Investigation of *in Silico* Modelling to Predict the Human Health Effects of Cosmetics Ingredients

Mohammed Aljallal

August 2019

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

Acknowledgements

First and foremost, I would like to thank my Director of Studies, Prof Mark T. Cronin, for his continuous support, guidance, and feedback throughout the course of my doctoral studies, as well as providing many of the initial ideas for this research. Similar thanks must go to my other supervisors Dr Judith C. Madden and Dr Steve Enoch.

I would also like to thank all my colleagues from the QSAR laboratory at Liverpool John Moores University for sharing their expertise during many useful discussions and providing a sounding board for ideas.

Additionally, I greatly appreciate the financial support provided to me by the Saudi Government represented by the Saudi Cultural Bureau and SFDA (Saudi Food and Drug Authority), without which this research would not have been possible. I hope to be a contributing and effective member of our dream as a Saudi youth community to build a high-quality regulatory system for safe drugs, food, cosmetics and medical devices to achieve the Saudi national vision to provide world-class government services that effectively and efficiently meet the needs of our citizens.

Special thanks go to my external supervisor Dr Qasim Chaudhry (University of Chester), for his valuable discussion and for sharing some of the data utilised in Chapter 5, for his continuous support and help from the first day in my research, and Dr Nick Price (Technology for Growth) for sharing some of the data used in Chapter 5.

Thanks, must also go to my beloved wife Fay and my daughter Norah, for their support and encouragement. Final thanks go to my parents who have provided immense emotional support throughout.

Abstract

Animal experiments have been the standard method to assess the safety of chemicals used in cosmetic products for decades. However, public opinion has continued to demand that *in vivo* hazard identification methods conducted on animals are replaced with alternative methods. Research on alternative methods to replace *in vivo* toxicity testing continually increased over the past few decades with different alternatives developed, such as *in vitro*, *in chemico* and *in silico* approaches.

Although different alternative techniques can be employed, no single technique can solely replace the complexity and an *in vivo* test, especially for chronic effects. Therefore, integrated testing strategies that can utilise the information from all available alternative testing approaches have been developed. Within the Adverse Outcome Pathway (AOP) paradigm, the molecular initiating event(s) MIE can be induced by several chemical key features which can be captured by structural alerts. When structural alerts for a MIE are compiled and supported by mechanistic and toxicity information confirming the induction of the same MIE, then they can be considered as an *in silico* profiler.

The overall aim of the work presented in this thesis was to assess the current *in silico* profilers for carcinogenicity (both genotoxic and non-genotoxic), mutagenicity and skin sensitisation through assessment using multiple high-quality experimental databases. The research presented herein demonstrates the ability to assess the positive predictivity of two types of structural alert, mechanism- and chemistry-based that pertain to the endpoints and proposes ways to improve the overall accuracy of these profilers. In this context, this study has given an insight to those alerts that may be found equally in endpoint-positive or negative compounds, and those which may be more effectively utilised to form groups of analogues for read across predictions. A detailed analysis of positive predictivity of the available mutagenicity, carcinogenicity and skin sensitisation structural alerts and profilers

within the OECD QSAR Toolbox against experimental data is presented. This investigation showed the structural alerts that are accurate as such, and those that may need further refinement, or their use may need to be reconsidered. In addition, the relationship between scaffolds of a range of diverse compounds and carcinogenicity showed that a total of 17 carcinogenicity scaffolds could be identified from the available databases and could be used as a base for an *in silico* profiler.

This work has also determined the need for further in-depth research in this area to study the suitability and merits of each of the alerts within the profilers currently included in the OECD QSAR Toolbox, and other *in silico* toxicity platforms, to identify the possibilities for improvement in their performance. This will, by implication, also improve the reliability of chemical read-across and grouping/categorisation for classification, labelling and risk assessment for regulatory use of the *in silico* methods.

Abbreviations

ADP	Adenosine Diphosphate
AOP	Adverse Outcome Pathway
ATP	Adenosine Triphosphate
CAS	Chemical Abstract Service
COSMOS	Integrated *in silico* models for the prediction of human repeated dose toxicity of COSMetics to Optimise Safety
ECEAE	European Coalition to End Animal Experiments
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EPA	Environmental Protection Agency
ER	Endoplasmic Reticulum
EU	European Union
GMPT	Guinea Pig Maximisation Test
GSH	Glutathione
HNEL	Highest No Effect Level
IATA	Integrated Approach to Testing and Assessment
TD50	Dose at which toxicity occurs in 50% of cases.
ITS	Integrated Testing Strategy
LEL	Lowest Effect Level
LLNA	Local Lymph Node Assay
LO(A)EL	Lowest Observed (Adverse) Effect Level
MIE	Molecular Initiating Event
NGC	Non-genotoxic carcinogen

NO(A)EL	No Observed (Adverse) Effect Level
OASIS	Optimised Approach based on Structural Indices Set
OECD	Organisation for Economic Cooperation and Development
PIF	product information file
PPV	Positive Predictive value
PETA	People for the Ethical Treatment of Animals
(Q)SAR	(Quantitative) Structure-Activity Relationships
RC_{50}	50% reactive concentration
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
ROS	Reactive Oxygen Species
RP	Responsible person
SA	Structural alert
SCCNFP	Scientific Committee on Cosmetic products and Non-Food Products
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SEURAT	Safety Evaluation Ultimately Replacing Animal Testing
SMARTS	SMiles ARbitrary Target Specification
SMILES	Simplified Molecular-Input Line-Entry System
S_N1	Unimolecular aliphatic Nucleophilic Substitution
S_N2	Bimolecular aliphatic Nucleophilic Substitution
S_NAr	Aromatic Nucleophilic Substitution
SOP	Standard Operating Procedure

Contents

Chapter 1: Introduction

Cosmetics and personal care products are amongst the everyday consumer products that are used by the vast majority of world's population. Whilst they offer a number of aesthetic benefits, the intimate nature of their application on the body, and repeated exposures due to frequent use, mean that the presence of any harmful substance in a cosmetic product can also pose a risk of adverse health effects to the user. Ensuring safety of cosmetics is therefore of utmost importance for both the industry and the regulatory authorities. Whilst the range of cosmetic products is seemingly endless, they can be broadly categorised as oral-care (e.g. toothpaste, mouthwash), hair-care (e.g. shampoo/conditioner, hair dye), skin-care (e.g. lotion, cream), make-up (e.g. mascara, lipstick), deodorant/antiperspirant, perfume and fragrance products.

1.1: Risk assessment and risk management

Risk assessment and risk management are crucial elements for the development of a cosmetic product that help to assess and mitigate possible adverse health effects to consumers. At the regulatory level, these two issues are kept independent of each other. The outcome of risk assessment, however, provides a basis for devising appropriate risk management strategies.

Generally, risk assessment of a cosmetic ingredient requires data and information on the following aspects:

- The physicochemical characterisation of the ingredients intended to be used in a cosmetic product. The knowledge of physicochemical properties of the substance may provide useful pointers to its potential behaviour, interactions and effects in biological systems.
- The identification and dose–response characterisation of toxicological hazards. The toxicological endpoints measured for risk assessment relate to both the local and systemic effects that may manifest over short, medium or long terms.

- An exposure assessment for reasonably foreseen conditions of use. This includes assessment of both external exposure and internal (systemic) exposure. The latter is usually derived from the external exposure on the basis of the level of absorption through relevant routes (dermal, oral, and/or inhalation) and distribution through the body.

All of the above data and information are used together to assess the overall risk to an average consumer, keeping in mind certain vulnerable groups - such as infants and children.

Amongst the toxicological endpoints that are studied for the safety assessment of cosmetics, carcinogenicity is one of the most difficult to measure or predict accurately. Historically, testing for carcinogenicity of cosmetic ingredients has been performed using the rodent carcinogenicity assay (OECD 2008), whilst organ level toxicity is studied in rodents using 28 day or 90-day using repeated-dose exposures (OECD 1995, OECD 1998). These studies are also used to derive a No Observed (Adverse) Effect Level (NO(A)EL), which is *"the highest exposure level at which there are no biologically significant increases in frequency or severity of adverse effects between the exposed population and it appropriate control*." (EPA 1995, Lewis *et al.* 2002). For instance, a 28 or 90-day inhalation, oral or dermal repeated dose study in rodents can provide data to derive a N(O)AEL value for use in risk assessment. Where some effects are observed in these tests, a Lowest Observed (Adverse) Effect Level (LO(A)EL) may, instead, be derived. The LO(A)EL is *"the lowest exposure level at which there are biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group*." (EPA 1995, Lewis *et al.* 2002). The NO(A)EL value (or the LO(A)EL value after applying appropriate safety factor) is used along with the estimates of internal (systemic) exposure to estimate the Margin of Safety (MoS) for the specific substance. In view of the possible toxicokinetics/ toxicodynamics differences between the test species (generally rodents) and humans, and the variability within the human population, an

uncertainty factor of 100 (10 x 10 respectively) is generally applied and substances with a MoS equal to, or greater than, 100 are considered safe for use in cosmetic products.

