. Increasingly model algorithms are incorporating QPRF as one of the output formats. It is specific to the toxicity endpoint and the substance tested on that model. It provides a stepwise summary of the various aspects considered by the model and/or the choice of decisions taken by the user (in the case of analog read across) in order to arrive at a particular prediction. This is particularly helpful not only to facilitate reproducibility of the prediction but also to determine its adequacy in mechanistic terms. For instance, the QPRF obtained from a typical global QSAR model may have limited information to offer in terms of mechanistic interpretation. A QSAR model for Ames mutagenicity may provide some mechanistic insight in terms of electronic/quantum mechanical descriptors that describe energy of frontier orbitals such as E_{HOMO} (highest occupied molecular orbital) and E_{LUMO} (lowest

unoccupied molecular orbital) which account for electron donating and accepting character. Such descriptors play an important role in interaction with nucleophilic sites on the DNA, a precursor event in the mutagenesis pathway.

5. ROLE AND IMPORTANCE OF MECHANISTIC CONSIDERATION / UNDERSTANDING IN THE DEVELOPMENT, ASSESSMENT, AND USE OF (Q)SAR APPROACHES

There are many possible (Q)SAR approaches and applications. This section focuses on the most frequently used approaches, including read across and category approaches. Most of these approaches center on using structurally similar substances with known carcinogenic activity to develop methods to predict the potential carcinogenicity of untested substances. The pros and cons of each approach are discussed along with discussion of the importance of mechanistic consideration in guiding and improving reliability and scientific soundness.

5.1 Chemical / structural class approach

The Chemical / Structural class approach has been used since the inception of (Q)SAR studies. This approach is best suited for data rich chemical classes with some mechanistic information available for in-depth analysis. In the 1970s to 1980s, a tremendous amount of systematic mechanism-based (Q)SAR studies were conducted on a variety of classes of chemical carcinogens by researchers in academia and US NCI/NTP through testing strategically synthesized analogs of selected carcinogens to test/verify mechanistic hypotheses. Most of these studies and findings were extensively reviewed in the series of books: “Chemical Induction of Cancer: Structural Bases and Biological Mechanisms” (Arcos and Argus, 1974a,b; Arcos et al., 1982; Woo et al., 1985,1988), and relevant data were systematically analyzed to develop class-specific characteristic structural and functional features that contribute to carcinogenic activity or inactivity. This approach is labor and time intensive and requires considerable expertise; but once completed, it can be used effectively. The book series along with more recent studies and nonclassified agency data from various program offices, were used by US EPA to develop knowledge rules and decision trees that eventually led to the development of the Cancer Expert System “OncoLogic” for predicting carcinogenic potential of substances by mechanism-based (Q)SAR analysis (Woo et al., 1995; Woo and Lai, 2005) . The System gives semi-quantitative assessment of concern level for carcinogenic potential of target substance together with scientific rationale. OncoLogic has been freely available for use by the general public for over 10 years (<http://www.epa.gov/oppt/sf/pubs/oncologic.htm>). Two recent external validation studies (Mayer et al., 2008; Benigni et al., 2012a) showed >90% accuracy for the two batches of substances tested. However, application of the System is limited to fibers, metals, polymers and 50 classes of organic substances and not all substituents are covered. An upgrading effort is currently under way.

5.2 Structural alerts approach

5.2.1 Overview of structural alerts:

A structural Alert (SA) can be defined as a substructural moiety that is known to be associated with a certain type of toxicity or biological property. A SA can be as simple as $-\text{CH}_2\text{-Br}$ or a complex substance grouping with molecular size or shape that may fit into a certain cellular receptor such as aryl hydrocarbon receptor (AhR). SAs can be recognized by expert scientists or searchable by predictive softwares (e.g., OECD QSAR Toolbox or Toxtree software) and can serve as an alert or flag for potential toxicity of the substance that contains the substructural moiety. The absence of SA, however, does not necessarily mean the substance is not toxic. Furthermore, it should be emphasized that the presence of SA merely indicates a potential and it is important to examine the rest of the molecule to consider modifying factors to reach a scientifically supportable decision. Some model algorithms (e.g. Multicase CaseUltra (Klopman and

Rosenkranz, 1994, Chakravarti et al. 2012)) assess the molecular structure for the presence of activating and deactivating/mitigating features that have statistical correlations with given activity/inactivity. The model output provides information on the number of known substances that possess these features along with their activity in support of the prediction. Derek Nexus is another rule-based structural alert system with a library of structural alerts for carcinogenicity, with most alert descriptions providing the metabolic/mechanistic basis for the alerts, information on structural constraints, and example chemicals. In most cases, SA should be considered as a toxicodynamics input that requires toxicokinetics inputs to complete the overall assessment. Indiscriminate use of SA may lead to false positives. For direct-acting SA, it is important to consider exposure scenarios. Another problem for inexperienced users is that some SA may be *masked*, i.e., the SA may be embedded within the molecule which requires hydrolysis, tautomerization (e.g., Figure 2), metabolism or photoactivation to be unveiled. The SAs used for identifying potential carcinogens are mechanism-specific; they may be classified as genotoxic SA and nongenotoxic SA.

5.2.2 Genotoxic structural alerts:

Genotoxic SAs have been used to identify potential genotoxic carcinogens for more than two decades. The underlying mechanistic basis for developing genotoxic SA is the unifying concept of James and Elizabeth Miller (Miller and Miller, 1947) that virtually all genotoxic carcinogens either are or can be metabolically activated to electrophiles which covalently bind to nucleophilic sites in DNA to initiate carcinogenesis. Ashby and Tennent (1991) were among the first to develop a list of genotoxic SAs that performed well in associating with genotoxic carcinogens in US National Toxicology Program (NTP) bioassays. Since then, the list of genotoxic SAs has been expanded by various authors (e.g. Woo and Lai, 2003, 2010; Enoch and Cronin, 2010; Benigni et al., 2013a). It should be cautioned that not all genotoxic SAs are alike with respect to their carcinogenic potential. Genotoxic SAs that are hard electrophiles (e.g., epoxide, carbonium ion) are more likely to bind to DNA than those that are soft electrophiles (e.g., sulfur-containing electrophiles, aldehyde) that prefer to bind to proteins (Figure 3). Also, for some highly reactive SAs (e.g., -O-CH₂-Cl) the site of contact (e.g. oral, dermal or inhalation) may pose the greatest concern. This topic will be further discussed in Sections 6.1 and 6.2.

5.2.3 Nongenotoxic structural alerts:

In contrast to the unifying concept for genotoxic mechanism of action, nongenotoxic carcinogens may act by a variety of mechanisms such as (a) receptor-mediated cell proliferation; (b) generation of reactive oxygen species and free radicals to cause oxidative stress and secondary DNA damage; (c) perturbation of DNA methylation leading to aberrant gene expression; (d) hormonal imbalance or disturbance of homeostatic status of cells; (e) cytotoxicity with subsequent compensatory regenerative hyperplasia; (f) persistent chronic inflammation; (g) inhibition of gap junctional intercellular communication; (h) disturbance of signal transduction; (i) reduction of programmed cell death (apoptosis); (j) mitogenesis; (k) tissue/cell overload with foreign bodies or certain metals; (l) inhibition of microtubulin polymerization; and (m) impairment of immune surveillance (e.g., Woo and Lai, 2003, 2010; Shah et al., 2011).

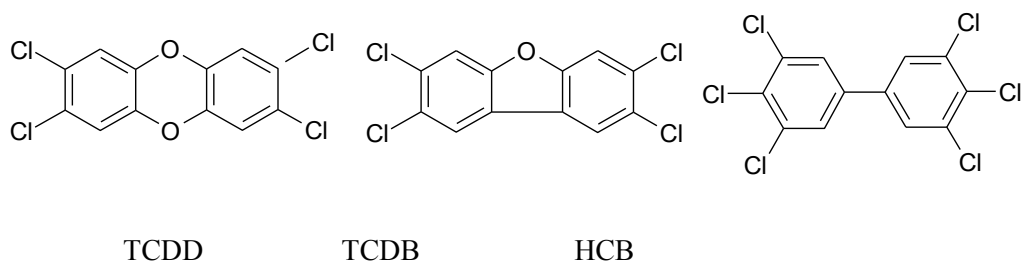
As may be expected from the multiple mechanisms, (Q)SAR analysis of nongenotoxic carcinogens is dependent on finding the specific mechanism(s) involved. Unlike genotoxic carcinogens which center on DNA as the common initial target molecule, the initial targets of epigenetic carcinogens may be distributed throughout the cell (e.g., nuclear receptor, cell membrane, cytoplasmic protein, organelles, etc.). Some mechanisms (e.g., signal transduction) may involve complex molecular and cell biology and therefore may

necessitate high throughput assays or toxicogenomics studies and computational biology for exploratory, supportive or confirmatory evidence (e.g., Shah et al., 2011; Sen et al., 2010; Kavlock et al., 2012). It is challenging for model developers to capture this vast diversity of mechanisms in a small subset of descriptors or fragments and build a realistic model.

By far the most extensively studied mechanism of nongenotoxic carcinogens is the receptor-mediated cell proliferation. Xenobiotic ligand induced activation of several nuclear receptors (aryl hydrocarbon receptor or AhR, pregnane X receptor or PXR, constitutive androstane receptor or CAR) for enzyme induction has been proposed to contribute to increased cell proliferation as the mode of carcinogenic action of a variety of hepatocarcinogens (e.g., Shah et al., 2011; Safe, 2006; Pascucci et al., 2008). Peroxisome proliferation activating receptor α (or PPAR α)-mediated cell proliferation and oxidative stress are believed to be the mode of hepatocarcinogenic action of peroxisome proliferators (Section 6.3).

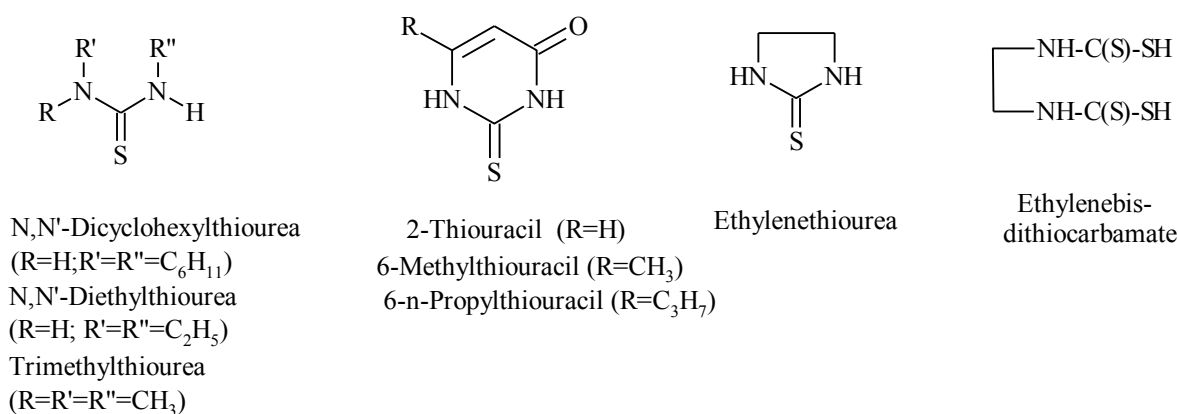
Mechanistically, since receptor binding is noncovalent and therefore reversible, (Q)SAR analysis of receptor-mediated epigenetic/nongenotoxic carcinogens essentially involves two components: (a) ability of the substance to bind to the receptor and serve as agonist/antagonist, and (b) long biological half-life allowing continuous or sustained binding/activation of the receptor. Receptor fitness can be studied by analyzing the structural features of active substances to find the favorable molecular size, shape and thickness/planarity or by computer-assisted 2D or 3D receptor modeling or docking studies. Biological half-life can be experimentally measured or deduced by looking for structural features suggestive of resistance to metabolism, e.g., fluorination (stable C-F bond), ω -1 branching of fatty acids (inhibition of β -oxidation), presence/absence of two adjacent ring positions in aromatic substances (which allows/discourages ring oxidation).

One of the most well-known nongenotoxic SAs is the one for the AhR receptor. The non-genotoxic carcinogen 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) and its isosteric analogs 2,3,7,8-tetrachlorodibenzofuran (TCDB) and 3,3',4,4',5,5'-hexachlorobiphenyl (HCB) all act via this receptor (Budinsky et al. 2014).



The positioning of Cl-atom at all the lateral positions of the three substances provides the best molecular configuration for receptor binding as well as discouraging ring oxidation. Both TCDD and TCDB are planar. For the chlorinated biphenyls, planarity is essential for binding to AhR. Chlorinated biphenyls with chlorination (or other bulk group) at position(s) next to the intercylic bond (i.e., 2,2', 6,6') can distort the planarity of the molecule and substantially reduce or abolish carcinogenic activity.

Another well-established non-genotoxic SA is thiourea / thioamide moiety and its precursors such as ethylenebiscarbamates. The molecular mechanism is not clearly understood but is believed to involve inhibition of thyroid hormone biosynthetic enzyme by the thiourea/thiocarbonyl moiety via formation of disulfide bridge.



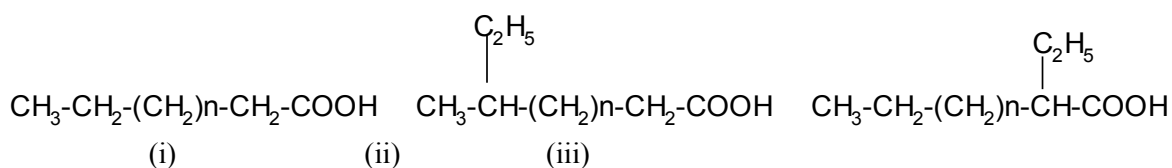
A number of other nongenotoxic SAs have also been identified by Benigni et al. (2013) and are now in Toxtree and OECD QSAR Toolbox. Like the genotoxic SAs, the nongenotoxic SAs should be used a starting point to consider the rest of the molecule to evaluate carcinogenic potential.

5.3 Category / Grouping approach

There are many different ways to categorize or group substances. For example, US EPA has developed a list of “Chemical Categories of Concern” based on chemical moiety (e.g., epoxide, acrylamide) or physicochemical properties (e.g., cationic surfactants) as alerts to avoid by industries during their development of new substances. Each category has a definition of chemical structure and limitation of members, hazard concerns, boundaries of size and route of exposure, and occupational exposure controls. The list has been continuously updated with the latest one in 2010 (USEPA, 2010).

The OECD has developed a guidance document for category/grouping approach to strategize and facilitate hazard assessment and toxicity testing (OECD 2014). In this approach, a substance category is defined as a group of substances whose physicochemical, toxicological and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity. The rationale for forming the category may be based on a range of characteristics such as common functional group, common constituents, or chemical classes with a defined chain-length, likelihood of having a common precursor and/or breakdown products or a particular reaction chemistry domain. The assessment of substances by using a category approach differs from the approach of assessing them on an individual basis because the effects of the individual substances within a category are assessed on the basis of the evaluation of the category as a whole, rather than on measured data for any one particular substance alone. The category approach is basically a weight of evidence approach (WOE), since it typically integrates both estimated and experimental data, and involves expert judgment. The biggest challenge in this approach lays in defining the category itself (its underlying rationale/mechanistic basis) and in particular its boundaries (ECETOC, 2012; Patlewicz et al., 2013a).

As an example of the importance of mechanistic understanding in determining category, consider the following three series of alkanolic (carboxylic) acids: (i) n-alkanoic acids, (ii) iso-alkanoic acids with branching at the end distal to the –COOH, and (iii) iso-alkanoic acids with branching at the carbon right next to –COOH (also known as the ω-1 position):



From the chemistry point of view, the three series of acids are not expected to have major differences in physicochemical (e.g., pKa) properties. However, the presence of branching at the sensitive ω -1 position can significantly prolong biological half-life of the chemicals and enhance their toxic potential. Whereas (i) and (ii) can readily undergo metabolism by β -oxidation, the ω -1 branching in (iii) can curtail β -oxidation. As a result, (iii) has a substantially longer half-life in the body than (i) and (ii). The longer half-life and the ability to bind to PPAR α receptor are believed to contribute to hepatocarcinogenicity (Section 6.3) as well as teratogenicity (Section 8.4) of some medium-size members of (iii) in rodents.

Category/grouping does not have to be restricted to structural similarity only. With the increasing information on mechanism of action of carcinogenesis, a common mechanism can also be used to categorize or group substances. For example, many peroxisome proliferators with a variety of chemical structures have been shown to be hepatocarcinogens in rodents. In section 6.3 an approach to find structural features for hepatocarcinogenicity out of such a category / group has been illustrated. With increasing studies on mode of action, adverse outcome pathway and transcriptional profiles, these mechanism-related commonalities can also be used as a basis for category/group approaches. The OECD QSAR Toolbox has been expanding various types of categories. Substances triggering multiple categories should deserve more attention for integrative consideration of potential hazard.

Product usage (e.g., flame retardants, plasticizers, pesticide inerts, etc.) is another category/grouping often used (Dionisio et al. 2015). The main purpose of such approach is to find safer and/or more economical alternatives or substitutes. Since usage-based categories/groups typically contain more heterogeneous compounds, extra caution must be exercised to determine whether they are suitable for (Q)SAR analysis. Sub-categorization may be needed to provide structural and/or mechanistic similarity.

5.4 Analog identification / comparison

One of the most common approaches for regulatory agencies to provide scientific support to substantiate a potential hazard, or for approximating quantitative risk assessment of a target substance is to find an appropriate analog or surrogate or source substance(s) with information on the toxicity endpoint of interest. The following steps may be useful for this task:

(1) The analog(s) must be expected to have the same/similar mechanism of action as the target substance for the toxicity endpoint of interest. With a very well defined mechanism of action, strong support can be achieved with as few as one analog (e.g., 2,4,6-trichlorophenol was considered a reasonable analog in an attempt to regulate 2,4,6-tribromophenol).

(2) If available, isomers and tautomers should be the first place to look because they can often give good SAR insight.

(3) Examine possible common breakdown products via looking for labile substructures such as acetal group ($\text{R}_2\text{C(OR')}_2$), formaldehyde releaser (e.g., $-\text{N-CH}_2\text{-N-}$, $-\text{O-CH}_2\text{-O-}$), Schiff bases (e.g., $-\text{N=C-}$).

(4) Examine for possible common metabolites via looking for precursors or easily metabolizable substructures such as terminal double bond, diarylazo, N-/O-dealkylation of small alkyl with α -H, small esters.

(5) Computer-assisted searches such as substructure search, OECD QSAR Toolbox, and USEPA's Analog Identification Methodology/AIM software (which also gives leads to look for available toxicity information) and Distributed Structure-Searchable Toxicity/DSSTox structure browser (which gives a number of analogs ranked by similarity index; see below).

It should be cautioned that analog-generating software based solely on structural similarity may not adequately take mechanism and metabolism into consideration. The suitability of the analogs must be examined with caution. For example, in searching for analogs for an aromatic amine compound $\text{CH}_3\text{-O-C}_6\text{H}_4\text{-NH-CH}_3$ in DSSTox predictive tool, 11 analogs with $>68\%$ similarity (based on Tanimoto coefficient) were identified. The "best" analog was 4,4'-dimethoxydiphenylamine and the second best was 4-anisidine (Figure 5). However, the "best" analog has an N-phenyl instead of an N-methyl group. The N-phenyl group is so resistant to be metabolized that the compound is not even classified as an aromatic amine. In contrast, through N-demethylation of the target substance and N-hydroxylation of 4-anisidine, the two substances can actually generate a common metabolite that can be further activated to reactive metabolite with potential to bind to DNA. Therefore, 4-anisidine should no doubt be the best analog. Users should be aware that similarity index must be used with mechanistic consideration for the selection of best analogs.

The screenshot shows the EPA DSSTox Structure Browser v2.0 interface. At the top, there are navigation tabs: "Search", "File Incidences", and "Search Details". Below the tabs is a "Details" section with a "Query" field containing a chemical structure of 4-methoxy-N-methylaniline. The "Results" section shows a table with columns: "Results Type", "Hits", and "Display". The table lists "Exact matches" (0 hits) and "Similarity $\geq 68.7\%$ " (11 hits). Below this is a table of search results with columns: "DSSTox Substance ID", "Similarity Score%", "Structure Match", "Substance Name", "CASRN", and "Substance Description".

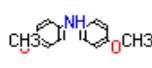
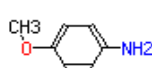
DSSTox Substance ID	Similarity Score%	Structure Match	Substance Name	CASRN	Substance Description
25093	85.4		4,4'-Dimethoxydiphenylamine	101-70-2	single chemical compound
24532	80.4		4-Anisidine	104-94-9	single chemical compound

Figure 5: Partial screen of a computerized search for analogs

After selection of a preliminary list of possible analogs, all the available information on physicochemical properties, toxicokinetics, toxicity profile and predictive tests for the toxicity endpoint of

interest must be assembled for a weight of evidence analysis to select the best analog(s). Final comparison between the target substance and best analog(s) for differences in substituents and their potential impact on important factors (e.g., electronic, steric, molecular size/shape, logP / solubility, half-life) that determine potential carcinogenicity should be conducted. It may not be always possible to find the perfect analog. In cases when a perfect analogy cannot be identified, it will be important to specify that the analog selected is expected to be more/less hazardous than the target substance for worst/reasonably-safe case scenario. The justification should be elaborated along with recommended goal-oriented short-term testing to ascertain the validity of the analogs.

5.5 Read across

Read across is currently one of the most actively used quasi (Q)SAR methods that estimates the potential toxicity of an untested substance based on structurally or functionally similar substances with known toxicity information. The principle of the read-across technique is that endpoint or test information for one substance is used to predict the same endpoint or test for another substance, which is considered to be similar by scientific justification (OECD 2014). Read across is generally the reason and the end-result of analog identification mentioned in section 5.4. In case of a chemical category the members or the source chemicals in the category may provide added support and/or establish a trend to determine possible cut-off points in terms of physical and chemical properties and also aid in defining the predictive domain. Read across has been increasingly used by regulatory agencies worldwide as an alternative approach for addressing the information requirements (i.e., filling data gaps). Ideally, read across should be mainly carried out between substances that share structural similarity as well as high likelihood of exerting a certain toxic effect via a similar mechanism of action. OECD guidance on read across and practical strategies to help develop scientifically supportable and valid read across are currently being proposed or developed (ECETOC, 2012; Patlewicz et al., 2013a,b; Enoch et al., 2011; Low et al., 2013).

Read across is easy and simple to do but difficult to do it correctly. Conceptually, it involves finding an acceptable group of related analogs with scientifically justifiable variable(s) such as specific chemical features or physicochemical properties etc. that can be associated / correlated qualitatively or quantitatively to robust data—on a specific toxicity endpoint of interest—of at least several members of the group. Then, using the variable(s), the potential toxicity of the target substance can be estimated to fill the data gap (Table II).

Table II: Conceptual analog read across matrix

Chemicals in category/cluster with commonality/link/rationale	Variable(s) that can be correlatable to toxicity	Toxicity (e.g., carcinogenicity)
C1	V1	T1
C2 (target substance)	V2	?
C3	V3	T3
C4	V4	T4

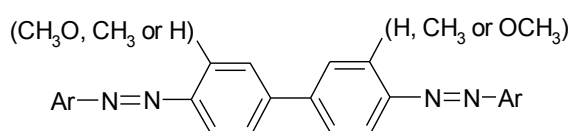
There are at least three major approaches for using commonality / link / rationale in forming category / cluster / group for read across. The most common approach used now is based on structural similarity which can be common functional group, homologous or congeneric series (i.e., chain length category), common chemical class or common precursors / metabolites / breakdown products (ECETOC, 2012). It will be ideal if the common feature contains a structural alert, either openly or embedded, that may contribute to the toxicity endpoint of interest (e.g., carcinogenicity) either directly or after metabolism. With increasing mechanistic studies and high throughput assays, functional or mechanistic similarity is gradually becoming an important commonality/link. One of the most often grouping approaches used by industries and regulators is product usage (e.g., flame retardants) to look for safer, greener or more

economical alternatives. Since such category often contains somewhat heterogeneous substances, caution must be exercised to subcategorize substances for use in read across if necessary.

Data of the variable(s) should be available in all or almost all members of the category. Depending on the target of the study, they can be physicochemical or toxicokinetics factors (e.g., number of carbons in chain, log P, solubility), metabolizability, biological half-life, or even biopredictive / bioinformatic data, etc. The variable(s) should be correlatable qualitatively or quantitatively to the toxicity endpoint but kept to a crucial minimum. To ensure that the target substance falls within the predictive domain of the read across study, the value of its variable (i.e. V2 in the table above) should be between at least two members (e.g., V1, V3) of the category to allow interpolation. If extrapolation is the only choice available, then some uncertainty should be expressed particularly if there is no indication of the possible trend.

The toxicity data of all members of the category used for read across should be of robust quality and include a spectrum of positive and negative data. The toxicity endpoint must be well-defined (e.g., carcinogenicity) and preferably include the exposure route(s). It should be cautioned that the prediction of one toxicity endpoint by read across is not necessarily applicable for another endpoint because of differences in mechanism of action. Reading across absence of toxicity tends to be more reliable and meaningful if the target substance is part of the tested negative structural domain populated by non-toxic substances supported by structural and/or physicochemical parameters as opposed to when the target substance is simply not a part of the tested positive structural domain. Moreover, this type of read across should include an assessment of bioavailability not only of target substance but also of the analog/category members to ensure that non-activity is not a result of non-bioavailability. After completion of the estimation of the toxic potential of the target substance, the scientific rationale and justification for the read across should be elaborated along with testing recommendations if the justification is not strong. More discussion on the pitfalls and uncertainties associated with read-across and strategies to address these are elaborated elsewhere (Blackburn and Stuard, 2014).

A good example of what could have been a great read across case is US NTP's benzidine dye initiative study (Morgan et al., 1994). The focus on this study was on the hundreds of benzidine derived dyes and pigments with the general structure shown below where Ar = aryl moieties with sulfonic acid, amino and various other substituents on the rings and metal chelation.



Generic molecular structure of a benzidine-based azo dye

All of them share a common link of having benzidine or its 3,3'-dimethyl or 3,3'-dimethoxy derivatives embedded in the chemical structure. Benzidine and its 3,3'-dimethyl or 3,3'-dimethoxy derivatives are known to be potent carcinogens (*rev.*, Woo and Lai, 2012). A number of water soluble benzidine dyes have been shown to be carcinogenic whereas the insoluble benzidine pigments are not carcinogenic. There is strong evidence that the carcinogenic dyes owe their carcinogenic activity to metabolic reduction of the azo (i.e., -N=N-) bond to release the embedded benzidine or benzidine congener moiety. Thus, metabolizability (which can be readily measured by assays or estimated by water solubility or extent of ring sulfonation) can be used as the variable for read across to assess carcinogenic potential of benzidine dyes. Benzidine Dyes Class (i.e., those that can be metabolized to benzidine) is currently listed by US NTP as a class of substances that are known to be human carcinogens (NTP, 2011). A 2010 study by US EPA (2010) listed 47 benzidine-derived (four unsubstituted, seventeen 3,3'-dimethyl- and twenty-six 3,3'-dimethoxy- derivatives) dyes as possible carcinogens.

The European Chemical Agency (ECHA) has recently developed a “Read –Across Assessment Framework (RAAF)” to provide a framework and guidance for consistent evaluation of the scientific aspects of a proposed read-across case, resulting in an output which is suitable for subsequent regulatory consideration of the read-across case (ECHA, 2015).

5.6 QSAR equations

Since the pioneering work of Corwin Hansch (1993) numerous QSAR equations have been developed to predict virtually any conceivable biological endpoints. The success of a QSAR equation to predict toxicity endpoints is dependent on a variety of factors such as (a) a well-defined endpoint, (b) the mechanistic understanding and complexity of the endpoint, (c) the availability of a group of structurally related compounds with potentially same mechanism of action, (d) the ability to find proper molecular descriptors, and (e) the ability to eliminate outliers justifiably. As mentioned in Section 2, mechanistically, cancer is one of the most complex toxicity endpoints. The quest to accomplish statistical association with mechanistic backing is particularly challenging for cancer endpoints but still achievable. One such example is the QSAR equation developed by Benigni and Franke (Franke et al, 2001) on carcinogenic potency of aromatic amines in mice.

$$\text{BRM} = 0.88 \times \log P \times I(\text{monoNH}_2) + 0.29 \times \log P \times I(\text{diNH}_2) + 1.38 \times E_{\text{HOMO}} - 1.28 \times E_{\text{LUMO}} - 1.06 \times \text{MR}_{2,6} - 1.1 \times \text{MR}_3 - 0.2 \times E_s(\text{R}) + 0.75 \times I(\text{diNH}_2) + 11.16$$

$$n = 37, \quad r = 0.907, \quad s = 0.381, \quad F = 16.3, \quad P < 0.001$$

where $\text{BRM} = \log(\text{MW}/\text{TD}_{50})$

$I(\text{monoNH}_2)$ and $I(\text{diNH}_2)$ = indicator variable (either 0 or 1);

E_{HOMO} and E_{LUMO} = energy of highest and lowest occupied molecular orbital;

$\text{MR}_{2,6}$ and MR_3 = molar refractivity of substituents at *ortho/meta* positions of amino group;

$E_s(\text{R})$ = Charton's substituent constants for substituents at the functional amino group

The overall equation showed that a separate equation is needed for aromatic amines with one and two amino groups. The gradation of potency of the carcinogenic amines first depended on their hydrophobicity, and secondly on electronic (reactivity, propensity to be metabolically transformed) and steric properties. On the contrary, the difference between carcinogenic and non-carcinogenic aromatic amines depended mainly on electronic and steric properties. These QSARs can be used directly for estimating the carcinogenicity of aromatic amines (Franke et al., 2010). The mechanistic backing was essentially in agreement with the key qualitative observations of the human experts on carcinogenic aromatic amines (Woo and Lai, 2003, 2010; see also Section 6.2).

Besides direct estimation of possible carcinogenic potency of substances, QSAR equations can also have flexible application to regulatory uses. Two important regulatory policies of many regulatory agencies are (i) to minimize the exposure of population groups to highly potent carcinogens and (ii) to ensure that widely used substances have low potential for risk. For such regulatory uses, QSAR equations can be modified to maximize the reliability of the prediction of negative compounds (i.e., safe substances) or optimized to focus on the potent carcinogenic substances to capture more effectively the characteristics of potent carcinogens (Franke et al., 2010; Benigni and Bossa, 2012).

5.7 Combining multiple (Q)SAR models/approaches

The complex multistep endpoint such as carcinogenicity cannot be perfectly modelled by one prediction algorithm. However, combining predictions from multiple (Q)SAR models /approaches or conducting a consensus modelling is capable of providing an enhanced mechanistic interpretability to the modelled data. Different (Q)SAR models are based on different algorithms and thus, come with their unique package of certainties and uncertainties. They differ in their underlying scientific rationale and thus tend to assess a given substance uniquely and may result in different predictions and should be assessed in an overall weight of evidence. On the other hand, similar predictions obtained from a variety of different models tend to be more convincing provided they look at the target structure in rational terms without having similar bias across their algorithms. For instance, structural alert based models scan the molecular structure of target substance (and analogs) and flag them for presence of key structural fragments/functionalities associated with genotoxic/non-genotoxic effects. Generally such models provide some degree of mechanistic information but are not designed to address the role of mitigating factors or modulators i.e. presence of fragments/functional groups that may neutralize the effect of activating factors. The mechanistic and endpoint specific profilers relating to genotoxic, non-genotoxic and carcinogenic activity found within the OECD QSAR Toolbox represent this category of algorithm. On the other hand, descriptor and/or fragment based statistical models examine electronic and/or structural and/or physicochemical attributes of a substance and consider its bioavailability and/or presence/absence of reactive centres for potential interaction with nucleophilic centres on DNA/protein molecules leading to genotoxic/carcinogenic activity. These models generally consider mitigating / modulating effects but provide limited input on mechanistic aspects. Expert systems such as DEREK Nexus (Sanderson and Earnshaw, 1991; Judson et al., 2003) assess the substance by applying certain knowledge-based rules/exceptions and provide mechanistic insight into the likely activity. Such models are bound by known interactions and thus, are not well-suited for ruling out activity of a substance. Hybrid models such as Oasis TIMES incorporate metabolism in their prediction algorithm. Such models analyse the target substance in terms of accessibility to potential metabolic pathways and also examine the role played by its metabolites. This method helps to determine the role of parent substance versus its metabolites in the toxicity. Application of read across approach helps to identify analogs that closely resemble the target substance in terms of structural features, physicochemical characteristics, mechanism of action and metabolism.

Frequently, the biological mechanism is activated by a specific tautomer of a substance. OECD QSAR Toolbox contains an algorithm for identifying potential tautomeric forms of a given substance. The genotoxic /non-genotoxic and carcinogenic potential of different tautomers could be generated by applying all the above mentioned (Q)SAR models / approaches.

Thus, each predictive model/approach examines a given substance with a unique set of tools. Considering an individual model prediction in isolation does not provide the entire picture and is not very informative for regulatory decision making. Building a matrix of all the predictions obtained from various types of (Q)SAR models / approaches can certainly provide a broader spectrum of potential scenarios on various aspects of the biological activity. This combination of predictions would offer deeper insight into the mechanism, metabolism, electronic, physicochemical and structural aspects of the substance and their influence/role in its activity. Thus, combining (Q)SAR predictions will definitely provide a more comprehensive basis to better interpret the complex biological endpoints such as carcinogenicity in the absence of empirical data.

A more formal consensus modelling approach as described in the NAFTA (Q)SAR guidance document can also be employed (NAFTA-TWG, 2012).

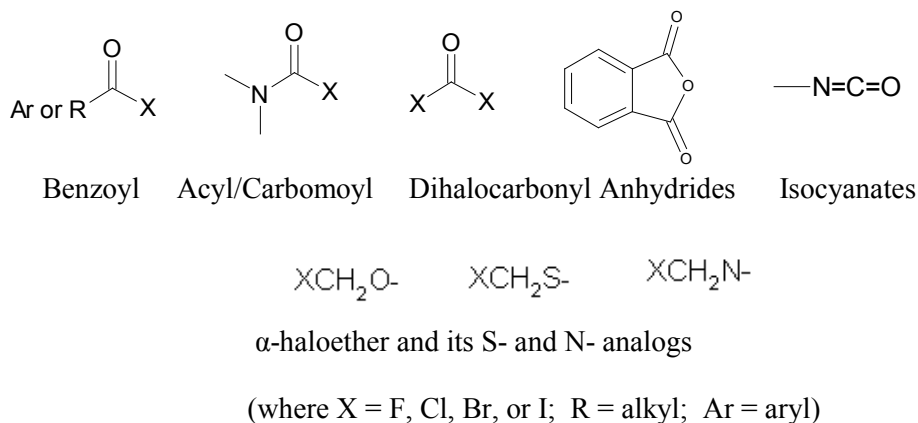
6. SPECIFIC MECHANISTIC CONSIDERATIONS FOR (Q)SAR ANALYSIS OF DIFFERENT CLASSES OF CARCINOGENS

One of the most important advantages of mechanism-based (Q)SAR analysis/approach is its flexibility. Knowledge/understanding of the mechanistic aspects of the particular toxicity allows identification of different relevant molecular descriptors and use in a customized manner to develop scientifically justified (Q)SAR approaches for different classes of substances. In this section, examples of mechanism-based (Q)SAR analysis/approaches for several mechanistically different classes of carcinogenic substances are discussed.