1.2: European Union regulation

In Europe, safety of cosmetics is regulated under the European Union's Cosmetic Regulation [Regulation (EC) No 1223/2009]. Article 2 of the Regulation regards a cosmetic product as a substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with the exclusive or main purpose of cleaning or perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours.

In Europe, safety assessment of cosmetics is carried out by industry, which is then assessed by the regulatory authorities. At the industry level, all the information relating to safety is maintained in a Product Information File (PIF) by the company's Responsible Person (RP). At the regulatory level, the safety of cosmetic ingredients is overseen by the European Commission, whereas that of the final products by competent authorities in the European Union (EU) Member States. The European regulatory framework for cosmetic safety requires pre-market notification of the intended use of any ingredients that fall within the regulated categories (the so-called Annex substances), assessment of the safety, regulatory approval and appropriate labelling of the final products.

The safety data on cosmetic ingredients submitted to the European Commission are reviewed by a committee of independent experts (the Scientific Committee on Consumer Safety (SCCS)), who advise the Commission on the safety of the substance. Depending on level of the assessed risk to the consumer, the Commission may allow an ingredient at the levels proposed by the

industry, allow it with restrictions on the use levels or the types of uses, or ban its use in cosmetic products. As such, the Commission may place a cosmetic ingredient in one of the Annexes of Regulation (EC) 1223/2009: Annex II (list of prohibited substances); Annex III (list of restricted substances III); Annex IV (list of colourants); Annex V (list of preservatives; or Annex VI (list of UV-filters).

Since 11 March 2013, the EU Cosmetic Regulation has banned animal testing of any cosmetic ingredient or a finished product in the EU, as well as the marketing of any cosmetic ingredient/product that has been tested on animals after the ban took force. This has placed a lot of emphasis on alternative non-animal methods to obtain data and information for risk assessment (Rogiers, 2019).

The Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) is another (and most influential) EU regulatory framework relating to safety of the workers and the environment from the chemicals that are produced and/or used at industrial scales. Since implementation in 2007, REACH has progressed in various phases on the basis of tonnage of the chemicals produced and now requires that substances imported or produced in the EU at one tonne per year or more should be registered with the European Chemicals Agency (EChA). Some cosmetic ingredients are produced or imported into the EU at high tonnages and hence will also be safety assessed under REACH based on data relating to physicochemical properties, toxicological effects and estimates of exposure. REACH also encourages the use of alternatives methods and the use of animal experimentation only as the last resort. Part of this is due to the commitment to the so-called '3Rs' principle (Reduction, Refinement, and Replacement of animals used in laboratory procedures) (Russell and Burch 1959).

1.3: Toxicological testing of cosmetic ingredients

Toxicology is an inter-disciplinary subject that brings knowledge together from chemistry, pharmacology and biology. The harmful effect(s) of a test chemical observed at the organism, organ, (sub)cellular or molecular level are termed the toxic endpoint or adverse effect/ outcome. Determining the nature and the extent of an adverse effect is generally based on:

a. Data from tests conducted *in vivo* on animals (e.g. rodents), as well as the information drawn from epidemiological studies or clinical trials on human volunteers. These data provide a basis for determining the levels below which the substance may be considered non-toxic (such as the NOAEL).

b. Data from tests carried out *in vitro* using cultures of bacterial, animal or human cells, cultured tissues or organoids.

c. Estimates drawn from computational (*in silico*) models that are based on structure-activity rules, algorithms and / or structural alerts for toxic potential that have been derived from experimental data on related group(s) of chemicals. Such models allow for the prediction of toxicity of untested substances.

In vivo testing has long been considered the most appropriate method for toxicological assessment of chemical substances to predict their potential effects in humans – albeit with consideration of the relevance of animal data to humans due to interspecies differences. Toxicological data for cosmetic safety assessment have also been historically derived from tests on animals in the form of measured values against specific endpoints that depict both short- and long-term effects in humans. These included:

- dermal/ percutaneous absorption; toxicokinetics; acute toxicity; irritation and corrosivity (skin and eye); skin sensitisation; mutagenicity/genotoxicity; repeated dose toxicity;

- where data indicated potential long term effects, further studies on carcinogenicity and reproductive toxicity could be required;
- data on photo-toxicity may be required for substances that are photo-reactive and the final products are intended for application on the skin exposed to sunlight;
- where available, data from exposure to humans, e.g. data from epidemiological studies or clinical trials are also taken into consideration.

1.4: Testing for carcinogenicity, mutagenicity or reproductive toxicity

Although the toxicological data required for safety assessment of cosmetic ingredients cover a range of short- and long-term endpoints, more emphasis is placed on identifying and avoiding the use of those substances that may be Carcinogenic, Mutagenic or Reproductive toxicants (so-called "CMRs"), or may be persistent and accumulative in the body. This is because acute and short-term toxic effects are relatively straightforward to detect and mitigate, unlike long-term effects, such as the risk of cancer. The current classification of CMR substances used in the EU is as follows:

- Carcinogenic substances are categorised either as 1A (known to have carcinogenic potential for humans), 1B (presumed to have carcinogenic potential for humans); or 2 (suspected human carcinogen).
- Mutagenic substances are categorised either as 1A (known to induce heritable mutations in the germ cells of humans); 1B (can induce heritable mutations in the germ cells of humans); or 2 (may cause concern for humans owing to the possibility to induce heritable mutations in the germ cells of humans).
- Reproductive toxicants are categorised either as 1A (known human reproductive toxicant); 1B (presumed human reproductive toxicant); or 2 (suspected human reproductive toxicant)

Under the current regulations, CMR 2 substances may be allowed in cosmetic products where they, in view of the exposure and concentration, have been found safe. CMR 1A or 1B substances are allowed only in exceptional cases that is when they comply with food safety requirements, *inter alia* as a result of their natural-occurrence in food, and that no suitable alternative substances exist, on the condition that such use has also been found safe (see Chapter 4 for more details).

1.5: Alternative (non-animal) methods

With the animal testing/marketing ban for cosmetic products under the EU Cosmetic Regulation, and emphasis on the 3Rs principles under other regulatory frameworks, there is an increasing focus on the use of alternative (non-animal) methods to obtain toxicological data for safety assessment (Balls, 2019). This is, in part, due to ethical reasons over, and public pressure opposing, the use of animals to assess the toxicity of chemicals (including cosmetic ingredients). In addition, scientific progress is driving the change with new approaches to toxicology that are more relevant to humans as well as environmental species. In particular, non-testing methods, such as *in silico* models and tools, provide a far cheaper and quicker option for the primary screening of chemicals for hazard identification compared to other methods. For instance, the average cost of a 90-day repeat-dose rodent study for a single chemical can be between $125,000-175,000 and requires the use of approximately 80 animals. Testing for long-term effects - such as carcinogenicity - may cost many times that amount. Thus, the use of alternatives non-animal methods offers the opportunity to reduce the cost of product development, the use of animals in toxicological studies, and can make the hazard identification process more rapid (Worth, 2019).

By banning *in vivo* testing of cosmetics under Cosmetic Regulation, the EU has opened the door for the use of scientifically-valid alternative methods. The main approaches used for this

purpose are based on *in vitro* tests using cultures of cells, tissues or organoids, *in chemico* approaches and computational (*in silico)* models that are based on structure-activity based rules, algorithms, and structural alerts (EC 2003, EC 2006, EC 2006). The capacity of a molecule to react and covalently link with significant biological macromolecules, such as proteins, is studied by *in chemico* testing. An example of this technique is the measurement of the capability of a compound to bind to a thiol group, such as contained on the amino acid cysteine, by the depletion of glutathione in a standard assay (Aptula *et al.* 2006). *In vitro* assays provide another non-animal route to assessment of toxicological hazards. The bacterial Ames Test is an example of an *in vitro* technique which is used to assess the mutagenic potential of compounds with the use of a mutant strain of the bacterium *Salmonella typhimurium* which is not able to generate histidine but will revert back to the wild type in the presence of a mutagen (Ames *et al.* 1973, OECD 1997). A range of *in silico* models and tools that can estimate different toxicological endpoints, e.g. mutagenicity, skin sensitisation, teratogenicity (Enoch *et al.* 2008a, Enoch *et al.* 2008b, Enoch *et al.* 2009, Enoch *et al.* 2011a) is now available. These are used to derive toxicity estimates based on (Quantitative) Structure-Activity Relationships ((Q)SARs), category formation (grouping) and read-across. The principles of structure-activity relationships (SARs) are based on the notion that biological activity (including toxicity) of a chemical substance is dependent on certain physicochemical and structural parameters. Thus, the toxicity of a yet-untested chemical may be predicted on the basis of models developed from SARs of groups of related substances, or through extrapolation of data from close structural analogues of the untested compound (the premise of category formation and read-across).