6.1 Direct-acting genotoxic carcinogens

Direct-acting genotoxic carcinogens are probably the most easily recognized carcinogens because of the well-spread use of genotoxic structural alerts (SA). However, genotoxic SAs are often incorrectly used to predict the carcinogenic potential of substances. As pointed out in Section 2, for a complete assessment, both toxicokinetics and toxicodynamics factors must be considered. Genotoxic SAs are essentially only toxicodynamics indicators. The presence or attachment of one or more of these groups in a molecule is suggestive of carcinogenic potential. Whether their presence may actually impart carcinogenic activity is dependent on a variety of factors such as (a) the nature of the SA (e.g., reactivity vs. stability), (b) the physicochemical properties of the molecule to which the SA is attached (e.g., impeding vs. facilitating the ability of SA to reach target tissue), (c) the micro environment surrounding the SA (e.g. steric hindrance vs. resonance stabilization), (d) the exposure scenario (particularly for highly reactive SA that can be readily detoxified), and (e) dosage and frequency of the exposure. Judicious use of SA is needed to avoid oversensitivity. Depending on the specific conditions of the SA-bearing substance, a different concern level for carcinogenic potential may be predicted. The following are some of the special situations that users of genotoxic SAs should be aware of:

Extremely short-lived, hydrolyzable SAs: A number of SAs can be readily hydrolyzed upon contact with water. They are very short-lived (in minutes or seconds) and therefore can only be of carcinogenic concern at portal of entry (e.g., via inhalation, injection). They are unlikely to be carcinogenic via drinking water or feeding studies. These SAs include (in the order of appearance in the following illustrations): benzoyl, acyl or carbamoyl halides, dihalocarbonyl compounds (e.g., phosgene), anhydrides, isocyanates, α -haloether (e.g., bis-chloromethyl-ether) and its S- and N- analogs. The last three SAs are unusual because normally C-F bond is quite stable. However, the proximity of halogen and heteroatom causes activation of the halogen so that even F can be a good leaving group.

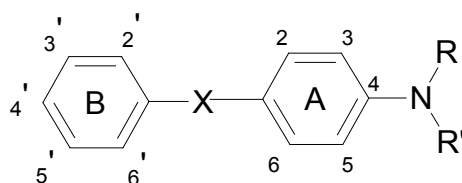


Readily detoxifiable SAs: Dialkyl sulfate and trialkyl phosphate are potential alkylating agents. However, the activity is limited to small alkyl groups such as methyl and ethyl. Beyond ethyl, the activity falls rapidly with increase in the size of the alkyl group. One important feature that may be ignored by the users is that only the first alkyl group is an alkylating agent. As soon as one alkyl group departs, the rest of the molecule (monoalkyl sulfate and dialkyl phosphate) is detoxified and should no longer be considered as a genotoxic SA. Aldehyde group is another readily detoxifiable SA because it is easily oxidizable either chemically or enzymatically. Therefore the most hazardous route is via inhalation. Unless attached to an α,β -double bond or aryl moiety, only small aldehydes (e.g., formaldehyde and acetaldehyde) are carcinogenic.

Situations that may enhance carcinogenic potential: There are many situations in which the combination of SA with other factors may enhance carcinogenic potential. Some of the examples are: (i) Presence of two or more SA, at terminal positions of a flexible molecule (e.g., linear alkyl chain but not aliphatic ring), 1-6 carbon atoms apart because of possible crosslinking between DNA strands or between DNA and protein. (ii) Attachment of SA to intercalating molecule (e.g., 3-ring planar) because of synergistic effect of DNA intercalation plus electrophilic reactivity. (iii) Attachment of SA to molecules resembling body constituents (e.g., nucleoside, amino acid) to facilitate transport of reactive group to target DNA. For example, the carcinogenic potency of nitrogen mustard can be increased by several folds by attaching nucleoside like uracil. (iv) Attachment of SA to resonance stabilizing moieties (e.g., α,β -double bond) to increase residence time in body as well as to add alternative reaction mechanism like Michael Addition.

6.2 Indirect-acting genotoxic carcinogens

To illustrate mechanism-based (Q)SAR analysis of indirect-acting genotoxic carcinogens, the Aromatic Amines class is used as an example. The SAR of aromatic amines has been extensively studied. The predominant activation pathway is oxidation of the amino group to generate electrophilic nitrenium ion which can be resonance-stabilized by the aryl moiety to make it stable enough to travel from the site of activation to reach and bind to DNA. The position of the amino group on the aromatic ring is very important. For linearly annulated two-ring (e.g., naphthalene) and three-ring (e.g., anthracene) system, the 2-position is almost invariably the best position to place the amino group for imparting carcinogenic activity. For two ring system with intericyclic group (see Table III), the 4-position is by far the best position for carcinogenicity. The intericyclic linkage must allow conjugation to facilitate resonance stabilization. Molecular planarity is also favorable for carcinogenicity due to ease of DNA intercalation and binding and more accessible to metabolic activation. The critical structural features can be best illustrated by the following chemical structure along with reasoning as noted in the table below:



Model aromatic amine

Table III: Effects of the nature and position(s) of substituent(s) on the carcinogenic potential of a model aromatic amine.

Critical Position/Factor	Effect on Carcinogenic Potential	Brief Reasoning
a. amino group	↑ if R/R' = H, CH ₃ ↓↓ if R/R' = <i>t</i> -alkyl	metabolic activation
b. position of amino group	relative activity: 4- >> 2- ≥ 3-	resonance stabilization
c. 3- and/or 5-	↑ if one CH ₃ ↓↓ if bulky group	favorable conformation flanking effect
d. intercyyclic group (X)	↑ if —, —O—, —S— ↓↓ if —CH ₂ —CH ₂ —	allows conjugation electron insulating
e. 2-, 2'-, 6-, 6'-	↓↓ if bulky group	distorts planarity
f. 4'-	↑ if -NH ₂ ↑ if -F ↓↓ if -COOH, -SO ₃ H	extended conjugation blocks detoxification ↓ absorption, ↑ excretion

These structural features can basically be used by the hazard / risk assessors as a guide to applying qualitative modification factors to consider carcinogenic potential of an aromatic amine compound. The modification factor(s) of a number of other chemical classes / subclasses have also been developed and summarized (Woo and Lai, 2003, 2010, 2015; Lai and Woo, 2012; see also, Klaunig, 2013).

6.3 Nongenotoxic carcinogens

It is generally recognized that structurally similar substances may have a much higher likelihood of sharing a common mechanism of action than dissimilar substances. It remains to be explored whether the reverse (i.e., whether mechanistically similar substances may share common structural features) is also true. This section shows that at least for rodent hepatocarcinogens that act via peroxisome proliferation, common structural features can be found despite heterogeneous chemical structure.

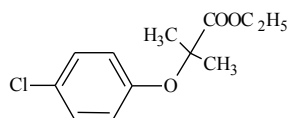
Peroxisome proliferators were first considered to be a novel class of chemical carcinogens by Reddy et al. (1980). Since then, many hepatocarcinogenic peroxisome proliferators have been found in pharmaceuticals, pesticides, industrial substances and environmental contaminants. Many of them are now known to act through the peroxisome proliferator-activation receptor α (e.g., Gonzalez, 2002; Corton et al., 2014) although a few may involve more than one receptor. The structure-activity relationship has been reviewed (e.g. Woo and Lai, 2003, 2010; Lai, 2004; Lake et al., 1993; Corton et al., 2014). Figure 6 shows the chemical structures of a variety of hepatocarcinogenic peroxisome proliferators (Woo and Lai, 2003).

Although at first glance, the chemical structures appear to be diverse, from the mechanistic point of view, there are two common features: (a) virtually all the compounds contain (either *per se* or after oxidation of a hydrolysis product) a hydrophilic group (in most cases, a carboxylic acid) at one end and a nonpolar moiety at the other end, and (b) most of the compounds contain structural features that are suggestive of resistance to metabolic detoxification. For example, for substituted phenoxy acid pharmaceuticals and pesticides, one of the key structural elements for peroxisome proliferative activity is chlorine substitution at the 4-position of the phenyl ring. When the chlorine atom is at the 2-, or 3- position of the phenyl ring, peroxisome proliferation is totally abolished. The 4-position is usually the most important position for detoxification. Chlorine substitution at the 4-position may discourage metabolic detoxification as well as exert some electronic effect. Most of the more potent compounds also have substitution at the ω -1 carbon (the carbon next to the terminal carboxylic acid).

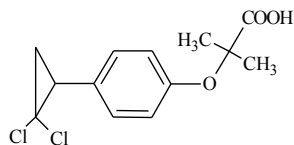
For the substituted *n*-alkyl carboxylic acids, most of the active compounds are also substituted at ω -1 carbon which renders the compound resistant to metabolism; the most effective substituent is an ethyl group. Several 2-ethylhexyl-containing esters such as di-(2-ethylhexyl) adipate (DEHA), di-(2-ethylhexyl)phthalate (DEHP) and di-(2-isononyl) phthalate (DINP) induce peroxisome proliferation because they are readily hydrolyzed to yield 2-ethylhexanol, which is further oxidized to 2-ethylhexanoic acid, an active peroxisome proliferator (Reddy and Lalwai, 1983). As compared to 2-ethylhexanoic acid, the 3- and 4-isomers are virtually inactive in peroxisomal responses (*cited in*: Lake et al., 1993). Perfluorooctanoic acid (PFOA), a rodent hepatocarcinogen with long biological half-life (Olsen et al., 2007) is also believed to owe its activity to resistance to metabolism because of the strength of the C-F bonds.

The human relevance of PPAR α -mediated rodent hepatocarcinogenesis has been the subject of intensive debate for more than a decade. Whereas the relevancy cannot be totally disregarded, considering the totality of the evidence, most regulatory agencies are supportive of the conclusion that this mechanism is unlikely to be relevant to humans (Klaunig et al., 2003; Lai, 2004; Corton et al., 2014). Nevertheless, the SAR features mentioned above can be effectively used to interpret rodent cancer data and assess the significance of human cancer risk as well as designing safer substances. Similar studies/exploration should also be extended to other mechanistically grouped substances to help finding structural features of importance.

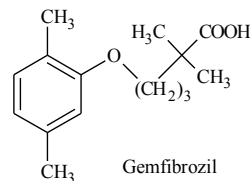
Endocrine disruptors, particularly estrogen receptor (ER) binding chemicals, are another group of mostly nongenotoxic chemicals that may induce cancer indirectly via causing hormonal imbalance. Some recent (Q)SAR studies of endocrine disruptors in general (Woo and Lai, 2015) and ER binding chemicals in particular have been highlighted in section 8

(1) Substituted Phenoxyacid Pharmaceuticals and Pesticides

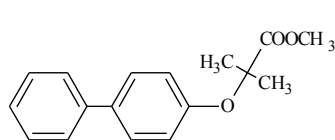
Clofibrate



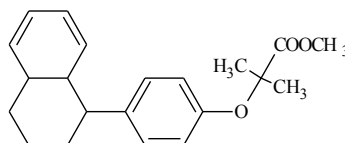
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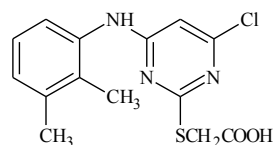
Gemfibrozil



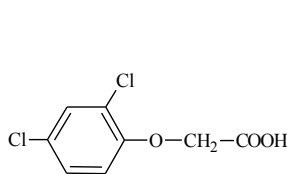
Methylclofenapate



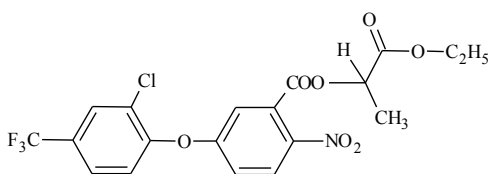
Nafenopino



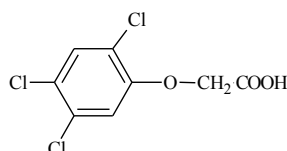
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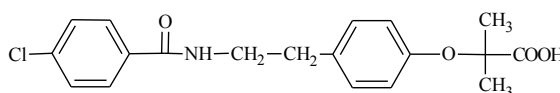
2,4-D



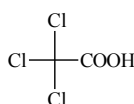
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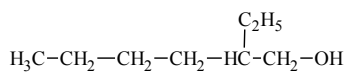
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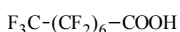
Bezafibrate

(2) Alkyl Carboxylic Acids and Precursors

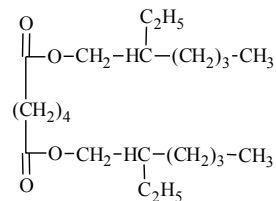
Trichloroacetic acid



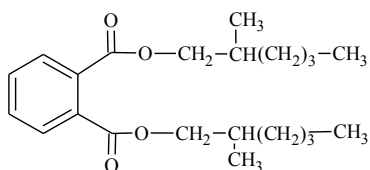
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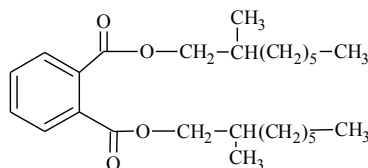
Perfluorooctanoic Acid



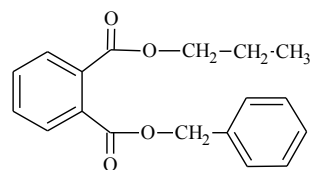
DEHA

(3) Phthalate Esters

Di(2-ethylhexyl)phthalate



DINP



BBP

Figure 6: Diverse chemical structures of PPAR α hepatocarcinogens

7. INTEGRATING CHEMOINFORMATICS, BIOINFORMATICS AND SHORT-TERM PREDICTIVE ASSAYS IN MECHANISM-BASED (Q)SAR ANALYSIS OF CARCINOGENICITY

7.1 Short-term biological tests providing mechanistic information for (Q)SAR analysis of carcinogens

There are numerous *in vitro* and *in vivo* short-term assays that can provide valuable mechanistic information for assessment of the carcinogenic potential of substances in an integrated (Q)SAR analysis. In this section, several of the most promising assays are discussed together with related recent findings and development. Results from these assays can be used to provide mechanistic support to the development, validation and interpretation of (Q)SAR studies in an integrated approach.

7.1.1 Genotoxicity assays - Perspective on their use to pre-screen potential carcinogens

Even though the existence of nongenotoxic carcinogens has been recognized decades ago (Chouroulinkov, 1988; Yamasaki et al., 1992) the somatic mutation theory of cancer has been by and large regarded as the predominant paradigm for the induction of cancer by substances, and has inspired the majority of studies. Two independent lines of research have been ongoing: a) the research by the Millers' pointing to the carcinogenic properties of the electrophilic substances, potentially able to react with the DNA; b) the research of genetic toxicologists on the ability of substances to induce mutation, thus being potentially able to elicit heritable genetic damage. A highly productive cross-fertilization between the two fields gave rise to: a) the theory that electrophilic substances (per se, or after metabolic transformation) are able to induce both mutations and cancer; and b) the generation of mutagenicity short-term tests (STT) (e.g., the Salmonella typhimurium or Ames test, incorporating metabolic activation) for identifying mutagenic / genotoxic substances (hence potential carcinogens). Prompted by the success obtained, subsequent major research efforts focused on the hypothesis "Mutation = Cancer": more than 100 STTs were developed, based on different genetic endpoints and types of cells, as to (hopefully) complement the Salmonella assay (Zeiger, 2004). The use of STTs for pre-screening of potential genotoxic carcinogens has been accepted by the scientific community and incorporated into regulatory schemes but the scientific basis/validity for some of these STTs may be questionable.

These regulatory schemes and strategies vary to a large extent, depending on the types of substances and intended use (e.g., whether the substances are industrial substances, pharmaceutical drugs, food additives or constituents); they also vary from one regulatory authority to another. However, a dominant trend can be recognized. Most often, a 2-tiered integrated testing approach is used. Tier 1 includes *in vitro* assays. In this tier, bacterial mutation assays (such as the Ames test) are used first, followed by tests based on *in vitro* mammalian cells (detecting gene mutations or chromosomal aberrations). Tier 2 involves the use of short-term *in vivo* studies (usually a bone-marrow cytogenetics assay) to assess whether any potential for mutagenicity detected at the Tier 1 *in vitro* stage is actually expressed in the whole animal. Thus, negative results *in vitro* are usually considered sufficient to indicate lack of mutagenicity, whereas a positive result is not considered sufficient to indicate that the substance represents a mutagenic hazard (i.e. it could be a false, or misleading positive). The above approach to mutagenicity testing has a fundamental theoretical unity, and has been recommended internationally as part of the strategy for predicting and quantifying mutagenic and carcinogenic hazard.