There are, however, certain limitations to each of the different alternative methods and as such they cannot entirely replace the results obtained from *in vivo* tests in a live functional animal. Despite much recent scientific progress, the assessment and prediction of many complex toxicological endpoints, especially chronic effects, is difficult. Thus, to obtain sufficient weight

of evidence for risk assessment a combination of several approaches need to be employed as a part of an integrated testing strategy (ITS) (Hartung *et al.* 2013).

1.5.1 Adverse Outcome Pathways and Molecular Initiating Events

The Adverse Outcome Pathway (AOP) paradigm provides a framework that enables the information provided by different testing methods to be integrated and organised cohesively and transparently (Ankley *et al.* 2010, OECD 2013, Vinken 2013, Vinken *et al.* 2013a, Vinken *et al.* 2014). The knowledge within an AOP provides a mechanistic link between data and information derived from different approaches. Knowledge can be provided for the upstream Molecular Initiating Event (MIE) and a potential downstream adverse outcome that may be relevant for risk assessment. AOPs are defined by a number of key testable events at different levels of biological organisation - including the organ, cellular or organism level (Ankley *et al.* 2010, Schultz 2010, OECD 2013, Przybylak and Schultz 2013). The progression of an AOP is towards the adverse event that is initiated by the interaction of a chemical and the site of action (the MIE), which is the primary event in the sequence. The primary interaction between the biological and chemical system may be obtained from the mechanistic information described by the MIE. The physicochemical properties and structural fragments of molecules that can interact via the MIE can be analysed and rationalised in terms of their mechanistic information and relevance. The sequential progression of toxicity from one level of biological complexity to another is represented in Figure 1.1. It is also recognised that the elicitation of the adverse effect in a biological system is a complex process which may have been provoked by multiple key effects at the cellular level following a single event upstream. Many, often unrelated, events in a pathway may combine to bring about the same adverse effect, as described by Vinken *et al.* (2013b) for the AOP for cholestasis. Other AOPs, e.g. for weak acid respiratory uncoupling, oestrogen receptor-mediated reproductive toxicity, voltage-gated sodium channel-mediated

neural toxicity, skin sensitisation and cholestasis have been developed accounting for a wide variety of adverse outcomes (Ankley *et al.* 2010, Schultz 2010, OECD 2011, Landesmann *et al.* 2012, OECD 2012). A more comprehensive list of the available AOPs is accessible from the AOP-Wiki via the AOP Knowledge Base (available from https://aopkb.org/, accessed 17.2.2017). To assist in their implementation, the AOP framework has been standardised in an OECD guidance document which indicates the process by which AOPs should be developed and assessed for reliability and robustness (OECD 2013).

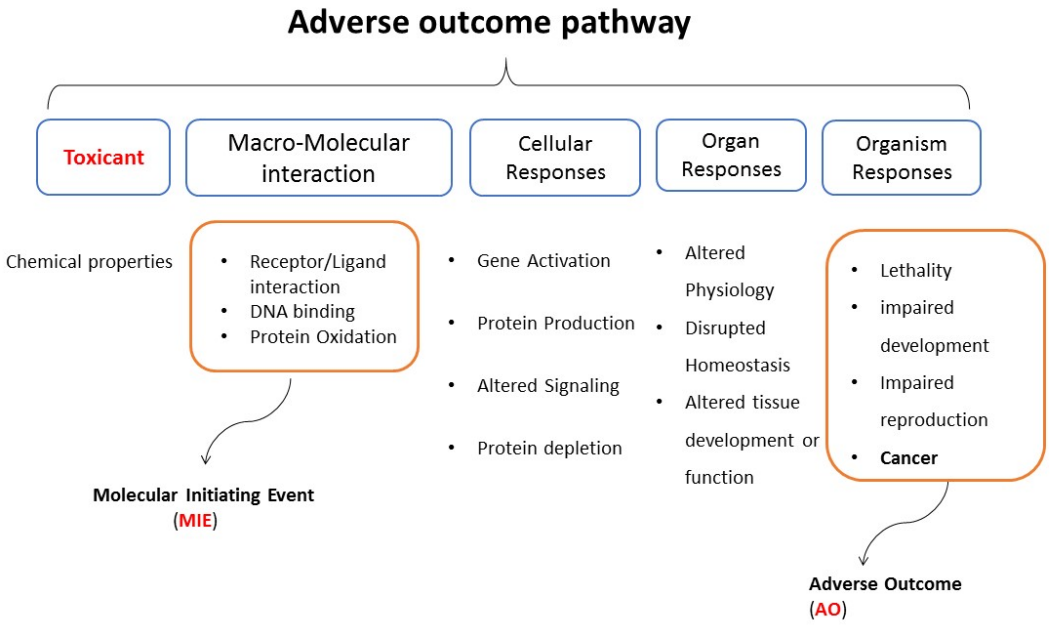


Figure 1.1. Summary of the steps within an adverse outcome pathway and examples of the type of effect or activity (adapted from Ankley *et al.* (2010))

1.5.2 In silico profilers

In silico methods can also be used to define and capture the MIE from an AOP pathway (Cronin and Richarz, 2017). The molecular fragments and chemical structures that are found to be responsible for inducing toxicity can be identified by these methods. One method of capturing 2-D information relating to, e.g. DNA or protein binding, is the use of structural alerts. A

collection of structural alerts that predict the same MIE have been considered to be used as an *in silico* profiler (Enoch and Cronin 2010, Enoch *et al.* 2011b). The profilers can be classified into two main types, namely mechanistic and non-mechanistic profilers (the latter described as chemistry-based profilers in this thesis). A profiler that is associated with a particular endpoint induced by a group of structural alerts related to an MIE is termed a mechanistic profiler. Thus, protein binding can be indicative of skin sensitisation as it is the MIE in this AOP. Ideally, structural alerts that are present in the mechanistic profiler should be associated with experimental data that exhibit the generation of toxicity as a result of the particular MIE. *In vivo*, *in vitro* and/or *in chemico* methods can provide such experimental data. These types of profiler are also used for the formation of categories and hence allow for read-across from tested analogous chemicals to fill data gaps for toxicity prediction (Enoch *et al.* 2011b).

A profiler for a particular endpoint that is based on a group of structural alerts related to chemistry is termed a non-mechanistic profiler. Such a profiler does not provide any mechanistic information about the initiation of toxicity. Instead, they may be based on cheminformatics, or a simple analogue/ homologue approach which indicates that a particular common structural group is responsible for the toxicity of the compound (Enoch and Cronin 2010). Even when the alerts are related directly to toxicity, the nature of the chemical alerts obtained by this approach does not identify the mechanism by which the observed toxicity is brought about. The reason for this is that small molecular fragments may initiate toxicity through different mechanisms whereas each profiler is used in an endpoint- and context-dependent manner. Chemistry-based profilers are nevertheless helpful in screening large datasets to identify which chemicals should undergo initial *in vitro* or *in chemico* tests (Cronin and Richarz, 2017).

1.5.3 Category formation and read-across

A set of chemicals having common properties may be assigned to a category according to the technique of chemical category formation (ECHA 2008, OECD 2011). The identification of chemicals having the same mechanism of action, or MIE, is one of the key means of forming a chemical category. Thus, a chemical category can be formed based on a structural alert for a particular mechanism where the same alert is present in the target chemical as well as its analogues. The Organisation for Economic Co-operation and Development's (OECD's) QSAR Toolbox software has been developed as a result of the need to form chemical categories for a wide range of toxicological endpoints with the aid of mechanistic profilers (available from www.qsartoolbox.org) (Schultz *et al*., 2018). The read-across approach uses appropriate data and assumes that the biological and chemical activity of similar chemicals will be similar (Jaworska and Nikolova-Jeliazkova 2007). This allows the prediction of activity of the target chemical with the help of toxicological data for chemicals belonging to the same category. This approach may allow for both qualitative and quantitative predictions by analysing the data available for analogous chemicals belonging to a specific category.

1.5.4 Profiling inventories for prioritisation

A library of information about a set of chemicals with their identities is termed a "chemical inventory". Chemical inventories are created and maintained for many purposes including regulatory use - such as the industrial chemicals registered under REACH. The inventories generally do not contain toxicological data associated with the chemicals but some free-access and commercial databases do provide such information. *In silico* profilers made up of relevant structural alerts can be used to screen inventories - for example to identify chemicals that may induce certain toxicity(ies). Although chemistry-based profilers lack mechanistic information, they are generally still useful for read-across (Alves *et al*., 2016). As such, chemistry-based

profilers may be used to screen inventories, large datasets and chemicals that contain one, or more, structural alerts. The presence of a chemistry-based alert in a cosmetic ingredient could indicate that the chemical would require *in chemico, in silico* and/or *in vitro* analysis to gain further insights into the mechanism of toxicity. Thus, the chemistry-based alert can lead to further investigations into the possible mechanistic basis (Limban *et al*., 2018).

1.5.5 Expert systems

In addition to the chemistry- and mechanistically-based profilers in the OECD QSAR Toolbox (and other freely available software such as Toxtree), there are a number of software packages termed expert systems that are available on a commercial basis e.g. ChemTunes, TIMES-SS and DEREK Nexus (formerly DEREK for Windows). Expert systems can be based on one or more different approaches to predicting toxicity, such as decision trees based on rules, structural alerts for particular toxicity endpoint and / or nested QSARs (Dearden *et al*., 1997). Structural alerts can identify endpoints, such as skin irritation/ sensitisation, mutagenicity and carcinogenicity. Using expert systems, a user can quickly identify chemicals that may have the potential to elicit a toxic effect. Expert systems are commonly used by cosmetics and pharmaceutical companies to screen datasets for toxicity at early stages of product development. Knowledge of the potential toxicity of the lead compound(s) helps to avoid safety issues at later stages of the R&D pipeline. Such information can help in the development of 'safer' compounds where fragments associated with potential toxicity can be substituted with other moieties (Limban *et al*., 2018).

1.6: Molecular initiating events for (sub)chronic repeat dose toxicity

Sub-chronic and chronic adverse effects of a particular substance can be identified with the help of repeated dose toxicity testing. In repeated dose toxicity testing, the organism is exposed

to the chemical to be tested either over a stipulated period of time (28 or 90 day) or for the complete lifespan of the organism (e.g. 2 year study in rat). Initiating events for some of the toxicological endpoints are known, e.g. covalent binding of the substance with a protein and DNA (de Groot and Noll 1983, Woodward and Timbrell 1984, Aptula and Roberts 2006, Aptula *et al.* 2006, Enoch *et al.* 2008a, Enoch *et al.* 2008b, Enoch and Cronin 2010, Enoch *et al.* 2011a, OECD 2012, Hewitt *et al.* 2013), however, for more complex endpoints relevant to this thesis, such as non-genotoxic carcinogenicity, there may only be limited clues to the MIEs.

1.7: Carcinogenicity - the use of SAR approaches to identify suspect carcinogens

In western countries, cancer is considered as one of the main causes of death after circulatory disease (Frankish, 2003). The National Cancer Institute (NCI) defined cancer as "*a term for disease in which abnormal cells divide without control and can invade nearby tissues and can also spread to other part of the body*". Whilst substantial effort and funds have been devoted to research into cancer in the recent decades, cancer is still one of the main diseases causing death. Numerous causes for cancer have been postulated, most notable amongst them are exposure to carcinogens in the environment, through diet, at the work place or due to lifestyle (Lichtenstein *et al*., 2000).

The potential of a chemical to elicit carcinogenicity and mutagenicity can be tested using a range of *in vitro* and/or *in vivo* test methods (Table 1.1). For genotoxicity testing, the endpoints are related mainly to gene mutations and chromosomal damage. *In vitro* methods in bacteria (i.e. the Ames test) or in mammalian cells are suitable and widely used to identify potential genotoxic chemicals. In the Ames test, the assay is usually performed both in the absence and the presence of an S9 fraction from rat liver to mimic the metabolic function of mammalian systems (Cartus and Schrenk, 2016).

A compound or its metabolite(s) may bind directly with DNA or exert indirect effects through interaction with the function of DNA leading to a positive outcome for genotoxicity. This may lead to an irreversible change in the DNA base sequence or a damage to the cellular genome. These changes, together with other effects in the cancer multistep process, e.g. suppression of apoptosis or DNA repair, are frequently linked to an increase in tumour rate. Rats and mice are primarily used to detect carcinogenic effects and general toxicity respectively, under chronic exposure conditions and over the lifespan of the animals. If there is a carcinogenic response *in vivo*, further *in vitro* and *in silico* investigations can provide crucial information as to whether the compound is carcinogenic as a result of genotoxic mode of action.

Table 1.1. A summary of selected *in vivo* and *in vitro* tests to assess the possible genotoxic effects of a compound according to OECD TGs (OECD, 2015)

Endpoint *In vitro* test methods	Test	Species
Mutagenicity (reverse mutation)	Ames test, Ames fluctuation test	Bacteria (*Salmonella typhimurium*, *Escherichia coli*)
Mutagenicity (forward mutation)	Hprt test	Mammalian cell lines
Mutagenicity (forward mutation)/Chromosomal damage	Thymidine kinase-/Mouse lymphoma assay	TK6 human lymphoblastoid cell line; L5178Y mouse lymphoma cell line
Chromosomal damage	Chromosome aberration test *in vitro*	Mammalian cell lines
DNA strand breaks	Comet assay	Cells and cell lines
In vivo test methods		
Mutagenicity	Transgenic rodent somatic and germ cell gene mutation assays	Transgenic rats or mice
Chromosomal damage	Micronucleus test *in vivo*	Mammalian erythrocytes/blood cells
Chromosomal damage	Chromosome aberration test *in vivo*	Mammalian bone marrow and mammalian spermatogonial cells

Although human cancer risk might be predicted from long-term carcinogenesis studies in rodents (mainly rat and mice), the cost and time required are quite high and there are ethical issues for the use of animals in such studies (Huff *et al.,* 1996). Attempts have therefore been made to develop alternative models - including short-term biological tests (such as the tests for mutagenicity), or structure-based *in silico* models (Benigni, 2012). Understanding the mechanisms of chemical carcinogenesis is crucially important to design prevention plans (Belpomme *et al*., 2007). As more details of the molecular basis of carcinogenic activity become known, the identification of potential carcinogens by SAR analysis is also becoming increasingly reliable (Benigni and Bossa, 2011).

From a mechanistic point of view, carcinogens can be regarded as being either genotoxic or non-genotoxic (epigenetic). As illustrated in Table 1.2, when chemicals or their metabolite(s) are capable of directly inducing cancer by altering genetic material in the target cells, they are classified as being a 'genotoxic carcinogen'. The term 'non-genotoxic carcinogen' is generally used for chemicals that are capable of inducing cancer by secondary mechanisms that do not involve direct damage to the genetic material. Whilst there are many data and much knowledge on the mechanisms leading to genotoxicity, it is more difficult to classify non-genotoxic carcinogens on the basis of mechanisms of action due to lack of specific mechanistic information (Hayashi, 1992). In fact, the differentiation between genotoxic and non-genotoxic carcinogenicity is rarely absolute as most potent genotoxic carcinogens also possess non-genotoxic activities that could act synergistically to lead to carcinogenic process. A unifying feature that can help identify genotoxic carcinogens is that they are either intrinsically electrophiles, or are transformed to electrophilic reactive intermediates. This, however, cannot be said for non-genotoxic carcinogens that can act through a range of different mechanisms that have no apparent unifying basis (Anastas *et al*., 2012).

Table 1.2. A summary of the main differences between genotoxic and non-genotoxic carcinogens.

Feature	Genotoxic carcinogen	Non-genotoxic carcinogen
DNA alteration	Direct	Indirect (secondary)
Mechanism	Known	Multiple
Structural Feature	Electrophiles	No unifying concept (several)

In order to gain a better understanding of the role of non-genotoxic carcinogenicity and its specific mechanisms of action, a thorough review is required. The outcome of this review is presented below as Section 1.8.

1.8: Non-genotoxic carcinogenicity

Predicting the carcinogenic potential of chemicals that act through non-genotoxic mechanisms is one of the major challenges in toxicology. Historically, events that could not be described by normal genetic principles of heritability were termed as being epigenetic. In a broad sense, epigenetic refers to the alteration of gene expression without changing the basic DNA sequence. Thus, non-genotoxic (epigenetic) carcinogenicity includes the actions of all natural or synthetic chemicals that may induce carcinogenic effects without involving mutation(s) in the DNA sequence. There has recently been huge growth in the scientific literature on the mechanisms of action of non-genotoxic (epigenetic) carcinogenicity; this is as a result of the greater importance being placed on gaining more knowledge about its mechanistic understanding and the molecular basis of chemical carcinogenicity (Benigni *et al.*, 2013). Unlike genotoxic agents, cancer induction by non-genotoxic carcinogens may occur through alteration of multiple pathways. Therefore, the activities of non-genotoxic carcinogens could include the molecular targeting of different cellular and extracellular constituents of various organs, but not DNA

(Yamasaki, 1995). Cytokines and hormones that operate through cell membrane receptors and intracellular communication processes - such as enhancing cell proliferation and abnormal cell cycle kinetics – may potentially alter sensitivity to a wide variety of cell growth mediators. As such, they have been considered the main determining factors for the carcinogenic response (Klein and Costa, 1997). Mechanisms of action of non-genotoxic carcinogens are mostly heterogeneous and often tissue, species and gender specific. The main non-genotoxic mechanisms, with an emphasis on those with features amenable to interpretation by SARs, are illustrated in the following section. The available information is used to support a unifying theory that can, at least in principle, be translated to an *in silico* tool for interpretation through predictive toxicology.