The evidence accumulated in more than 30 years of genotoxicity testing permits to draw conclusions on the scientific hypotheses that have given shape to the pre-screening strategies for carcinogens. It appears that the original hypothesis "mutation = cancer" is only valid within the limited

area of the DNA-reactive substances: these induce cancer, together with a wide spectrum of mutations. For these substances, the best predictor of carcinogenicity is the Ames test (Zeiger, 1998; Benigni et al., 2010); hundreds of genotoxic carcinogens have been correctly predicted or verified by the Ames test. However, it should be cautioned that false negatives may occur if the necessary activating enzyme(s) of the test chemicals is(are) not present in the S-9 mix of the Ames test. For example, aryl azo compounds and cycasin require preincubation with azo reductase (Prival et al., 1988) and β -glucosidase (see Cycasin chapter in Woo et al., 1988), respectively. Safrole, methyleugenol and related alkenyl benzene compounds require sulfotransferase (SULT) activation to acquire mutagenic activity. SULT-enabled strains of *Salmonella typhimurium* have been developed and shown to give positive result for hydroxylated metabolites of methyleugenol and related compounds in the Ames test (Herrmann et al., 2012). Several low molecular weight carcinogens (e.g., ethyl acrylate, allyl chloride) yielded negative or inconclusive Ames tests (Emmert et al., 2006). Ames test has also been considered inappropriate for biocidal compounds and antibiotics due to their high cytotoxicity for bacteria. Substances with low bioavailability that require endocytosis/phagocytosis for cellular uptake (e.g, nanomaterial and larger particles, insoluble crystalline metals/metal compounds, microcystin, etc.) may also be inappropriate due to the lack of endocytosis/ phagocytosis capability in bacteria. For such cases, a gene mutation test in mammalian cells (e.g., hprt test, mouse lymphoma assay) is considered more appropriate (EC_SCCS, 2014).

In addition to the Ames test, *in vitro* micronucleus tests for both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations are also recommended for screening purpose (EC_SCCS, 2014; EURL_ECVAM, 2014). These tests may increase the sensitivity by expanding coverage from the mechanistic point of view by including chromosome aberrations. However, they are often prone to yielding false positive (Zeiger, 1998; Benigni et al., 2010) particularly if rodent cell lines are used (Fowler et al., 2012). Fowler et al. (2014) recently showed that the predictive performance of micronucleus tests can be substantially enhanced by using human lymphoblastoid cells (TK6) or peripheral blood lymphocytes (HuLy) with functional p53. More efforts are needed to develop or validate *in vitro*, mutagenicity-based STTs that may efficiently complement Ames test for predicting genotoxic carcinogenic potential of untested chemicals.

The other working hypothesis was that *in vitro* positives should be confirmed through an *in vivo* genotoxicity assay; However, it has been demonstrated that existing *in vivo* tests are insensitive and may give a majority of false negative results for many clearly genotoxic carcinogens (Benigni et al., 2012b). For most regulatory agencies, a negative *in vivo* genotoxicity study of a substance---particularly for low production/exposure substances--- is often equated to lack of genotoxic carcinogenic concern with subsequent reduction or elimination of regulatory requirement of a standard 2-year cancer bioassay.

The above weakness of genotoxicity assays for predicting carcinogens is now largely recognized, and operational improvements are sought by trying to manipulate the assay systems to, for example, reduce the sensitivity of *in vitro* complements to *Salmonella* and to improve the sensitivity of the *in vivo* systems (Kirkland et al., 2007; Kirkland and Speit, 2008). One of the major reasons for insensitivity of the widely used bone marrow micronucleus assay is limited ability for unstable mutagens or short-lived reactive metabolites to reach the bone marrow due to detoxification along the way. Alternative *in vivo* micronucleus assays have been developed using hepatocytes or peripheral erythrocytes; the potential improvement remains to be systematically studied. One possible way to reduce false negatives is to include a positive control using a known chemical carcinogen that is structurally related to the substance of interest to ensure that the assay is sensitive enough for the substance class. Alternative assays such as *in vivo* DNA binding may be helpful in finding potential false negatives. Chemicals that may have organ-specific *in situ* reactivation (e.g., re-activation of GSH-/cysteine-conjugate of halogenated alkanes/alkenes by β -lyase in kidney, β -glucuronidase reactivation of glucuronidated aromatic amines in urinary bladder) are likely to give false negatives in most *in vivo* tests that do not involve the potential target organs (Kadlubar et al. 1977; MacFarlane et al. 1989).

7.1.2 Type/Quality of DNA adduct data

Covalent binding of an electrophilic substance to nucleophilic sites in DNA to form DNA adducts has been widely recognized to be an important step to tumor initiation. DNA adducts have now been consistently used as a biomarker of exposure to genotoxic substances but its role as a marker for cancer risk and molecular epidemiology is still subject to debate (e.g., Taningher et al., 1990; Hemminki et al., 2001; Swenberg et al., 2011). Some organizational approaches for the assessment of DNA adduct data (e.g., type of adduct, frequency, persistence, type of repair process) in concert with other relevant data, such as dosimetry, toxicity, mutagenicity, genotoxicity, and tumor incidence, to inform characterization of the mode of action were initiated with the ultimate goal of developing a framework for use of DNA adduct data in risk assessment (Jarabek et al., 2009; Himmelstein et al., 2009). Much of the uncertainty appears to be associated with *in vivo* persistence of DNA adducts. A recent study by Naiman et al. (2012) provided firm evidence that the induction of urinary bladder cancer in rats by 2-methoxyaniline (*o*-anisidine) can be correlated to the target-specific *in vivo* persistence of DNA adducts. As much as 39% DNA adducts still remained in the bladder 36 weeks after treatment, whereas no persistent DNA adducts were observed in the non-target organs liver, kidney and spleen.

DNA adduct data, if available, can be used to support (Q)SAR predictions of individual substances (e.g., Woo et al., 1998). Given the complexity of adduct persistency and repair it is unlikely to be used directly as a quantitative predictor of carcinogenic potency. However, together with the covalent binding index, DNA adduct data can be useful as a qualitative indicator for mechanistic support for carcinogenicity. Depending on the quality of the DNA adduct data (see Table IV) different confidence / concern level may be assigned.

In general, unless the substance is known to be highly unstable and can only have portal of entry effect, *in vivo* DNA binding data may be considered to generate more concern than *in vitro* data. For the highly unstable substances, the *in vivo* data should include portal of entry tissues. The nature of the DNA adduct is also mechanistically important. Some adducts (e.g., the cyclic etheno adducts) may be more stable than others.

Table IV: Type/Quality of DNA adduct data

Type of DNA adduct data	Carcinogenicity Concern Level*
<i>In vitro</i> DNA binding	LM
<i>In vivo</i> DNA binding	LM/M
<i>In vivo</i> DNA binding with stable adduct(s) <u>or</u> binding at position(s) that affect base pairing	M

<i>In vivo</i> DNA binding with stable adduct <u>and</u> binding at position(s) that affect base pairing	M/HM
<i>In vivo</i> DNA binding with stable adduct <u>or</u> binding at position(s) that affect base pairing <u>and</u> evidence of persistence	M/HM
<i>In vivo</i> DNA binding with stable adduct <u>and</u> binding at position(s) that affect base pairing <u>and</u> evidence of persistence	HM/H

*LM = Low Moderate, M = Moderate, HM = High Moderate, H = High

Adducts with covalent binding at nucleoside position(s) that interfere with base pairing (e.g., O⁶ position of guanine) are expected to be more promutagenic than those that do not affect base pairing (e.g., N-7 position of guanine). As mentioned earlier, availability of evidence of persistent DNA adducts is another critical reason to have high concern for carcinogenicity potential. The proposed carcinogenicity concern level of each of the different types of DNA adduct data is summarized in the Table IV.

7.1.3 Cell transformation assays (CTAs)

Cell transformation assays (CTAs) were developed several decades ago to study chemical carcinogens (e.g., DiPaolo et al., 1969). The CTAs, particularly the Syrian Hamster Embryo or SHE (e.g., Isfort and LeBoeuf, 1995; Vasseur and Lasne, 2012; Benigni et al. 2015) and Bhas 42 (Sakai et al., 2011; Sasaki et al., 2015) assays, have been used to predict carcinogenic potential of substances as well as for mechanistic studies. The SHE cells are primary cells that can mimic all the three stage of carcinogenesis process whereas Bhas 42 cells are non-transformed BALB/c 3T3 cells that have been transfected with human *ras* oncogene and can act as initiated cells. There has been a surge in the interest for using CTAs because of their ability to detect both genotoxic and nongenotoxic carcinogens as well as metal carcinogens. Based on predictive performance and evidence for intra- and inter-laboratory reproducibility, the merits of SHE and Bhas 42 assays are currently under consideration by OECD. The SHE CTA has recently been accepted as a potential screening test (OECD, 2015) but the testing guideline remains to be finalized. The CTA using the C3H10T1/2 cell line was considered to be useful to elucidate molecular mechanisms of cell transformation at the genomic and transcriptomic level with possible prediction of some carcinogenic mechanisms such as cell proliferation via ornithine decarboxylase induction, peroxisome proliferation (Landkocz et al. 2011; Vasseur and Lasne, 2012). Benigni and Bossa (2011) showed that a tiered strategy, with inexpensive and fast tests in Tier 1 (e.g. the Ames test or structural alerts) and the Syrian hamster embryo CTA in Tier 2, is able to identify up to 90% of carcinogens. Expanding the previous investigation by (i) including results of cell transformation assays on inorganics, together with an additional assay (Bhas 42), and (ii) considering new structural alerts for nongenotoxic carcinogenicity, Benigni et al. (2013b) were able to identify both genotoxic and nongenotoxic carcinogens, with an estimated 90-95% sensitivity. The above tiered approach was also demonstrated to be able to identify almost all the human carcinogens in IARC classes 1 and 2 (Benigni et al., 2013c).

7.1.4 Toxicogenomics

Pivotal roles of toxicogenomics in mechanisms-based (Q)SAR: As may be expected, the scientific reliability of mechanism-based (Q)SAR is dependent on the accuracy of the known or proposed mechanism of action and supportive data. There are cases in which the mechanism may have to be hypothesized. Toxicogenomics studies can be a perfect tool to explore or confirm the mechanism of action. With major advancements in microarrays and bioinformatics, it is now possible to mine gene expression changes across thousands of genes to derive expression signatures that can identify relevant biological pathways and elucidate mechanism/mode of action. Beyond mechanistic input, toxicogenomics studies can also provide valuable information for test design, internal dosimetry, dose-response, risk assessment, and human relevancy of animal toxicants (e.g., Sen et al., 2010; Thybaud et al., 2007; Thomas et al., 2009; Ellinger-Ziegelbauer et al., 2009; Sanderson, 2012; Low et al., 2011). Closer collaboration between toxicogenomics and (Q)SAR researchers, particularly in the selection of substances for testing, may lead to even more useful findings.

Toxicogenomics as a predictive tool for carcinogenic potential of chemicals: The feasibility of using short-term gene expression/transcriptional profiles to predict long-term toxic effects has been actively pursued in the past several years. Among the various toxicity endpoints, cancer is one of the most targeted endpoints for toxicogenomics-based predictive models because of the potentially tremendous saving in cost, time, labor and animals.

Thus far, most of the studies have focused on predicting rat hepatocarcinogens. Nie et al. (2006) were able to predict nongenotoxic rat hepatocarcinogens with an accuracy of 88.5% based on the gene expression data of 6 genes signature in male rats treated for 24 hr. In these studies, the molecular mechanisms of nongenotoxic rat hepatocarcinogenesis appeared to all be linked to the well-known oncogene, c-myc. Ellinger-Ziegelbauer et al. (2008) identified separate characteristics in 14-day gene expression profiles for genotoxic and nongenotoxic carcinogens and were able to predict a set of independent validation compounds with up to 88% accuracy. Auerbach et al. (2010) developed a set of seven gene expression models based on a combination of rat 2-, 14-, and 90-day exposure data to a collection of 4 positive and 3 negative hepatocarcinogens and used the models to predict hepatocarcinogenic potential of alkenylbenzene flavoring agents. The models differentiated between known hepatocarcinogenic (estragole and safrole) and nonhepatocarcinogenic (anethole, eugenol and isoeugenol) alkenylbenzenes. In the case of safrole, the models correctly differentiated between carcinogenic and non-carcinogenic dose levels. Two untested alkenylbenzenes, myristicin and isosafrole, were predicted to be possible (but somewhat weak) hepatocarcinogens and recommended for testing with high priority. Liu et al. (2011) showed that a 5-day toxicogenomics animal model was able to predict nongenotoxic hepatocarcinogens with a predictive accuracy of 82% using 62 substances from NCTR liver cancer database. Two Japanese groups independently developed 28-day gene expression models for predicting potential rat hepatocarcinogens using large-scale databases. Matsumoto et al. (2009, 2011) developed the “CARCINOscreen” model using 47 carcinogens (both genotoxic and nongenotoxic) and 26 noncarcinogens. The predictive accuracy was close to 95% with some showing apparent dose dependency. Uehara et al. (2011) developed the “Genomics-Assisted Toxicity Evaluation System” trained with 6 nongenotoxic hepatocarcinogens and 54 noncarcinogens for 3, 7, 14, and 28 days (3 different doses) of treatment. The model gave prediction accuracies for hepatocarcinogenicity of 99% sensitivity, 97% specificity in the training data set with virtually no false positives. Pathway analysis of feature genes revealed that the mitogen-activated protein kinase p38- and phosphatidylinositol-3-kinase-centered interactome and the v-myc myelocytomatosis viral oncogene homolog-centered interactome were the 2 most significant networks. Overall, there appears to be sufficient evidence that models based on gene expression profile, particularly those with multiple time points and doses can be predictive of hepatocarcinogenic potential of substances in rats (Thomas et al., 2013; Jackson et al., 2014). It will be

interesting to see whether there are any inter-laboratory variations and whether some standardization will be needed.

In contrast to the liver, there is much less information on the use of toxicogenomics to predict carcinogenic potential of substances in other target organs. Thomas et al. (2009) used 90-day short-term gene expression profile biomarkers to predict the carcinogenic potential of 26 substances of diverse chemical structures in mouse lung. The substances were given by diverse routes (feed, gavage, and inhalation). The predictive accuracy was 79.3% with a sensitivity of 71.4% and a specificity of 86.3%. Attempts to use statistical models to predict dose-response increases in tumor incidence for two substances yielded acceptable results for methylene chloride but over-predictive results for naphthalene. Toxicogenomics studies of some kidney carcinogens are available (e.g., Vettorazzi et al., 2013) but work on predictive models has not yet been published in the open literature.

Development of *in vitro* toxicogenomics approaches: The high cost of *in vivo* toxicogenomics studies and the need for developing high throughput methods have stimulated *in vitro* toxicogenomics approaches. Comparison of *in vivo* and *in vitro* toxicogenomics data by Van Kesteren et al. (2013) showed similarities in transcriptomics response in xenobiotic metabolism, lipid metabolism, oxidative stress. However, the detection of cancer-related pathways in the *in vitro* study was more problematic. The authors emphasized the need to consider toxicokinetics when modeling a complex *in vivo* endpoint with *in vitro* models. Using Syrian Hamster Embryo (SHE) cells, Landkocz et al. (2012) identified a set of genes involved in the organization of cytoskeleton that may be predictive of hepatocarcinogens by peroxisome proliferators like di-(2-ethylhexyl) phthalate. Schaap et al. (2012) demonstrated that gene expression profiling in primary mouse hepatocytes is a useful approach to categorize non-genotoxic carcinogens according to their modes of action. The approach was effective for detecting peroxisome proliferators, metalloids and skin tumor promoters but not all non-genotoxic carcinogens. A new development of using mouse embryonic stem cells as a second *in vitro* test system to complement hepatocytes was promising in improving categorization of non-genotoxic as well as genotoxic carcinogens (Schaap et al., 2014/5). Another new high content *in vitro* toxicogenomics approach with whole genome microarray has been shown to offer pertinent biological data to support predictions of *in vivo* hepatotoxicity/hepatocarcinogenicity potential (De Abrew et al. 2015).

7.2 Integrative approaches and tools

The need to integrate chemistry-based predictive profilers and/or biology-based predictive assays for the most effective (Q)SAR analysis of chemical carcinogens has long been recognized. This is particularly important for the cancer endpoint because of the multi-stage, multi-component process of chemical carcinogenesis. In this section, a number of integrative approaches are discussed.

7.2.1 OncoLogic Functional Arm as an example of a conceptual integrative approach specific to carcinogenicity analysis

One of the first formal, available integrative approaches was the “functional arm” of OncoLogic cancer expert system which uses a mechanism-based approach to organize and integrate all available non-cancer short/medium-term predictive test/data of a substance as a tool/basis for predicting carcinogenic potential of that substance (Woo and Lai, 2005; Woo et al., 1998). In this tool, over 20 categories of predictive tests/data points were selected. These include: (a) inducers/inhibitors of oncogenes and tumor suppressor genes, (b) transgenic rodent assays, (c) genotoxicity-related physicochemical properties and assays, (d) epigenetic assays, and (e) available subchronic toxicity data indicative/suggestive of carcinogenicity. Each test / datum is assessed in terms of its contribution to the initiation, promotion, or progression stage of carcinogenesis (Figure 7). Depending on the type of test/datum (e.g., *in vitro* vs. *in vivo*), the dose/concentration eliciting positive/negative effect and the severity of the effect, a scoring

system has been assigned by expert judgment. Out of a full total score of 100, the maximum allowable scores for the initiation, promotion, and progression stages are 40, 30 and 30, respectively.

When assessing a test substance, the top three scores in each of the initiation, promotion and progression stages are used. Both positive and negative tests/data are taken into consideration. If multiple tests/data are available in the same mechanistic stage, then the weight given to redundant test/data gets progressively lower. The scores of all the selected tests/data are integrated to give a final score that, in turn, can be converted to concern level. The output of the “functional arm” can be used in hazard assessment by itself or to double check the result from the SAR analysis. In cases in which the substance in question does not have all the tests/data available for all three stages of carcinogenesis, then a partial score will be given together with a summary of data gaps/deficiencies. The functional arm is freely downloadable at US EPA’s Sustainable Future program website (<http://www.epa.gov/oppt/sf/pubs/oncologic.htm>) for use by anyone.

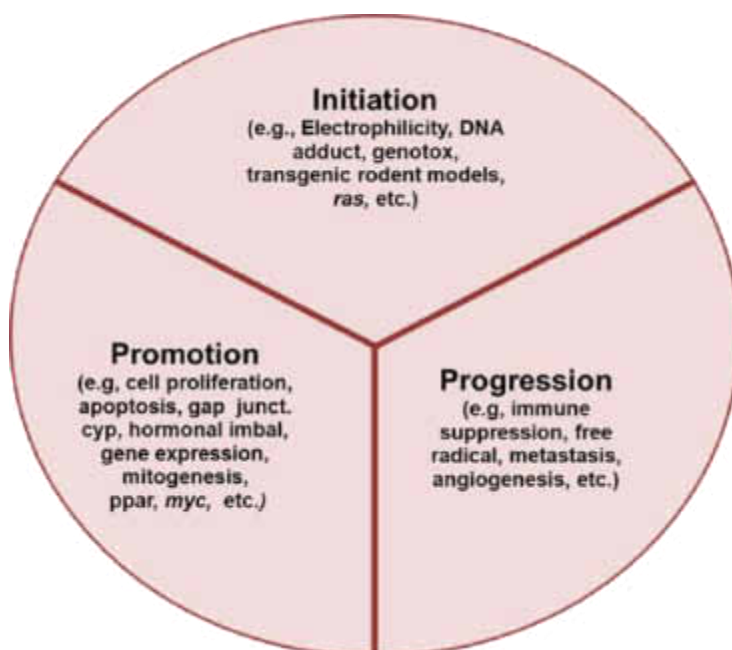


Figure 7: Functional arm of OncoLogic: Integrating predictive tests based on mechanism

7.2.2 OECD QSAR Toolbox – a simple tool allowing complex analyses

The OECD QSAR Toolbox is a platform that allows the user to profile a target substance structure in terms of toxicological mechanisms, metabolism and physicochemical properties (Diderich, 2010; Dimitrov and Mekenyan, 2010). It also aids in the process of identification of analogs that possess empirical toxicity data and have matching mechanistic profiles. In addition, the Toolbox has features that help identify tautomers of a substance. As mentioned earlier, the Toolbox consists of various built-in mechanistic profilers relating to genotoxic and non-genotoxic carcinogenicity (Table V).

Each profiler is built on a specific set of structural alerts that comprises of functional groups and structural features with known associations with specific genotoxic/non-genotoxic carcinogenicity endpoints. Presence of a profiler flag on a substance does not necessarily indicate activity and similarly its absence may not rule that out completely because profiler algorithms are based only on available knowledge. However, the profiler information is more useful when used in combination with (Q)SAR predictions since they provide mechanistic justification for the toxicological endpoint being examined. For

instance, if a substance is predicted positive for Ames mutagenicity by (Q)SAR models and also flagged for DNA binding then there is an increase in weight of evidence. The other use is in the evaluation of suitability of an analog or composition of substance category members in terms of mechanistic similarity with respect to toxicological endpoint. For instance, substances that share common structural features, have similar physicochemical characteristics, have identical DNA binding profiles, generate similar metabolites and are predicted positive for Ames mutagenicity by (Q)SAR models may be considered suitable for the formation of a substance category.

The Toolbox contains various metabolic simulators that generate potential metabolites for a given target substance (Figure 8). In certain situations the simulators prove extremely useful. For instance, when the parent substance structure is predicted Ames positive by (Q)SAR models but is not flagged for any potential mutagenic activity by, for example DNA binding profiler, then in this situation it is useful to examine the potential biological activities of its metabolites. The simulators also help to determine if two or more substances that are being scrutinized for their analogous behaviour share a common metabolite or common metabolic pathway. This piece of mechanistic information adds confidence in the selection of the analog(s).

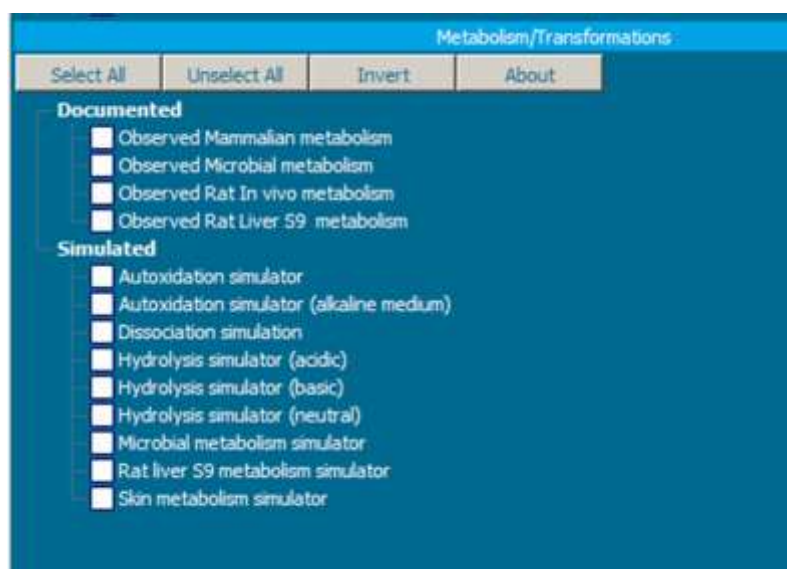


Figure 8: Metabolism feature in OECD QSAR Toolbox showing metabolic simulators (adapted from OECD QSAR Toolbox v3.3)

The Toolbox contains a feature to examine the presence of tautomerism in a given chemical structure. In addition, there is an Empiric profiler called 'Tautomers unstable' that can identify if a tautomer is unstable or stable. Tautomerism is defined as the existence of two or more substances that are capable of facile interconversion, in many cases merely a simple proton transfer in an intramolecular fashion forming a covalent bond. Unlike other classes of isomers, tautomers exist in mobile equilibrium with each other. It is important to note that tautomers have the same molecular formula but different SMILES notation. From the perspective of structure-activity relationship, this may have important consequences on the nature of profiler flags, analog selection and (Q)SAR prediction. Since tautomerism can play such an important role in physical, chemical and biological properties of a substance, it is very useful to consider this aspect in analog selection for building categories and read across.

Table V: Profilers relating to genotoxicity/carcinogenicity built within OECD QSAR Toolbox

Name of profiler	Brief description
DNA binding by OASIS v1.2	Based on the 78 structural alerts known for interaction with DNA analyzed in Ames Mutagenicity model. These are separated into 7 mechanistic domains and each is further into more than 12 mechanistic alerts.
DNA binding by OECD	Based on 60 mechanistic structural alerts known for binding of organic compounds to DNA. The alerts cross six broad organic chemistry mechanisms.
Protein binding by OASIS v1.2	Based on 101 structural alerts responsible for interaction with proteins. These are separated into 11 mechanistic domains, each of which is further separated into more than 2 mechanistic alerts.
Protein binding by OECD	Based on 16 mechanistic alerts covering 52 direct acting structural alerts based on theoretical organic chemistry.
Carcinogenicity (genotox and nongenotox) alerts by ISS	Based on 55 structural alerts. Includes 35 SAs for genotoxicity and 20 SAs for nongenotoxic carcinogenicity. The SAs are molecular functional groups or substructures known to be linked to the carcinogenic activity of chemicals.
DNA alerts for AMES, MN and CA by OASIS v.1.2	Based on the 78 structural alerts responsible for interaction with DNA, especially related to Ames mutagenicity, Chromosomal aberration and Micronucleus tests. This profiler accounts for incapability of some chemicals having an alert to interact with DNA due to electronic and steric factors. This is explicitly defined by inhibition masks associated with some alerts.
<i>in vitro</i> mutagenicity (Ames test) alerts by ISS	Based on 30 structural alerts associated with mutagenic activity of chemicals.
<i>in vivo</i> mutagenicity (Micronucleus) alerts by ISS	Based on 35 structural alerts known to be linked to the induction of effects in the rodent <i>in vivo</i> micronucleus assay.
Oncologic Primary Classification	Consists of molecular definitions that mimic the structural criteria of chemical classes of potential carcinogens. Used solely for the purpose of categorization based on the definition of an OncoLogic™ class.

7.2.3 ToxCast and ‘Hallmarks of Cancer’ approach

In vitro high throughput screening (HTS) approaches are being developed in the US EPA’s ToxCast (Toxicity foreCasting) project to prioritize substances for targeted testing program and to identify gene targets/molecular pathways relevant to human cancer progression (Kavlock et al., 2012). In the recently completed Phase I study of the ToxCast project, Kleinstreuer et al. (2013) developed a model for predicting rodent carcinogenicity based on data from 292 substances tested in 672 HTS assays targeting 455 genes, proteins, pathways, and cancer-related processes, including the “cancer hallmark processes” described by Hanahan and Weinberg (2000, 2011). The hypothesis is that the more cancer hazard processes a substance perturbed, the more likely the substance may be carcinogenic. The model was trained on a subset of 232 pesticides. A simple scoring function was generated to identify substances with significant *in vitro* evidence that was predictive of *in vivo* carcinogenicity in different rat tissues and organs. This scoring function when applied to an external test set of 33 pesticides was able to differentiate between possible/probable and negative/unlikely carcinogens ($p = 0.024$). However, there are a number of notable false negatives. In particular, the well-known multi-target carcinogens, ethylenethiourea and primicarb, were inactive in all the assays. Several critiques of the limitations and problems associated with the use of phase I *in vitro* data to predict *in vivo* toxicity endpoints in general (e.g., Benigni et al., 2010; Thomas et al., 2012) and carcinogenicity in particular (Benigni, 2013) have been published. It is recognized that the phase I study was preliminary in nature and that capability of HTS to explore mechanistic insight is highly useful for mechanism-based (Q)SAR analysis. The Phase II study is currently under way.

7.2.4 Mode of Action (MOA)/Human Relevance (HR) and Adverse Outcome Pathways (AOP)

The MOA and AOP are essentially similar frameworks that mainly differ in their origin (MOA for human health community; AOP for ecological and QSAR community). Efforts are under way to harmonize the terminology (http://aopkb.org/aopwiki/index.php/Main_Page). Both frameworks provide a structured approach for organising and describing biologically plausible mechanistic information on specific adverse outcomes (toxicity endpoints) through sequences of events commencing with initial interactions of a chemical or group of similar chemicals with a biomolecule in a target cell or tissue (i.e., molecular initiating event), progressing through a dependent series of intermediate events and culminating with an adverse outcome ((Seed et al., 2005, Boobis et al., 2006, 2008, Meek et al., 2013, Corton et al., 2014). They describe key cytological and biochemical events that are both measurable and necessary for the progression of a specific pathway to an observed effect.

The plausibility a specific MOA/AOP (including these for carcinogenicity) is dependent on the analysis of the linkages between the molecular initiating event, the intermediate key events and an adverse outcome in terms of: (1) strength, consistency and specificity of the association (http://aopkb.org/aopwiki/index.php/Main_Page), (2) temporal relationships, (3) dose-response relationship, and (4) evaluation of possible competing alternative MOAs (Seed et al., 2005, Boobis et al., 2006, 2008, Meek et al., 2013, Corton et al., 2014). In a way, the MOA and/or AOP can be considered as the ultimate integrative approach which provides a transparent and scientifically-based framework to present the progression of a toxicological response from the initial interaction of the substance to the adverse outcome.

Specific MOA and AOP can provide supportive or confirmatory evidence for mechanism-based (Q)SAR if the structural and/or activity aspects or a substance of interest can be related to that of substances in established MOA or AOP. Even an incomplete MOA or AOP can be helpful if they contain supportive information—such as knockout mice studies, reporter assays, enzyme induction or TSH data—that can reinforce postulated mechanisms. They can also help to uncover limitations in some conventional (Q)SAR assumptions. For example, MOA studies by Cruzan et al. (2013) showed that, contrary to

assumption, the epoxide of styrene is not the ultimate carcinogenic intermediate. On the other hand, (Q)SAR can help MOA and AOP studies by providing mechanistic chemistry, guidance on selection of analogs to explore mechanism of action, and toxicokinetics information that can help define range of activity or cut-off points.

7.2.5 Strategic testing using a combined *in silico* + *in vitro* approach

Since untested chemicals make up a very large majority of all existing chemicals (e.g., Judson et al., 2008), it is not possible to assess their hazard/risk without some form of prediction of carcinogenic potential based on simplified alternative approaches. From what presented above, it appears that some approaches (e.g., the (Q)SAR approach, or the tiered approach based on the appraisal of DNA-reactivity and tissue disruption/ disorganization) are more promising than others. At the same time, it should be emphasized that this process-----as internationally accepted---- is based on the integration of different tools, both computerized and experimental: strategic testing as part of an integrated testing strategy to maximize information and avoid the use of animals where possible is fast becoming the norm with the advent of new legislations. However, two opposite types of skepticism inhibit a wider use of alternative approaches (for example (Q)SARs). Regulatory hazard assessors are suspicious of negative model predictions for the non-carcinogens, because of concerns about possible false negatives from the prediction models. Industrial safety assessors are suspicious of positive model predictions for the carcinogens because of possible false positives from the prediction models. Both of the above concerns tend to encourage continued default use of the rodent carcinogen assay. Among others, this has the consequence that chemicals under investigation may remain in the market for a further 10 years, even when proven to be carcinogenic at the end of the rodent bioassay. This may have deleterious consequences for human health. At the present-state-of-art, the apparent dilemma concerning false negatives and false positives probably cannot be solved simultaneously: classifying untested chemicals as carcinogen or noncarcinogen are different scientific issues and require separate solutions. Taking as an example the tiered approach including DNA-reactivity and Cell Transformation assays (CTAs), the issue of false negatives in classifying non-carcinogens is mainly a (Q)SAR issue which can be eliminated through the use of Structural Alerts conservatively designed to remove all known carcinogens from the queue of untested chemicals. When necessary, the CTAs can help in identifying carcinogens missed by (Q)SAR. In this way, a probably large range of chemicals can be classified as “safe” based on limited data (SAs and / or Ames, SHE and/or Bhas42), and without animal experimentation. The issue of false positives, on the other hand, is a Weight-Of-Evidence issue which can be eliminated through sequential testing driven by mechanistic hypotheses for the chemical structure, without the use of the rodent carcinogen assay at least for initial classification (Veith et al., 2012). Relatively simple toxicokinetics considerations and/or studies can also help to minimize false positives.

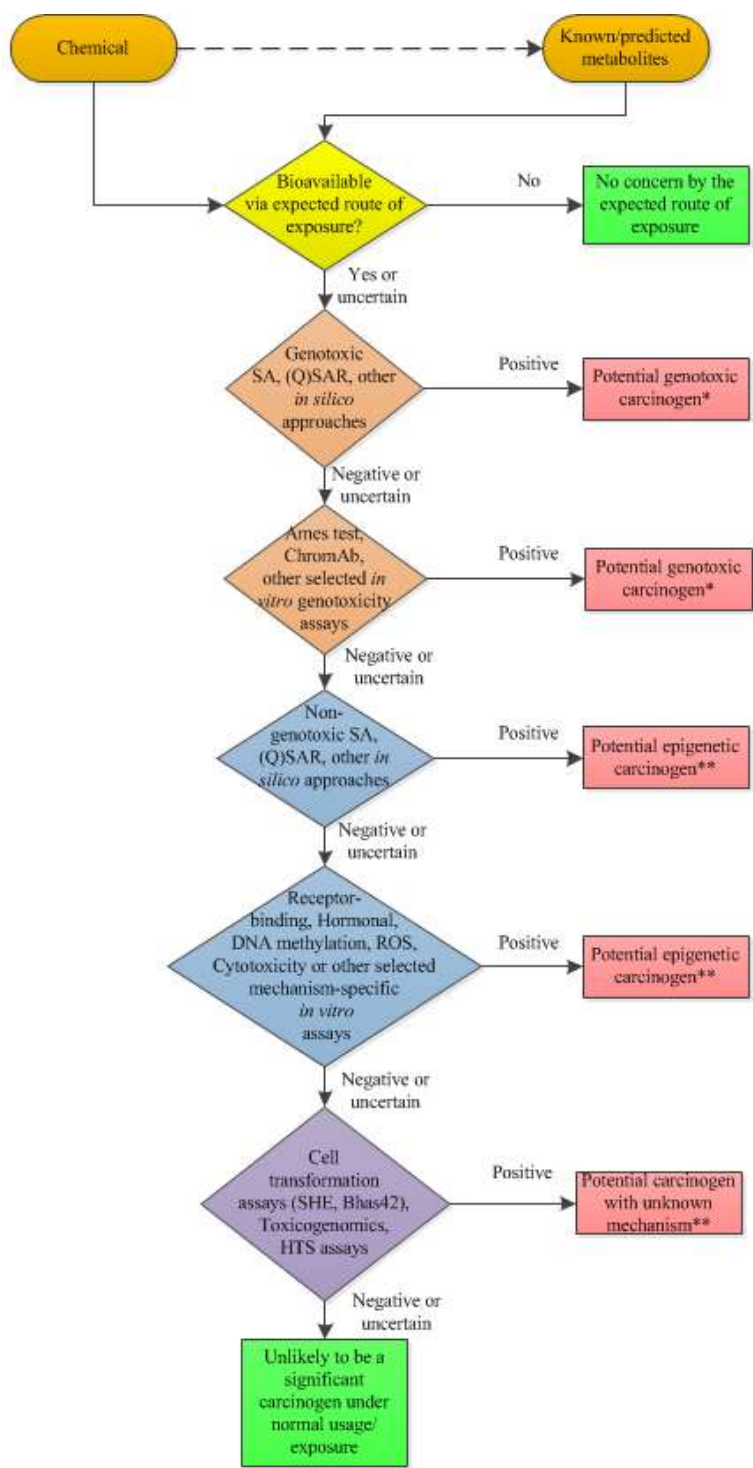
The integrative approach of using (Q)SAR and selected predictive tests as a basis to assess carcinogenic potential of chemicals with reduction in animal testing was the subject of two recent International QSAR Foundation workshops. The presentations given at the workshop can be viewed from the foundation website (<http://www.qsari.org/index.php/workshops>). One of the major goals of the workshops was to develop a combined *in silico* + *in vitro* approach to pre-screen chemicals for potential carcinogens. A prototype of such an approach was developed by Benigni (Benigni, 2014). In the present document, this prototype has been expanded to screen untested chemicals for potential carcinogens as well as to predict whether the carcinogens may be genotoxic (Figure 9). For some predicted nongenotoxic carcinogens, there is possibility of predicting possible mechanism of action to determine relevance to humans.