As noted above, there is a need to identify the key mechanisms of action of non-genotoxic carcinogenicity. Those that are known so far, along with examples of structural alerts and exemplar compounds that may be of use, are discussed below:

1.8.1 Peroxisome proliferation

One of the main groups of non-genotoxic carcinogens comprises a group of diverse chemicals that have been collectively termed as "peroxisome proliferators" (PP). Almost all eukaryotic cells contain cytoplasmic organelles called peroxisomes (or microbodies), that vary between tissues in terms of size, number and tissue profile (de Duve, 1983). Peroxisomes play an important role in β-oxidation of very-long-chain fatty acids and in the biosynthesis of cholesterol and bile acid (Mannaerts and van Veldhoven, 1993). The possibility to induce noticeable peroxisome proliferation was first demonstrated in rodent liver cells after administration of the hypolipidemic drug clofibrate (Paget, 1963). Since then, a number of other compounds have been identified as peroxisome proliferators. These include herbicides, solvents, plasticisers, leukotriene antagonists, as well as natural compounds (Gonzalez *et al*.,

1998). There is a noticeable increase in both the size and the number of peroxisomes and hepatomegaly after administration of the non-genotoxic agents to rodents due to hypertrophy and enhanced cell proliferation (Reddy *et al*., 1986). Administration of peroxisome proliferators over longer periods of time to rats and mice has been shown to result in the development of hepatocellular carcinomas (Rusyn *et al*., 2006).

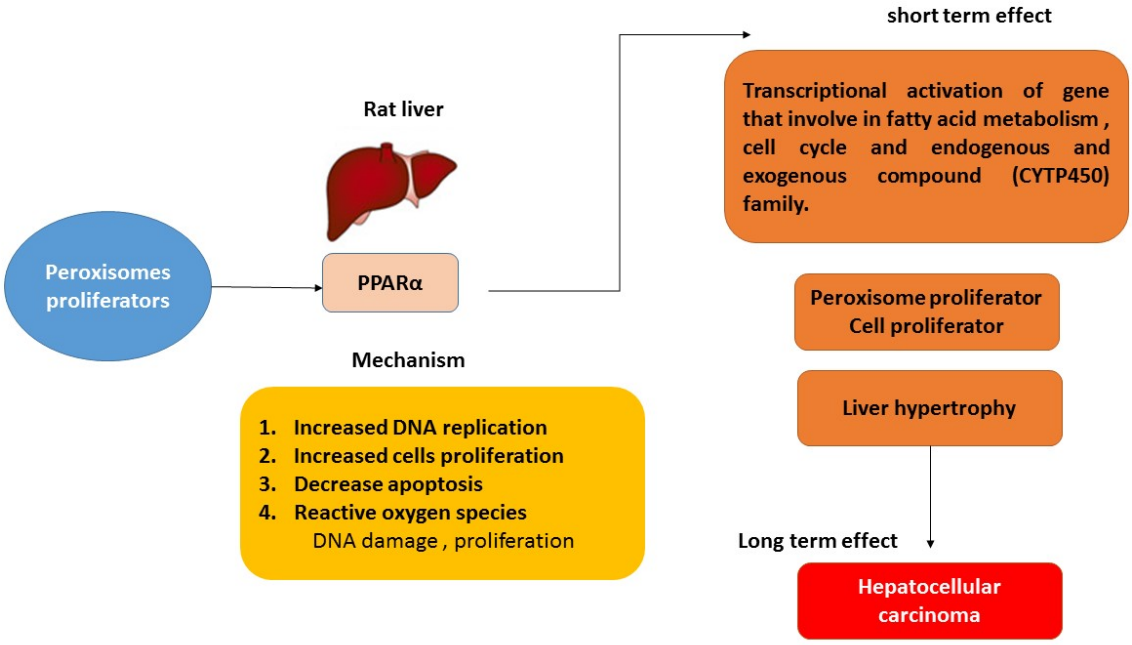


Figure 1.2. Peroxisome proliferation: consequences of PPARα activation in the liver and the proposed underlying mechanisms (adapted from Michalik *et al.,* 2004).

As shown in Figure 1.2, the exact process of how peroxisome proliferators cause tumours in rodent liver it is not fully understood. However, two factors are thought to be the main inducers of hepatocarcinogensis in rodents:

(i) induction of oxidative stress that leads to DNA damage; and

(ii) enhanced cell proliferation or decreased apoptosis that alters the growth control of hepatocytes (Corton *et al.,* 2000).

The imbalance between the production and degradation of hydrogen peroxide (H_2O_2) resulting from oxygen radical generation is the main cause of oxidative injury. H_2O_2 is a by-product of acyl-CoA oxidase, the level of which increases by 10-30 fold during the induction of peroxisomes, while there is only a two-fold increase in the level of catalase, which is not sufficient to degrade all of the H_2O_2 produced (Reddy *et al*., 1986). Additionally, it is believed that peroxisome proliferation increases the rate of fixation of DNA in the genome leading to changes in gene expression, such as increased expression of oncogenes or silencing of the tumour suppressor genes.

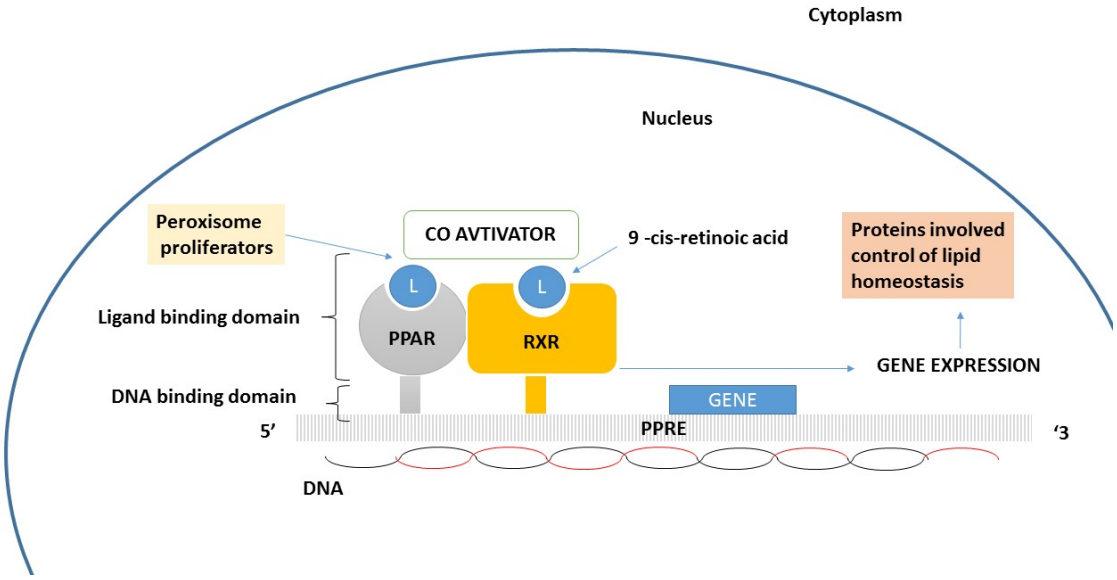


Figure 1.3. Mechanism of action of peroxisome proliferation.

Peroxisome Proliferator Activated Receptors (PPARs) were identified in the early 1990s as novel members of the steroid receptor superfamily (Schmidt *et al*., 1992). Figure 1.3 shows that binding of peroxisomes proliferators to PPARs leads to dimerisation with the Retinoid X Receptor (RXR). This heterodimer (PPAR-RXR) binds to DNA in a specific sequence element called the Peroxisome Proliferator Response Element (PPRE) that initiates gene expression and the production of proteins involved in fatty acids metabolism (Green and Wahli, 1994).

Collectively, each isotype of the PPAR family has a specific function in lipid homeostasis, as there are different isotypes of PPAR including PPARα, PPARβ/δ, and PPARγ (Green, 1995). Of particular chemical interest is the mediation of biological effects for peroxisome proliferators, which is mostly performed by PPARα. The primary natural ligands of PPARα are saturated and unsaturated fatty acids and it is highly expressed in liver, heart, kidney and muscle which all have higher rates of mitochondrial fatty acid oxidation. It is essential to have PPARα to mediate the carcinogenic response to a peroxisome proliferator in rodents, but its relevance to humans has been debated intensively. Noticeable differences in the response of peroxisome proliferators among species have been reported ranging from being highly susceptible in rats and mice to being highly intractable in dogs, guinea pigs, non-human primates and humans to short term effect of PP exposure (Bentley *et al*., 1993). In the case of humans, it is not entirely understood how resistance to peroxisome proliferators occurs while there is functional PPARα. The differences in PPARα expression is likely to be a probable explanation for the species-specific effects of PP (Tugwood *et al*., 1996). It was concluded that peroxisome proliferators are unlikely to cause human liver cancer at the expected exposure levels (Cattley *et al*., 1998). However, it cannot be disregarded that there is a dependency between PPARα mediated rodent liver cancer and differential PP exposure (Lai, 2004). The following sections discuss two special classes of the effects that have been attributed to peroxisome proliferators: (a) inhibition of gap junction intercellular communications and (b) DNA methylation.