In the first step, possible metabolites are identified or predicted by expert judgment or available metabolism software models. For both parent chemical and metabolites, the potential bioavailability (see section 2.1.1) by the expected route of exposure can be assessed to see whether the concern for the

chemical can be eliminated, at least for that particular route; this step can cut down the number of chemicals for assessment. Then the parent chemical and selected metabolites (activated metabolites should be given priority while detoxified metabolites should be given lower priority unless there is evidence of organ-specific reactivation mechanisms; see section 2.1.1) are sequentially examined through (a) the genotoxic *in silico* module, (b) the genotoxic *in vitro* testing module, (c) the epigenetic/nongenotoxic *in silico* module, and (d) the epigenetic/nongenotoxic *in vitro* testing module to determine qualification for listing as a potential genotoxic or epigenetic/nongenotoxic carcinogen. In any of the sequential steps (a) – (d), if there is any doubt or uncertainty about the justification for positive finding, the chemical should be passed on to the next step for evaluation. If potential carcinogens are not found among the parent or the metabolites after (a) – (d) steps, they can then be tested in (e) cell transformation assays (e.g., SHE, Bhas42; see section 7.1.3) and, if possible, in toxicogenomics (see section 7.1.4) and HTS assays (see section 7.2.3) in order to catch potential carcinogens with unknown or undefined mechanisms. This should make this logical evaluation exhaustive enough for most regulators to accept a “classification” of “no known potential to cause cancer” or “unlikely to be a significant carcinogen under normal usage/exposure”. In this case, no further tests (either *in vitro* or *in vivo*) would be required.

For the two *in silico* modules, genotoxic and nongenotoxic structural alerts (SA), (Q)SAR screening, various different models and expert systems --- encoding expert knowledge regarding the chemical induction of cancer (e.g., Toxtree, Oncologic, etc.) --- can be used. The use of SA is particularly versatile and easy because of computer automation. Mechanistic chemistry has helped to uncover or discover new SAs (e.g., Enoch and Cronin, 2010). Genotoxic SA has proven to have a high degree of success in predicting potential genotoxic carcinogens. The development of nongenotoxic SA is relatively new but already quite promising (see section 5.2.3; see also Benigni et al., 2013a; Woo and Lai, 2010, 2015) especially for those involving receptor-mediated mechanisms. When using genotoxic or nongenotoxic SA, it is important to couple with toxicokinetics considerations to improve assessment and minimize false positives. In addition to the above approaches, structural similarity analysis --- with mechanistic backing whenever possible --- can select categories of analogs which have been tested, and the argument for cancer potential begins to be weighted by association with data for chemicals in the same category. Until the science is confident enough to have distilled all possible carcinogenicity mechanisms into a (Q)SAR model / expert system, (Q)SAR is mainly seen as a tool to generate initial hypotheses regardless of the availability of test data. Chemicals that are “positive” to the above screening, i.e., with structural profiles for known pathways of carcinogenesis, would be tested to confirm the hypothesis suggested by the models.

For the genotoxic *in vitro* testing modules, as pointed out in section 7.1.1, with few exceptions, Ames test is by far the most predominant and best test for gene mutation with high success rate for predicting potential carcinogens. For substances with low bioavailability that require endocytosis/phagocytosis for cellular uptake (e.g., nanomaterial and larger particles, insoluble crystalline metals/metal compounds), a gene mutation test in mammalian cells (e.g., *hprt* test, mouse lymphoma assay) is considered more appropriate (EC_SCCS, 2014). Besides the Ames test, some selected *in vitro* micronucleus tests for both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations may be used for screening purpose (EC_SCCS, 2014; EURL_ECVAM, 2014) but with caution that they may be prone to yielding false positive (see section 7.1.1) particularly if rodent cell lines are used (Fowler et al., 2012). Fowler et al. (2014) recently showed that the predictive performance of micronucleus tests can be substantially enhanced by using human lymphoblastoid cells (TK6) or peripheral blood lymphocytes (HuLy) with functional p53.



*Subject to toxicokinetics considerations; **Subject to toxicokinetics considerations; some mechanisms may not have human relevance

Figure 9: Pre-screening of potential carcinogens using a combined *in silico* + *in vitro* approach

The nongenotoxic *in vitro* testing module may be the most challenging to assemble because there is little or no unifying concept for the vast variety of nongenotoxic mechanisms (see Section 5.2.3). More mechanism-specific predictive assays are needed for screening potential epigenetic carcinogens. The recent surge in interest in mode of action/human relevance (MOA/HR) studies has led to the development of *in vitro* receptor binding assays, receptor-mediated reporter assays and biochemical assays that can be used to predict potential epigenetic carcinogens. For some of these receptor-mediated mechanisms (such as CAR, PPAR α receptors), human relevance consideration may be used to modify the concern of predicted epigenetic carcinogens for humans. *In vitro* assays of cell proliferation, intercellular communication, immunosuppression and indicators of indirect DNA damage (e.g., 8-hydroxy-2'-deoxyguanosine), as well as imbalance/disruption of various endocrine systems and DNA methylation may also help to screen potential epigenetic carcinogens.

For the chemicals that reach the final module, cell transformation assays (SHE, Bhas42, see section 7.1.3) can be used to “catch” the remaining potential carcinogens. Both SHE (e.g., Isfort and LeBoeuf, 1995; Vasseur and Lasne, 2012) and Bhas 42 (e.g., Sakai et al., 2011; Sasaki et al., 2015) assays have been shown to mimic the key stage(s) of the *in vivo* carcinogenesis process and can detect both genotoxic as well as nongenotoxic carcinogens. It is debatable whether the two CTAs can serve as the final gatekeeper. Unless the chemical is of substantially different characteristics (e.g., nanomaterial), the combination of exhaustive negative CTA plus (Q)SAR analysis and other testing should justify the consideration of the chemical as “unlikely to be carcinogenic under normal usage/exposure”. As pointed out in section 7.1.3, the two CTAs have high positive predictivity and should be able to serve as a gatekeeper to “catch” potential carcinogens conservatively. Using Ames test, genotoxic and nongenotoxic SAs and CTAs, Benigni et al. (2013b,c) were able to identify both genotoxic and nongenotoxic carcinogens, with an estimated 90-95% sensitivity and correctly identify almost all the human carcinogens in IARC classes 1 and 2. Besides CTAs, *in vitro* toxicogenomics studies (see section 7.1.4) can be used to provide input on finding the carcinogenic potential of chemicals as well as their possible mechanisms although the cost and labor are expected to be substantially higher. Likewise, Tox21 HTS assays and the ToxCast program (see section 7.2.3) may also provide input to the gatekeeper as their predictive capabilities can be unveiled from new data analyses.

Overall, the strategic combined *in silico* + *in vitro* testing approach, depicted in Figure 9, integrates most of the major (Q)SAR and predictive tests that are effective in predicting carcinogenic potential of chemicals with mechanistic consideration. It should be capable of screening potential carcinogens from untested chemicals conservatively. It can also provide support to evaluate potential carcinogenic hazard/risk for chemicals that are banned for animal testing (e.g., cosmetics in EU). As mentioned in section 6.3, even if identified as carcinogenic, some nongenotoxic carcinogens are not necessarily of human concern because the underlying mode of action may not be applicable to humans. For chemicals that require more thorough evaluation, the stepwise assessment of various types of predictive information can help to organize weight of evidence approach. For proper evaluation of the role (Q)SAR structural alerts, the user should have basic knowledge of interplay between SA and toxicokinetics to contribute to toxic potential (see Section 6). For chemicals with little or no ADMET information or reliable predictive models, it is advisable to conduct at least some basic toxicokinetics studies or delineate salient data gaps and research needs.

8. BRIEF OVERVIEW OF MECHANISTIC (Q)SAR ANALYSIS OF OTHER TOXICITY ENDPOINTS

Besides carcinogenicity, mechanistic consideration has also been increasingly used in the (Q)SAR analysis of chemicals that may induce other toxicity endpoints. In this section, a brief overview of selected recent studies on mechanistic (Q)SAR of chemicals for several other toxicity endpoints will be presented with emphasis on comparing/contrasting with carcinogenicity and inter-relationship among different endpoints. No attempts have been made to review these endpoints in detail.

8.1 Skin sensitization

Skin sensitization, the immunological priming which may lead to allergic contact dermatitis (ACD), is one of the most extensively studied toxicity endpoint. Skin sensitization, mutagenicity and skin carcinogenicity share a common mechanistic basis of having electrophilic chemical/intermediate as the proximate/ultimate toxicant in the initiating event. Skin sensitization was at one time proposed as a possible predictor of carcinogenicity (Ashby et al., 1993) but the correlation seemed to be limited (Albert and Magee, 2000) and there is increasing evidence that most potent skin sensitizers tend to be soft electrophiles while the preferred electrophiles for carcinogenicity tend to be harder electrophiles (see Section 2.2.2). Some skin sensitizers that predominantly act by reacting with –SH group (e.g., isothiazolone pesticides, see case study 1 in Appendix) do not appear to be carcinogenic probably because DNA does not contain –SH groups. In fact, there is some evidence that skin sensitization may counter potential carcinogenic effect by competing for electrophilic intermediates needed for cancer initiation.

From the mechanistic point of view, skin sensitization is a relatively well defined endpoint with protein binding as the principal toxicodynamic step; however, the subsequent steps to allergic contact dermatitis are more complicated. The biology, structural requirements and mechanistic (Q)SAR of skin sensitizers have been reviewed (e.g., Karlberg et al., 2008). The basic requirements are low M.W. (<1000Da) chemicals that are reactive (for binding to and thus haptening protein) and have appropriate lipophilicity ($\log P \sim 2$) that may allow penetrating epidermis. There are three types of skin sensitizers: (a) electrophilic chemicals per se or chemicals that can be metabolized/activated to electrophiles, (b) radical-forming chemicals (e.g., hydroperoxides), and (c) metal ions (e.g., Ni, Co, Cr) capable of forming stable complexes with protein. Most skin sensitizers are electrophiles that bind to protein via SN2 reactions, SNAr reactions, Schiff base formations, Michael additions or acylation reactions. Their main nucleophilic targets are thiols (-SH) of cysteine and primary amines (-NH₂) of lysine in skin protein. Other nucleophilic sites include histidine (=N-), methionine (-S-) and tyrosine (-OH). The relative alkylation index (RAI) has been used to quantify the relative degree of haptening as a function of dose given. The use of RAI should be limited to series of compounds reacting by the same mechanism and does not fully consider skin penetration (see Karlberg et al., 2008). A knowledge + statistics hybrid expert system, the TIMES-SS (TIssue Metabolism Simulator for Skin Sensitization), was developed as a promising tool to predict skin sensitization potential of chemicals (Patlewicz et al., 2014).

Some notable recent mechanistic studies include development of chemical categories with well-defined applicability domain using structural alerts, mechanistic chemistry supplemented by glutathione reactivity assay and *Tetrahymena pyriformis* cytotoxicity assay (Enoch et al., 2011-2013; Nelms et al., 2013), quantitative mechanistic models using QSAR supplemented with glutathione reactivity assay (Enoch et al., 2012) or cysteine-based peptide reactivity assay (Roberts and Aptula, 2014), and adverse outcome pathway approaches (van der Veen et al., 2014; Patlewicz et al., 2014).

8.2 Endocrine disruption – Estrogen Receptor (ER) Binding

Endocrine disruptors have long been shown or suspected to cause adverse health effects in humans and wild life (e.g., Kavlock et al., 1996). Exposure to endocrine disruptors can lead to a variety of toxic effects such as ecotoxicity, reproductive and developmental toxicity and carcinogenesis (e.g., Birnbaum and Fenton, 2003). The US EPA initiated the Endocrine Disruptor Screening Program (EDSP) in 1998 and has continued to screen and test the “universe of chemicals” by various approaches (e.g., Hornung et al., 2014; Huang et al., 2014). The OECD has published a series of documents on Testing for Endocrine Disruptors under their Series on Testing and Assessment (e.g., Reports No. 111, 118, 135, 174, 181).

Endocrine disruptors may cause hormonal imbalance by a variety of means (e.g., manipulation of tropic hormone, precursors, synthesis, agonist/antagonist at receptor, secretion). Among the various approaches to study endocrine disruptors, estrogen receptor (ER) binding is one of the most actively studied. Estrogen receptors are believed to be the ancestral precursor to the evolution of steroid receptors in all vertebrate (Thornton, 2001). The binding of estrogenic chemicals to ER is believed to be the molecular initiating event (MIE) of various possible adverse outcome pathways (AOPs) that lead to toxicity in humans and wild life. The two main isoforms of ER are ER α and ER β ; they share a multi-domain architecture consisting of a DNA binding domain, a ligand binding domain, and an activation domain.

Mechanistic studies of ER binding include 3D-QSAR (e.g., Waller 2004; Serafimova et al., 2007), docking studies (e.g., Halgren et al., 2004), and relative binding affinity (RBA) to estradiol by displacement assay (e.g., Schmieder et al., 2014). The OECD QSAR Toolbox currently has:

(a) an extensive ER binding affinity database that has compiled RBA (expressed as % relative to estradiol) data of a large number of chemicals tested by four international contributors (500 tested using human ER, 147 using trout ER, 232 using rat ER, and 864 using ERs from a variety species),

(b) a mechanistic profiler capable of classifying chemicals (based on molecular weight and structural characteristics) into: (i) very strong binders (M.W. 200 – 500 with two rings with a –OH group connected to each of them, (ii) strong binders (M.W. 200 – 500 with at least one 5-/6-membered carbon ring with an unhindered –OH or –NH₂ group), (iii) moderate binders (M.W. 170 – 200 with at least one 5-/6-membered carbon ring with an unhindered –OH or –NH₂ group), (iv) weak binders (M.W. <170 with at least one 5-/6-membered carbon ring with an unhindered –OH or –NH₂ group), and (v) nonbinders (chemicals that do not fit any of the above characteristics), and

(c) an expert system developed by US EPA (Schmieder et al., 2014) to predict RBA based on a logic rule-based decision tree that encodes human experts’ mechanistic understanding with respect to both the chemical and biological aspects of the well-defined endpoint or the ER bioassay domain.

Owing to the structural diversity of chemicals that can bind to ER, RBA or structural similarity alone is often insufficient to make accurate prediction of the estrogenic and downstream toxicity potential of chemicals. Furthermore, most industrial or environmental chemicals under study have very low RBA. Various strategies of developing local models that combine RBA with mechanistic grouping and collection of *in vitro* data optimized to detect low RBA chemicals have been developed (e.g., Serafimova et al., 2007; Hornung et al., 2014; Schmieder et al., 2014). The binding domain of ER has subpockets or specific sites that allow chemical-receptor interactions. The lower RBA chemicals can only bind to one site (e.g., alkylphenols to site A and alkylanilines to site B) whereas the higher RBA chemicals such as steroids have multiple H-bonding groups and can bind to both A and B sites. Approaches to optimize screening or prioritization studies of the low RBA chemicals of environmental and regulatory concern (e.g., food use inert, FI and antimicrobial pesticides, AM) using high quality data and expert systems have been suggested (Hornung et al., 2014; Schmieder et al., 2014). Beyond mechanism-based (Q)SAR studies,

more toxicogenomics studies (e.g., Medak-Erdogan et al., 2013; Gong et al., 2014) and high throughput assays (e.g., Huang et al., 2014) should be conducted to provide integrative and confirmatory support.