1.8.1.1 Inhibitors of Gap Junction Intercellular Communication:

Gap junction intracellular communication has been shown to be inhibited by several non-genotoxic carcinogens including agonists of PPARα (Upham *et al*., 2008). Adjacent cells are connected internally by the channels formed by the plasma membrane, termed Gap junctions (Klaunig *et al*., 2003). The channels have the same structure irrespective of which tissue cells

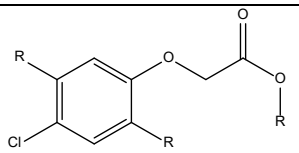
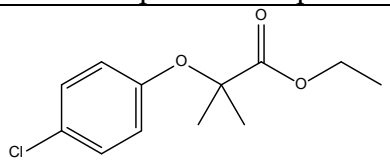
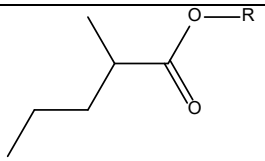
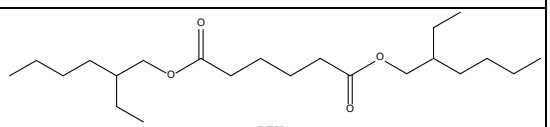
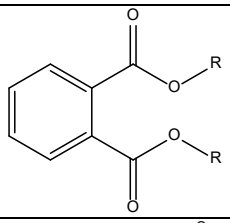
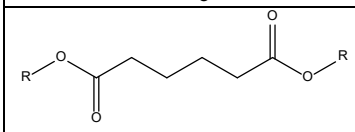
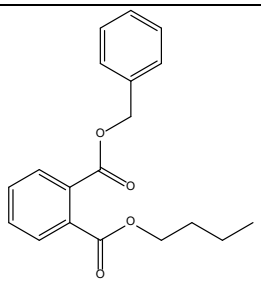
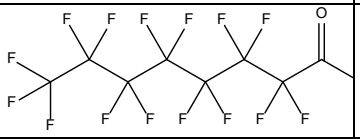
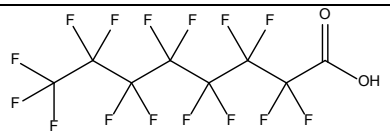
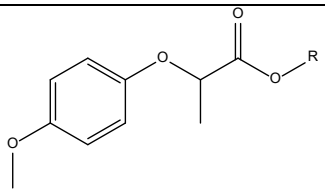
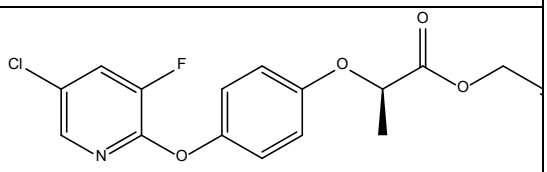
they are present in. In a typical gap junction, six subunits of connexin protein form a hemichannel connexon. Two connexions, one from each cell, dock to form a gap junction. Small ions and molecules such as calcium, glucose or growth regulators can diffuse directly through the gap junction between the cells and this process is known as Gap Junction Intercellular Communication (GJIC) (Yamasaki, 1990). GJIC is a normal phenomenon required for homeostatic maintenance in multicellular organisms, normal growth, development and tissue differentiation. Gap junction regulation can occur at the translational, post-translational or transcriptional level. Stable or transient up or down regulation of GJIC can occur at any of these three levels by exogenous or endogenous chemicals involving many mechanisms. It has been observed that stable normal gap junction regulation is linked with tumour suppressor genes and abnormal gap junction regulation is linked with activated oncogenes. GJIC has also been shown to be reduced by many carcinogens (Trosko, 1998). Heterologous or homologous alteration (either connexins localisation or aberrant expression) of GJIC has been reported in almost all malignant cells (Yamasaki, 1990). Although the mechanisms underlying the relationship between GJIC inhibition and carcinogenic response are ambiguous, GJIC is still considered to be important in tumour promotion and hence carcinogenesis.

1.8.1.2 DNA Methylating Agents:

Altered DNA methylation patterns have been reported following exposure to peroxisome proliferators such as dibutyl phthalate, dichloroacetic acid, Wy-14, trichloroethylene, trichloroacetic acid and gemfibrozil (Tao, 2000). DNA methylation is an epigenetic modification which affects regulation of transcription. It is covalent addition of a methyl group at the 5^{th} position of the cytosine ring within the CpG island (which is the region within DNA with a high frequency of CpG sites, i.e. the area where a cytosine nucleotide (C) is followed by guanine nucleotide (G) within the linear sequence of bases). DNA methylation is considered

to be a non-genotoxic mechanism involved in the promotion and initiation of carcinogenesis (Watson and Goodman, 2002). In both tumour tissues and cells, the altered DNA methylation patterns have been observed as compared to normal cells. It has been observed in the genome of various animal and human cancers that hypermethylation (region specific) and hypomethylation (global) can coexist. The most important clue is that the CpG islands are hypermethylated in tumour cells, however, there is no methylation at all in the CpG islands in normal cells. The CpG islands have more CpG regions as compared to other regions of the genome which are associated with coding regions or promotors of genes. Hypermethylation of these regions may result in transcriptional alteration or silencing of associated genes which, in turn, may result in inactivation of tumour suppressor genes. Alternatively, proto oncogenes, such as c-Jun or c-Myc can be abnormally activated by hypomethylation. Moreover, methylcytosine to thymine deamination can also occur by hypermethylation, which can result in cytosine to thymine point mutations (Robertson, 2000). In short, different changes after DNA methylation, such as hypermethylation, hypomethylation, altered gene expression, and mutation may lead to carcinogenesis. A number of structural alerts have been identified for peroxisome proliferation as shown in Table 1.3.

Table 1.3. The main structural alerts for peroxisome proliferation (Benigni *et al.,* 2013)

N	Alert name	Structural alert	An example molecular containing the alert that elicits peroxisome proliferation
1	Substituted phenoxy acids		 clofibrate
2	Substituted N alkyl carboxylic acid		 DEHA di-(2-ethylhexyl) adipate
3	Phthalate (or butyl diesters and monoesters)	 	 butyl benzyl phthalate
4	Perfluorooctanoic acid (PFOA)		
5	Phenoxy herbicide		 clodinafop-propargyl

1.8.2 Inducers of oxidative stress

Oxidative stress has been implicated in carcinogenesis in many ways (Halliwell, 2007). It is caused by the imbalance between the antioxidant capability of the target cell and the production of Reactive Oxygen Species (ROS). The short-lived ROS - such as $^{\cdot}OH$ (hydroxyl), O_2^{-} (superoxide anion) and non-radical oxygen derivatives such as H_2O_2 (hydrogen peroxide) - are highly reactive towards many biological entities, including lipids, proteins, nucleic acids and

membranes. A decrease in cell's antioxidant resistance leads to increase in the level of ROS and consequently to oxidative stress and oxidative damage. Both endogenous sources (peroxisomes, inflammation in cell and mitochondria) and exogenous sources (radiation, industrial chemicals, drugs and environmental agents) can produce ROS (Klaunig *et al.,* 2009). Oxidative metabolism in mitochondria and enzymes, such as cyclooxygenases, xanthine oxidases, lipoxygenases and NADP oxidase endogenously produces superoxide anions (Valko *et al*., 2004). The enzyme superoxide dismutase (SOD) depletes superoxide anions and the resulting H_2O_2 is removed by glutathione peroxidases and catalases. However, in the presence of metal ions, hydrogen peroxide can be converted to the hydroxyl radical through The Haber-Weiss (Fenton) reaction. The hydroxyl radical is more reactive and aggressive in terms of modifying DNA and the production of several oxidation products (Valko *et al.,* 2005). However, the hydroxyl radical cannot diffuse within cells because of its high reactivity. It is hypothesised that free radical hydrogen must be produced from hydrogen peroxide in the immediate vicinity of DNA and can easily cross cell membranes (Klaunig *et al*., 1995).

ROS can damage DNA in several ways including DNA cross-linking, at apurinic/apyrimidinic sites, deoxyribose modification, breakage in single or double strands and deoxyribose modification. Normal in cellular DNA, repair machinery mends this damage by nucleotide excision and base excision repair. Where this is left unrepaired before replication, it could lead to genome instability, cell death or DNA mutation (Cooke, 2003). 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is the most studied oxidative DNA lesion that is produced by the hydroxyl radical at the C-8 position of deoxyguanosine residues. It is also a commonly used biomarker of oxidative stress along with its keto-enol tautomer 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Valavanidis *et al.,* 2009). In addition, 8-OHdG is highly mutagenic due to mispairing of adenine in the replication process (Cheng *et al*., 1992). High

levels of 8-OHdG have been observed in many studies on human cancers and animal tumours (Klaunig *et al*., 1995).

ROS have also been found to induce genetic changes, such as chromosomal rearrangements and DNA mutations, which are the basis for the initiation of cancer (Cooke, 2003). ROS cannot only cause cell damage, but they can also affect cell regulation and intracellular signalling (Allen and Tresini, 2000). The stress activated signalling cascade starts in cells due to a change in the redox potential of cells by oxidative stress, which in turn activates transcription factors related to redox potential (Adler *et al*., 1999). Mitogen activated protein kinase (MAPK) pathways are the signalling pathways initiated by ROS. These pathways activate many factors including hypoxia-inducible factor-1(HIF-1), nuclear factor of activated T cells (NFAT), nuclear factor (NF) -κB, p53, and activator factor-1(AP-1). The expression of many DNA damage protective genes is controlled by these pathways, including the genes involved in DNA repair, induction of apoptosis, damage cell proliferation arrest and the ability of the immune system to repair damage (Valko *et al*., 2006). ROS start signalling initially by the release of intracellular calcium, which in turn activates the protein C kinase, a serine threonine kinase which regulates cell survival, migration, death and proliferation. These epigenetic effects play an important role in tumour promotion (Gopalakrishna and Jaken, 2000).

Many epigenetic carcinogens (phenolic compounds such as o-phenylphenol, pentachlorophenol, and quercetin-type flavonoids) cause cancer by the induction of oxidative stress. A common pathway in drug transformations is oxidation of the phenols by CYP450 enzymes that lead to the production of hydroquinone, which is oxidised into quinone. The semi-quinone radical formed by the reduction of one electron is followed by superoxide anion

formation by autoxidation in the presence of oxygen and ultimately quinone regeneration (Bolton *et al*., 2000). This redox cycle produces ROS, which could lead to oxidative stress (Kovacic and Jacintho, 2001).

Oxidative stress is also an important mode of action for toxic metals including copper, cobalt, aluminium and iron. These metals are collectively involved in hydroxyl radical production *in vivo* by activation in Fenton reactions and are thus termed carcinogenic metals (Valko *et al.,* 2005). Other metals such mercury, lead and cadmium increase ROS production indirectly by depleting thiol containing enzymes and antioxidants which are the major cellular antioxidants (Leonard *et al.,* 2004). Chromium exists in three oxidative states - Cr(0), Cr(III), and Cr(VI). Cr(III) compounds are not carcinogenic to humans (Hopkins, 1991). The International Agency for Research on Cancer (IARC) classified Cr(VI) compounds as group 1 carcinogens to humans (Straif *et al*., 2009). Cr(VI) uses the anion channel to enter the cells as chromates interact with physiological phosphate and sulphate (Zhitkovich, 2005). Inside the cells, biological reducers (cysteine, glutathione and ascorbate) reduce Cr(VI) into Cr(III) (Standeven *et al.,* 1992). The reductive reactions produce several products including radicals based on carbon, sulphur and Cr(V) (O'Brien, 2003). Moreover, Fenton type reactions also produce hydroxyl radicals by Cr forms (Shi *et al.,* 1993). Oxidative stress is produced by two main factors: the oxidising abilities of Cr(V) and the formation of ROS. Besides ROS, the carcinogenicity of Cr(VI) is more dependent upon its DNA mutagenesis ability (Zhitkovich, 2011). These compounds can cause damage to DNA in different ways, including DNA-DNA cross linking, oxidative damage, Cr-DNA adducts and DNA protein cross-linking (O'Brien, 2003). The reduction of Cr(VI) to Cr(III) is required for the interaction with DNA, Although that there are some recent studies have shown the cellular uptake of reduced Cr which was produced by extracellular redox

reactions (Valko *et al.,* 2005). Cr(III) compounds are poorly permeable to the cell membrane and are therefore non-toxic (Costa, 1997).

Arsenic is also a Group 1 carcinogen to humans (Shi *et al.,* 2004). Inorganic arsenic, such as arsenate V and arsenite III, is methylated to form MMA (monomethylarsonic) and DMA (dimethylarsinic) in the body as a result of detoxification process (Vaskenaposhian, 2004). Trivalent arsenite can interact with thiol containing enzymes and proteins in their reduced state and inhibit several biochemical pathways. Pentavalent arsenate is less active than trivalent arsenite (Huang *et al*., 2004) but it may be reduced to arsinite in the body after absorption (Rosen, 2002). Arsenite has been found to cause multilocus deletion mutations in human-hamster hybrid cells (Hei *et al.,* 1998). Dose-dependent transformation of BALB/3T3 cells and Syrian hamster embryo cells was induced by sodium arsenite and sodium arsenate (Bertolero *et al*., 1987; Lee *et al*., 1985). Ultraviolet radiation and inorganic arsenic have also been reported to cause co-mutagenicity and carcinogenicity (Hughes, 2002). A number of mechanisms have been proposed for arsenic carcinogenesis in humans. Several studies have indicated different types of ROS production during arsenic metabolism including singlet oxygen ($^1O_2$), nitric oxide (NO•), arsenic-mediated generation of superoxide (O_2•−), peroxyl radical (ROO•), dimethylarsinic radical [$(CH_3)_2As$•], dimethylarsinic peroxyl radical [(CH_3) 2AsOO•] and hydrogen peroxide (H_2O_2) (Shi *et al.,* 2004). The mode of action of arsenic carcinogenesis has been linked to other effects including altered DNA repair, possibly as a result of oxidative stress, enhanced cell proliferation, gene amplification, cell progression/promotion, p53 suppression and DNA methylation (Hughes, 2002).

1.8.3 Inducer of hormonal imbalance

Epigenetic carcinogenesis has also been linked to increased expression of trophic hormones due to hormonal imbalance. Increases in both exogenous and endogenous hormones can stimulate cell proliferation which, in turn, can result in the formation of tumours by greater cell division and random genetic errors (Henderson *et al*., 1988). There is a negative feedback regulation mechanism amongst trophic hormone target tissues and hypophysis; this acts to stop target gland secretion and the elimination of secreted hormones which together overproduce the respective pituitary hormone. If this mechanism continues for a long time it results into the development of tumours in over-stimulated target glands or over-reactive hypophysis.

Many goitrogenic xenobiotics are associated with non-genotoxic mechanisms through hormonal imbalance. These chemicals may disrupt any biosynthesis steps including thyroid hormone biosynthesis, metabolism and secretion, and induce thyroid tumours from follicular cells (Capen, 1992). Xenobiotics use several methods to decrease thyroid activity by either increased excretion of the thyroid hormone in bile, interference with thyroid secretion and synthesis in the thyroid gland, T4 to T3 conversion disruption and hepatic mixed-function induction (Capen, 1992). The pituitary gland negative feedback system regulates thyroid stimulating hormone (TSH) secretion and synthesis, and results in sustained expression of TSH. Stimulation of TSH results in hyperplasia, hypertrophy and neoplasia in rodents by proliferation of follicular cells (Hill, 1989). However, it is believed that thyroid pituitary disruption mechanisms of tumorigenesis are less relevant in humans than tumorigenesis in rodents (Hill *et al*., 1998).

Hypothalamo-pituitary-testis (HPT) axis disruption and hormonal imbalance in rodents are the epigenetic mechanisms which lead towards the induction of tumours in Leydig cells (interstitial cells that produce testosterone). These cells are stimulated by leuteinising hormone (LH) to produce testosterone. Negative feedback of testosterone results in the production of LH from

the pituitary gland (Clegg *et al*., 1997). In rodents this feedback is blocked by different chemicals with different mode of actions including oestrogen agonism, androgen antagonism, testosterone biosynthesis inhibition, gonadotropin releasing hormone (GnRH) agonism, 5α-reductase inhibition, dopamine agonism and aromatase inhibition. The chemicals that are involved in HPT axis disruption, with the exception of dopamine agonists and GnRH, may pose a risk to human health. Rodents are more sensitive than humans to Leydig cell tumours induced by chemicals (Cook *et al*., 1999).

The carcinogenic action of oestrogens is an epigenetic mechanism as well as having genotoxic effects. Cells expressing receptors for oestrogen, i.e. in the breast, liver and endometrium, start proliferating as a result of prolonged expression of oestrogen from exogenous and endogenous sources. The organs, whose normal growth is under hormonal control, develop hyperplasia to neoplasia as a result of proliferation induced by oestrogen (Henderson *et al*., 1988).