The potential use of RBA or ER binding to predict potential carcinogens remains to be explored. There is strong evidence that ER binding can play an important role in estrogen-induced carcinogenesis, but the molecular mechanisms are still not clearly understood. The most widely accepted theory holds that estradiol, acting through estrogen receptor alpha (ER α), stimulates cell proliferation and initiates mutations arising from replicative errors occurring during pre-mitotic DNA synthesis. The promotional effects of estradiol then support the growth of cells harboring mutations. Over a period of time, sufficient numbers of mutations accumulate to induce neoplastic transformation (Yue et al., 2013). Besides the promotional and indirect genotoxic effects, estradiol (and its interconvertible metabolite estrone) can also exert genotoxic effects after being metabolized to catechols and then to reactive quinones that can form DNA adducts and contribute to oxidative DNA damage by reactive oxygen species (Russo and Russo, 2004; Chen et al., 2008; Yager, 2014). It is uncertain whether weak ER binders which lack direct genotoxic activities can be carcinogenic. There is also evidence that ER binding may require co-effectors/co-modulators (e.g., Katzenellenbogen and Katzenellenbogen, 2000) for transcriptional and other responses; it is not clear whether the same processes operate for all ER binders.

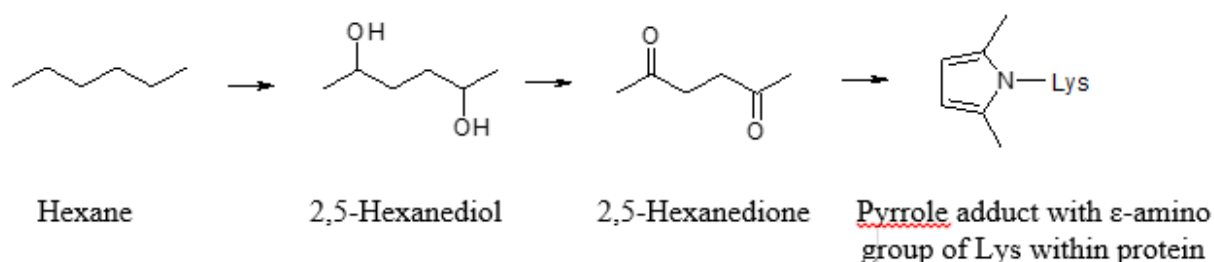
Rotroff et al. (2014) developed an ER Interaction Scoring system by integrating 13 different ToxCast *in vitro* assays for ER signaling (including binding, agonist, antagonist and cell growth response) using data from 1814 chemicals. For 36 reference chemicals, an ER Interaction score >0 showed 100% sensitivity and 87.5% specificity for classifying potential ER activity. When applied to a broader set of chemicals with *in vivo* uterotrophic data, the ER Interaction Scores showed 91% sensitivity and 65% specificity.

8.3 Neurotoxicity

Neurotoxicity is a complex endpoint with many possible manifestations such as nerve damage, nerve degeneration and neurological deficit. The molecular targets of neurotoxicants include axonal protein, enzymes (e.g., acetylcholinesterase), ion channels (e.g., sodium channel, ligand-gate chloride channel), receptor (e.g., nicotinic, GABA, etc.) and mitochondrial complex (LoPachin and Decaprio, 2005; LoPachin and Gavin, 2012; Costa, 2008). With a few exceptions as discussed below, the mechanisms of action of neurotoxicants are either unknown or poorly understood.

Protein adduct formation is a common molecular mechanism in neurotoxicity of at least three different groups of chemicals. For organophosphorus pesticides and related compounds, the phosphorylation of the –OH group of serine residue at the “esteratic site” of acetylcholinesterase, with subsequent irreversible inhibition, is generally regarded to be the mode of action of these chemicals (e.g., Fest and Schmidt, 1973). Organophosphates with small alkyl groups are also moderately active alkylating agents or potential carcinogens (e.g., Arcos et al., 1982). The competition between these two electrophilic sites is often the cause for unusual structure-activity relationships.

2,5-hexanedione (HD), the common γ -diketone metabolite of human neurotoxicant n-hexane and methyl-n-butyl ketone, has been known to initiate neurotoxic effect by binding to the ϵ -amino group of lysine residues in axonal protein to form stable 3,5-dimethylpyrrole adducts that eventually lead to central-peripheral distal axonopathy.



The mechanistic reasons for the specific neurotoxic effects of HD have been attributed to its chemical properties as a weak and hard electrophile. It is ideal for reacting slowly with the ε-amino (hard nucleophile) group of lysine. This coupled with the slow turnover of axonal protein allow accumulation of HD adducts to a toxic threshold concentration that causes cumulative γ-diketone neurotoxicity (Lopachin and Decaprio, 2005; Lopachin and Gavin, 2014). The neurotoxicity of HD is a perfect example of the importance of mechanistic understanding in (Q)SAR analysis of structurally closely related chemicals. Within the entire n-alkane homologous series, HD is the unique member that is best suited for its ability to provide the γ-spaced diketone. The 2- and 5-positions are sterically the most favorable positions for enzymatic hydroxylation to secondary alcohols that can be oxidized to ketones.

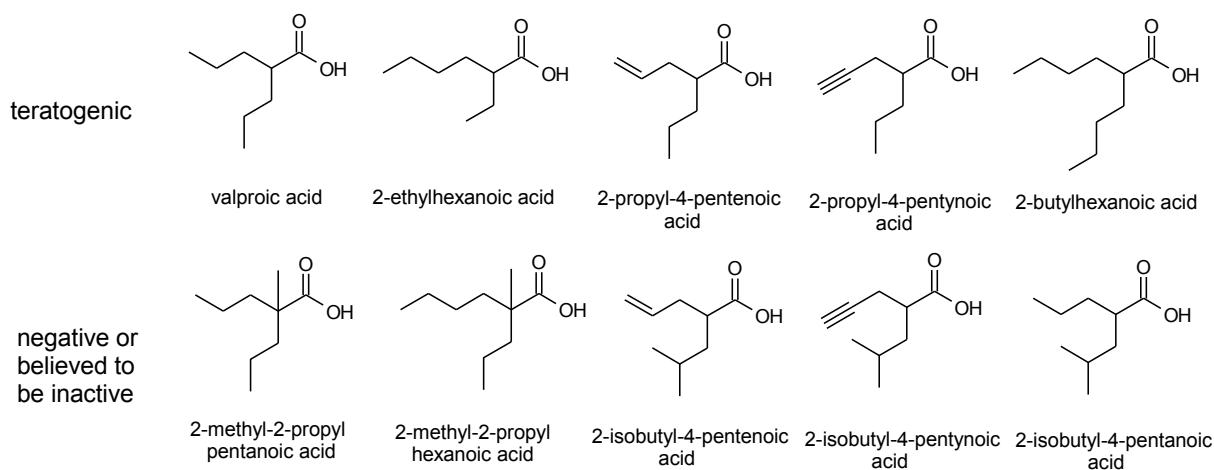
Besides HD, acrylamide (ACR) is the prototype of protein binding chemical as neurotoxicant. The molecular mechanism of ACR neurotoxicity has been studied by LoPachin et al. (LoPachin and Decaprio, 2005; LoPachin and Gavin, 2012). ACR is a soft electrophile belonging to the α,β-unsaturated carbonyl or type-2 alkene chemical class. It produces cumulative neurotoxicity in exposed humans and animals through a direct inhibitory effect on presynaptic function. *In vivo* proteomic and *in chemico* studies demonstrated that ACR formed covalent adduct with nucleophilic (soft nucleophile) cysteine thiolate groups located within active sites of presynaptic proteins leading to inactivation/disruption of nerve terminal processes and impaired neurotransmission.

8.4 Developmental toxicity

Developmental toxicity is another toxicity endpoint with complicated mechanisms. In fact, there are many time-dependent mechanisms and multiple endpoints. Compared to other toxicity endpoints, (Q)SAR of developmental toxicity is not as advanced. An International Life Science Institute workgroup pointed out a number of difficulties and challenges in constructing (Q)SAR models (especially statistically based) for developmental toxicity (Julien et al., 2004): (a) endpoints may be subjective and not always reproducibly interpreted, (b) difficulty in “scoring” for an activity, (c) the dynamic nature of embryonic development can lead to ambiguity in the relationship between structure and activity, and (d) scarcity of data with limited chemicals and chemical classes. Hewitt et al. (2010) evaluated the performance and limitations of various (Q)SAR modelling, structure alert-based expert system prediction and chemical profiling studies of developmental toxicity data. In general, there was limited success of current modelling methods when used in isolation. When used in combination, in a weight-of-evidence approach, better use may be made of the limited developmental toxicity data available with potential improvement in predictivity. A number of useful structural alerts for developmental toxicity have been identified (Blackburn et al., 2011) and organized into decision tree type of framework (Wu et al., 2013).

One of the most interesting classes of teratogens is the relatively simple branched aliphatic carboxylic acids. The most well-known members of the class are valproic acid and 2-ethylhexanoic acid which have been used as anticonvulsant and commercial products, respectively. The SAR of this class as teratogens has been reviewed by DeVito (2012). As can be seen from the list below, small changes in chemical structure can lead to significant changes in their teratogenic activity. The main structural requirements for

high teratogenic potency of valproic acid related aliphatic acids include: (a) a free carboxylic acid (corresponding esters that can be metabolically hydrolyzed to free acids are also active), (b) branching at carbon C-2 (also known as the omega minus one carbon in other terminology) with one hydrogen at C-2, no double bond between C-2 and C-3, and one linear (unbranched) alkyl group larger than methyl at C-2, (c) total number of carbon atoms optimal around 8, and (d) the stereochemistry at the C-2 carbon is important for some members of the class (e.g., (R)-2-ethylhexanoic acid is highly teratogenic but its (S)-enantiomer is inactive) but not consistently so for all.



The exact molecular mechanism of teratogenic action remains to be studied. It is interesting to note 2-ethylhexanoic acid and several related branched acids are also hepatotoxic and may induce liver tumors in animals (see Section 6.3) probably via involvement of peroxisome proliferation.

9. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This document has summarized different (Q)SAR approaches to assess the potential carcinogenic hazard/risk of substances. In each of these approaches, mechanistic understanding can make a significant impact on the reliability and scientific confidence of the assessment. Mechanistic information/consideration can also help to predict/assess potential route-/target- specificity and human relevancy which are often overlooked by traditional (Q)SAR predictive tools. To the extent possible, all the key components of mechanism-based (Q)SAR analysis—toxicokinetics, toxicodynamics, and bioinformatics input—should be carefully evaluated and integrated for an optimal analysis (Figure 10). For (Q)SAR analysis in which not all important components can be considered, any information/data gaps crucial to the reliability of the study should be identified and articulated for users to decide whether the assessment can be used for their specific purposes.

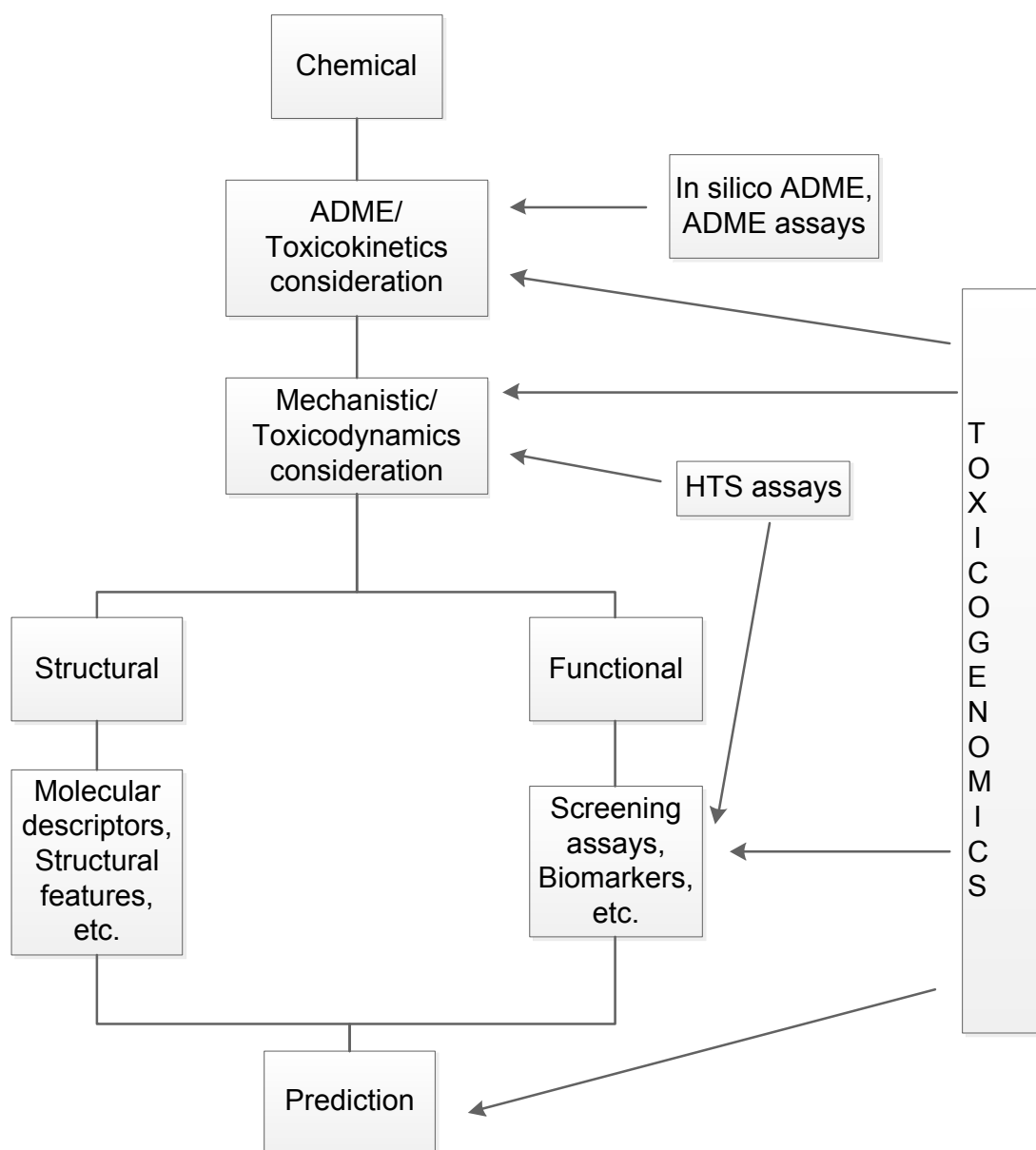


Figure 10: Integration of chemistry-based (Q)SAR and biology-based predictive assays

With the decreasing trend for conducting long-term cancer bioassays due to economic and ethical considerations, regulatory agencies and chemical manufacturers may increasingly have to resort to (Q)SAR analysis for the assessment of carcinogenic hazard/risk potential. Since the reliability of (Q)SAR analysis is dependent on the availability of data, information and knowledge of adequate number of structurally related analogs; mechanistic considerations may play an important role in this respect. Some (Q)SAR approaches such as “category approach” and “read across” are further strengthened with mechanistic understanding. The regulatory and (Q)SAR communities should be on the alert for setting standards to determine reliability of (Q)SAR analysis of data-poor substances particularly if they are expected to have substantial human exposure.

One bright point for the mechanism-based (Q)SAR field is the increasing availability of mechanistic information. The reliability of (Q)SAR analysis can be substantially enhanced by supportive mechanistic data. Short term *in vitro* and *in vivo* predictive assays such as DNA binding, cell transformation assays and toxicogenomics are particularly helpful (see Section 7). The capability of *in vitro* HTS assays for predicting carcinogenic potential is still not quite clear. One of the major roadblocks for *in vitro/in vivo* correlation may be the vast difference in toxicokinetics. Difference in repair mechanisms may be another factor for complicated endpoints such as carcinogenicity. Although the HTS assays can cover a large number of mechanistic endpoints, it is unlikely to be able to cover all the important and relevant endpoints. Specific chemical classes may require specific mechanistic and toxicokinetics considerations for optimal prediction of carcinogenic potential. In this respect, it will be helpful to have collaboration between HTS and (Q)SAR communities to determine what works well for the respective fields and the possibility of combining complimentary strategies to develop optimal integrative approaches for assessing carcinogenic potential of all classes of chemical substances.

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APPENDIX

Case Studies from NAFTA (Q)SAR Guidance Document

Case Study I: Use of a weight of Evidence (WOE) approach, including SAR information, to waive the chronic toxicity/carcinogenicity study requirement in a biocide reregistration decision.

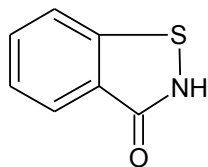
1, 2-benzisothiazolin-3-one (BIT) is a member of the isothiazolone class of biocides. Some of the registered uses of BIT involve chronic/long term exposures (e.g., use in metal working fluids). To address this type of potential exposure scenario, chronic and/or cancer studies would usually be required.

The chemical structures of the isothiazolone biocides can be divided into two sub-classes (Figure (i)):

1. General isothiazolone class: Isothiazolone pesticide substances without a benzene ring attached at the 4-5 position of the isothiazolone ring. For example: Kathon RH287, Kathon RH886 and/or OIT, which have been registered by OPP.

2. 1,2-Benzisothiazolone class: Isothiazolone pesticide substances with a benzene ring attached at the 4-5 position of the isothiazolone ring. In this case, BIT is the substance being discussed.

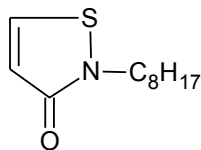
Isothiazolone Pesticidal Chemicals with benzene ring



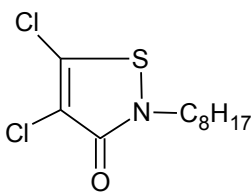
BIT

1,2-Benzisothiazolin-3-one

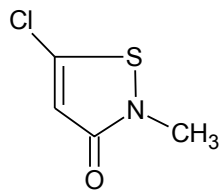
Isothiazolone Pesticidal Chemicals without benzene ring



Octhilinone

2-n-Octyl-4-isothiazolin-3-one
or OIT

Kathon RH287

4,5-Dichloro-2-n-octyl-4-
isothiazolin-3-one or
DCOIT

RH 886 (Mixture of CMIT and MIT)

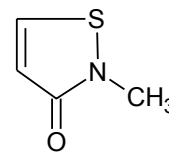
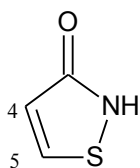


Figure (i): Chemical Structures of the Isothiazolone Biocides

All isothiazolone biocides contain an isothiazolone ring as depicted below.



The issue to be discussed in the example case is whether chronic/cancer studies can be waived based on existing conditions. The issue is discussed from following different aspects.

• **Pesticidal mode of action:** BIT, CMIT, MIT, OIT, and DCOIT all share a common pathway for antimicrobial activity:

- All inhibit cell respiration
- All inhibit the same class of dehydrogenase enzymes

These biocides react with microbial cells through cleavage of the S-N bond to form an S-S linkage with the thiol group on target enzymes. Biocidal activity is a function of the inhibition of cell respiration.

• **Structure Activity Consideration:** According to the pesticide mode of action consideration, the antimicrobial activity for all isothiazolone classes is due to the isothiazolinone ring and the sulfur nitrogen bond in the isothiazolinone ring plays a key role for efficacy as a biocide. The current issue is whether the benzene ring will be a concern for potential toxic effects of BIT. Based on SAR information, the benzene ring may prolong the biological half-life of the metabolic intermediate moieties in the body.

• **Toxicity Profile for BIT:** There are no carcinogenicity or chronic toxicity studies for any of the benzene ring-isothiazolone substances (such as BIT). BIT is not mutagenic as all acceptable guideline mutagenicity studies were negative. The toxicity profile of BIT shows that it is an irritant following oral and dermal exposures, and this is the effect observed following repeated dosing in subchronic toxicity studies.

In two oral subchronic rat studies, gastrointestinal irritation was reported at 10 mg/kg/day (lowest dose tested), and there were no other treatment-related systemic effects.

In a 90-day rat dermal study, skin irritation and histopathology were noted at all doses of 100, 300 and 1000 mg/kg/day, while systemic toxicity was only reported at the limit dose (1000 mg/kg/day). Gastrointestinal irritation/histopathology was also reported at 100 mg/kg/day which may be attributed to grooming.

The repeated-dose metabolism and disposition study indicates the metabolites associated with BIT exposure may remain in the body much longer than the parent compound. The evidence from this study indicates benzene containing metabolites may accumulate in the liver, kidneys and thyroid gland.

• **Toxicity Profile for the non-benzene ring isothiazolone pesticides:** The mutagenicity data for the non-benzene ring containing isothiazolones were largely negative except for a few positive observations *in vitro* with CMIT/MIT and DCOIT. Three chronic/carcinogenicity studies are available for non-benzene ring isothiazolone pesticides and all were negative for carcinogenicity, although two of these were found to have major deficiencies for the chronic toxicity portion of the studies.

One study was conducted using drinking water administration of a 14.2% CMIT/MIT mixture at doses of 2.0/3.1, 6.6/9.8, and 17.2/25.7 mg/kg/day in rats males/females. This study reported hyperplasia of the GI tract but no other systemic effects.

The second study used dermal administration of a single dose of 400 ppm CMIT/MIT to the skin of mice for 30 months and the only significant finding was dermal irritation.

A carcinogenicity study was conducted using dietary administration of OIT. There were no reported carcinogenic effects following oral exposure to up to 1000 ppm in the diet for 78 weeks. Although some tumors were reported, the incidences were within the ranges for the control animals.

All of the isothiazolones produced toxicity at the site of contact, i.e. irritation of the gastrointestinal tract, skin and respiratory tract, when administered at high doses. These biocides produce minimal to no significant systemic toxicity; no histopathological change distant from the site of dosing was observed, which appears correlated with rapid metabolism and excretion for these substances. Based on read-across comparison, it is concluded that:

Skin irritation: Similar findings in all dermal studies (BIT, CMIT/MIT, OIT) although at different dose levels.

Skin histopathology: Similar for BIT and OIT, none found in rabbit study on CMIT/MIT

Similar clinical chemistry findings with BIT and OIT and similar to BIT oral dog study

Severe skin irritation in BIT dermal study

The relative potency is as follows:

Skin Irritation: CMIT, DCOIT > OIT, MIT, BIT

Skin Sensitization: CMIT > DCOIT > OIT > MIT > BIT

• **Risk assessment considerations:** As a class, the isothiazolone pesticides are irritants by all routes of exposure, and are dermal sensitizers. For BIT, gastrointestinal irritation provides the basis for points of departure for short, intermediate, and chronic/long-term exposure scenarios.

Final Recommendation:

Based on a read-across comparison, and weight of evidence (WOE) approach, the chronic toxicity / carcinogenicity study for BIT is not required at this time if the risk assessment is protective of irritation. This recommendation was based on the following considerations: 1) available cancer studies for the isothiazolone pesticides are negative; 2) there is a lack of mutagenicity concern for BIT, and the other isothiazolone pesticides; 3) BIT and the other isothiazolones are irritants following oral, dermal and inhalation exposures and produce similar effects following subchronic exposures; 4) the isothiazolones as a group have a known mode of action for antimicrobial activity; 5) irritation is the predominant effect and is the basis of the PODs; 6) although the metabolism study for BIT showed an increased half-life and accumulation of radioactivity in thyroid compared to other isothiazolone substances, these observations were determined to be not of toxicological significance, as the toxicological effects of BIT up to 90 days were not different than the effects observed with the other isothiazolone substances.

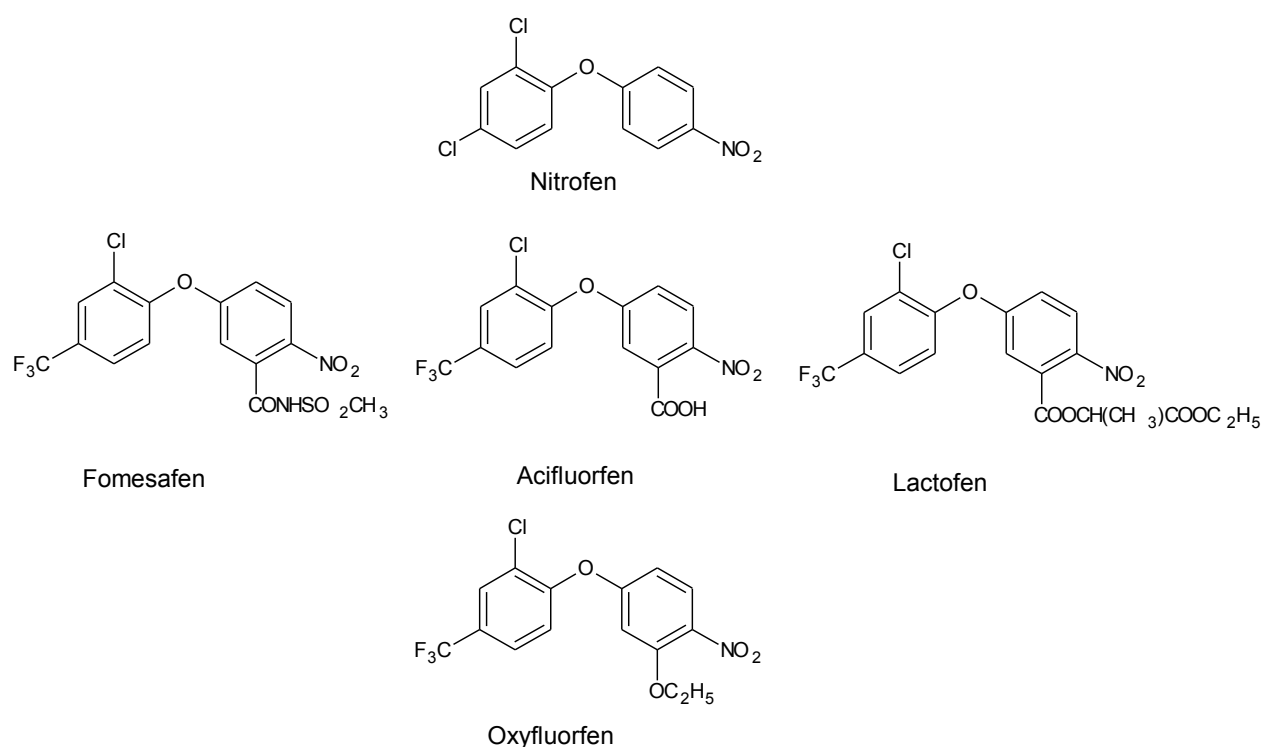
It is recommended that the available data be evaluated to inform the need for a UF to account for subchronic to chronic exposure durations for BIT.

Case Study II: Fomesafen cancer assessment and mode of action: use of mechanism-based SAR**Description of the case:**

Fomesafen, a diphenyl ether herbicide, was submitted to OPP's Cancer Assessment Review Committee (CARC) for re-evaluation of its carcinogenic potential to humans. The herbicide was previously shown to be a mouse hepatocarcinogen by the submitter and classified as a Category C possible human carcinogen by OPP. The new data provided by the submitter included: (a) consistent negative genotoxicity data, (b) some evidence of involvement of peroxisome proliferator-activated receptor alpha receptor (PPAR α) as a possible nongenotoxic mode of action for carcinogenicity, and (c) metabolism data. No SAR study was attempted. The Committee concurred that the pesticide should be nongenotoxic but considered the PPAR α evidence inadequate.

SAR approaches conducted:

Several structurally related diphenyl ether pesticides with carcinogenicity data were identified. Among these, Nitrofen, Lactofen, Acifluorfen and Oxyfluorfen were considered the closest. Like Fomesafen, all four were hepatocarcinogenic in mice with Oxyfluorfen being weakly/marginally active. The chemical structures are shown below.



The presence of a nitro group in aromatic ring is generally considered a genotoxic structural alert. Indeed, there was some evidence that Nitrofen was positive in the Ames test but the evidence was complicated by the presence of impurities. In addition to mouse liver tumors, there was some evidence that Nitrofen may induce pancreatic tumors in the rat. The mode of action of Nitrofen has not been thoroughly studied.

The mode of action of rodent hepatocarcinogenesis for both Lactofen and Acifluorfen (HED MTARC; TXR #s 0051907 and 0052006, respectively) has been extensively studied and shown to involve PPAR α -mediated peroxisome proliferation. Lactofen can be readily hydrolyzed by esterases to yield Acifluorfen as its primary metabolite. Structure-activity relationships studies have shown that one of the major structural requirements/alerts of most peroxisome proliferators is the presence of an acidic functional group (e.g., carboxylic, sulfonic) either in the parent compound or a metabolite (Woo and Lai 2003). The key question is whether Fomesafen can be hydrolyzed to a carboxylic acid metabolite. In general, the amide (-CO-NH-) bond is quite resistant to enzymatic hydrolysis. However, in Fomesafen, the presence of a sulfonyl group adjacent to the amide linkage can significantly facilitate hydrolysis. Indeed, a metabolism study by the submitter showed that up to 10% of Fomesafen may be hydrolyzed to yield a carboxylic acid metabolite as the most significant metabolite. Thus, Fomesafen, Acifluorfen, and Lactofen may actually have common carboxylic acid metabolite(s). It is interesting to note that, despite structural similarity, Oxyfluorfen, which cannot be metabolized to a carboxylic acid metabolite, is only weakly/marginally active as a hepatocarcinogen. Attempts to demonstrate possible PPAR α -mediated activity were unsuccessful for Oxyfluorfen. Overall, these findings strengthen the biological plausibility of PPAR α mode of action for Fomesafen-induced liver tumor formation in mice.

Outcome of the SAR study

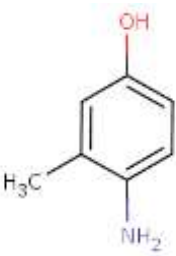
The SAR study provided significant support to the weight of evidence of a PPAR α mode of action of Fomesafen-induced mouse liver tumors. Based on the current scientific understanding of peroxisome proliferation (e.g., Klaunig et al., 2003) and previous US EPA decisions on structurally related herbicides

(e.g., Lactofen and Acifluorfen), the level of confidence in this assessment is high. While the proposed mode of action for liver tumors in mice is theoretically plausible in humans, it is quantitatively implausible and unlikely to take place in humans based on quantitative species differences in PPAR α activation and toxicokinetics. In accordance with the US EPA Final Guidelines for Carcinogen Risk Assessment (March 29, 2005), the CARC classified Fomesafen as “Not Likely to be Carcinogenic to Humans”.

Case Study III: A chemical with no carcinogenicity data was assessed (at a prioritisation level) for carcinogenic potential within the Australian Inventory Multi-tiered Assessment and Prioritisation (IMAP) by NICNAS.

Use of weight of evidence (WOE) approach, including (Q)SAR information was used to determine the carcinogenicity potential of the chemical Phenol, 4-amino-3-methyl- (CAS No. 2835-99-6). The chemical has been identified as used in hair dye preparations in Australia. No animal toxicity data are available on the carcinogenicity of the chemical.

Chemical Identity

Synonyms	4-amino-m-cresol 2-amino-5-hydroxytoluene 4-hydroxy-o-toluidine 2-methyl-4-hydroxyaniline 4-hydroxy-2-methylaniline
Structural Formula	
Molecular Formula	C7H9NO
Molecular Weight	123.15
SMILES	<chem>c1(N)c(C)cc(O)cc1</chem>

Genotoxicity data

Based on in vitro and in vivo genotoxicity studies, the chemical is not considered to be genotoxic.

All in vitro studies with the chemical gave negative results:

a bacterial gene mutation assay (OECD TG 471) using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 (3–5000 μ g/plate plate incorporation test; 1–1000 μ g/plate

preincubation test without metabolic activation; and 3–2500 µg/plate preincubation test with metabolic activation) (SCCP, 2005);

a bacterial gene mutation assay using *S. typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 with or without metabolic activation (up to 600 µg/plate; alone or mixed equally with 6 % hydrogen peroxide) (CIR, 2004);

a thymidine kinase (tk) gene mutation test (OECD TG 476) with L5178Y mouse lymphoma cells at 0.048–6.25 µg/mL doses without metabolic activation; 0.391–47.5 µg/mL (first experiment) and 0.5–40 µg/mL (second experiment) with metabolic activation. In this study, a slight increase in mutation frequency was reported at 25 and 47.5 µg/mL with metabolic activation in the first experiment and a marginal effect at 40 µg/mL in the second, but these findings were not considered sufficient to demonstrate a 'clear and/or reproducible induction of gene mutations at the tk-locus' (SCCP, 2005); and

two unscheduled DNA synthesis (UDS) tests (non-guideline) using rat hepatocytes (up to 100 µg/mL) (SCCP, 2005; CIR, 2004).

All in vivo studies with the chemical gave negative results:

a micronucleus assay (OECD TG 474) in bone marrow cells of NMRI mice exposed to the chemical (dissolved in sodium chloride) by intraperitoneal (i.p.) injections at 20, 100 or 200 mg/kg bw (SCCP, 2005);

a micronucleus assay (non-guideline) in which NMRI mice were exposed by gavage to the chemical (dissolved in DMSO) at 100, 333 or 1000 mg/kg bw (SCCP, 2005; CIR, 2004);

a sister chromatid exchange (SCE) assay in which Chinese hamsters were dosed with the chemical in aqueous solution, either orally at 100, 300, 1000, 1500 or 2000 mg/kg bw or, i.p. at 10, 30, 100, 300 or 400 mg/kg bw (CIR, 2004);

an UDS test (non-guideline) in which Wistar rats were orally administered a single dose of the chemical at 60 or 600 mg/kg bw (for 16 hours) and at 1000 mg/kg bw (for four hours), prior to culturing hepatocytes for analysis after four hours (CIR, 2004; SCCP, 2005).

QSAR modelling information

QSAR modelling, using OASIS–TIMES, resulted in positive predictions for in vitro genotoxicity and negative predictions for in vivo genotoxicity. However, it should be noted that the chemical structure was out of the applicability domain of the model. If a prediction is out of the applicability domain of the model, it indicates that there is a greater uncertainty about the model's reliability since the performance statistics of the data in the model may not be applicable to the chemical.

Mechanism of action information

Primary aromatic amines undergo metabolism to reactive electrophiles as an initial step in the carcinogenic mechanism of action. This usually involves N-hydroxylation of the aromatic amines to an N-hydroxylamine and eventual formation of pro-carcinogenic nitrenium ions. The highly reactive nitrenium ions can covalently bind to DNA, provided that they are sufficiently stabilised so as not to undergo further reactions. The stability of the nitrenium ions is correlated with mutagenicity, for example in the Ames test, with metabolic activation (Benigni & Bossa, 2011). However, the presence of two or more electron-donating groups, particularly in the ortho- and/or para-positions, reduces the metabolic N-hydroxylation

and inhibits the formation of the nitrenium ions (Vance and Levin, 1984; Shimizu and Yano, 1986; Serafimova et al., 2007). This is the case with the chemical due to the presence of the hydroxyl group in the para-position and another electron-donating group (methyl) at the ortho-position.

Conclusion

The QSAR model predictions were considered to not outweigh the negative test results for genotoxicity. In addition, the presence of electron-donating functional groups at the ortho- and para-positions mitigated the carcinogenic potential of the chemical.

Based on the weight of evidence analysis of the available information, the chemical is not considered to be carcinogenic.