1.8.4 Agonists and antagonists of the aryl hydrocarbon receptor.

Aryl hydrocarbon receptor (AhR) mediation by different natural and synthetic chemicals can have many toxicological and biological effects on cells, including carcinogenesis. AhR belongs to the superfamily of basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) proteins and is a ligand activated transcription factor. AhR agonists consist of halogenated and planar aromatic hydrocarbons including biphenyls, heterocyclic plant constituents, dibenzofurans and related chemicals and polychlorinated dibenzo-p-dioxins (Denison *et al*., 2002). Until now, no authentic AhR high affinity endogenous agonists have been identified, although there are studies which indicate that endogenous physiological ligands are involved in the activation of AhR itself and the signalling pathways of AhR. Unbound AhR complexed with co-chaperons

and chaperone HsP90 has been found in cellular cytosol, which translocates into the nucleus after binding with the ligand. In the nucleus, HsP90 and other chaperons are released and it associates with Ah receptor nucleus translocator (Arnt) forming an AhR:Arnt heterodimer (Bock and Köhle, 2006). Special DNA binding sites called Ah-responsive element (AhRE), dioxin responsive element (DRE), or xenobiotic responsive element (XRE) present in the target genes regulatory regions are recognised by this heterodimer and it binds to this site (Rowlands and Gustafsson, 1997). The target genes may include proliferation regulatory genes, genes involved in differentiation and development and Phase 1 and 2 biotransformation enzymes coding genes (Beischlag *et al*., 2008).

Enhanced DNA binding results in corepressors and coactivators recruitment, chromatin structure remodelling and target genes transcriptional machinery activation. Constant exposure to agonists (for example exposure to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)) leads towards toxic tissue- and species-specific effects including teratogenicity, chloracne, wasting, liver tumour promotion, carcinogenicity and immunotoxicity (Bock and Köhle, 2006). Although the role of AhR in producing these responses is observed, the underlying molecular mechanisms are still unknown. It is hypothesised that inappropriate or sustained activation of AhR results in the TCDD mediated toxicities and deregulated physiological functions (Poland and Knutson, 1982). In another experiment on rodents expressing mutant AhR, the role of this receptor in hepatocarcinogenesis promotion was demonstrated (Moennikes, 2004). It has been hypothesised that at the tumour initiation stage sustained AhR signalling is involved which facilitates the genotoxically injured cells selective survival. Nuclear proteins and signalling factors, AhR/Arnt crosstalk have also been described (Puga *et al*., 2009). Specifically, crosstalk with the oestrogen receptor.

1.8.5 Summary of the findings on mechanisms of non-genotoxic carcinogenicity

As described in Section 1.8, most carcinogenic chemicals associated with a non-genotoxic of action have been reported to act through one or more of the following mechanisms:

1. Peroxisome proliferation, which may result from either induction of oxidative stress; enhanced cell proliferation or decreased apoptosis; inhibition of gap junction intercellular communication; or DNA methylation.
2. Induction of oxidative stress, that may result from either an increase in the production of oxyradicals, or a decrease in the cell's antioxidant capacity that may lead to DNA damage in several ways - such DNA cross-linking, at apurinic/apyrimidinic sites, breakage in single or double strand and deoxyribose modification.
3. Induction of hormonal imbalance
4. Agonist and antagonist of aryl hydrocarbon receptor (AhR), which is a ligand activated transcription factor.

Knowledge of these key mechanisms of action has the capability to provide a basis for further evaluation and *in silico* analysis of these important effects

1.9: The potential use of in silico tools for the identification of non-genotoxic carcinogen

With regard to developing *in silico* tools for the prediction of the toxic effects of cosmetics ingredients, there has been a great deal of research undertaken. For instance, Safety Evaluation Ultimately Replacing Animal Testing-1 (SEURAT-1) was a cluster of European projects created in response to the Seventh Amendment of the final deadline for the Cosmetic Directive in January 2011. It comprised a collaboration of one co-ordination project and six research projects encompassing 70 European Universities, commercial companies and research

institutes. The SEURAT-1 Cluster operated for five years (2011-2015) with the aim of reducing the reliance on *in vivo* repeated dose toxicity tests by beginning the process of replacing it with *in silico* and *in vitro* methods. The COSMOS Project was one of the six research projects of SEURAT-1; the main objectives of COSMOS were as follows:

a. The formation of an inventory of cosmetic ingredients including their chemical structures as well as, where possible, toxicological data.

b. The collection and compilation of recent sources of the toxicological data from the literature and regulatory sources.

c. The development of new software to be applied in the analysis of the repeated dose toxicity of the cosmetics towards humans.

The safety of cosmetics ingredients within the European Union was supported by the development of new tools with the aid of the COSMOS project and other collaborative projects associated with SEURAT-1. It was also envisaged that the results of the six research projects could help provide a basis for alternative techniques and tools in the identification of toxicity of chemicals used in pharmaceutical industry (Cosmostox.eu, 2019).

The main outputs from the COSMOS Project were the development of the COSMOS database (https://cosmosdb.eu/cosmosdb.v2/) which focussed on repeated dose toxicity data for cosmetics ingredients, amongst other endpoints and data. The data were used to enrich and enhance the datasets available for derivations of Threshold for Toxicological Concern (TTC) values (Yang *et al*., 2017). In addition, a number of innovative computational approaches for the assessment of chronic toxicity endpoints (notably liver toxicity) were developed in the COSMOS Project. The new modelling approaches were developed around the use of knowledge of MIEs for endpoints such as hepatic steatosis (Mellor *et al*., 2016) and PPARγ dysregulation (Al Sharif *et al*., 2017). Overall, the COSMOS Project illustrated the possibility

of modelling a complex endpoint when appropriate and mechanistically based *in silico* models were developed.

Taken as a whole, the SEURAT-1 Cluster enabled better use of the data from new methods. There were at least two major contributions including development of a set of read-across case studies proposed by Berggren *et al.,* (2015). The case studies provided a number of learnings for the development collation of data and justification of similarity hypotheses (Schultz and Cronin, 2017) as well as the definition of uncertainties in read-across (Schultz *et al*., 2019). In addition, the SEURAT-1 Cluster developed a workflow, or strategy or safety assessment in the case of no data, with decisions made on an exposure basis in the first place, then leading to use of new types of data. The type of sequential approach was termed an *ab initio* chemical safety assessment workflow and demonstrated the utility of combining together different types of information (Berggren *et al*., 2017). Overall the SEURAT-1 philosophy, and COSMOS Project in particular, demonstrated that information on chemistry can be used to make assessments of complex and subtle toxicities, especially when combined into integrated frameworks of data gathering.

1.10: Context and research aims of this thesis:

The safety of cosmetic products is of utmost importance in relation to consumer health because of the large variety of products, the frequency of use and the intimate nature of applications on the body. In Europe, ensuring safety of cosmetics is jointly undertaken by industry and regulatory authorities. The European regulatory framework for cosmetic safety requires pre-market notification of the intended use of any ingredients that fall within the regulated categories, assessment of safety, regulatory approval, and appropriate labelling of the final products. At the regulatory level, safety of cosmetic ingredients is assessed by an independent committee of experts (the Scientific Committee on Consumer Safety, SCCS) before they are

allowed by the European Commission to be used in cosmetic products. The safety assessment of final products is overseen by the competent authorities in the EU Member States. Ensuring overall safety of the final products placed on the market, nevertheless, remains responsibility of the industry.

Safety assessment of cosmetic ingredient/products requires detailed information and data relating to the physicochemical properties and toxicological hazard of ingredients, as well as the possible route(s) and the extent of consumer exposure. The toxicological data for hazard identification/ characterisation are generally drawn from a systematic scheme that involves testing against set endpoints that can provide information on potential acute (short-term) and chronic (long-term) adverse effects. The most difficult endpoints to measure accurately in this regard are those that are indicative of long-terms effects, such as reproductive and developmental effects and carcinogenicity. The available tests for gene mutation and DNA damage can indicate the potential of a cosmetic ingredient to be a genotoxic carcinogen. However, identifying non-genotoxic carcinogens is a particular challenge due to the lack of a single mode of action. As non-genotoxic carcinogens can act through alteration of multiple pathways, and generally without a change in DNA sequence, predicting the potential carcinogenicity of a non-genotoxic chemical is one of the current major challenges in toxicology, although as shown in Section 1.8 the main mechanisms can be defined.

The EU Cosmetic Regulation (EC) No 1223/2009 has also resulted in a ban on animal testing of cosmetics ingredients since 11 March 2013. This means that all toxicological data for cosmetic ingredients and products need to be drawn from alternative (non-animal) methods. This has made testing of cosmetic ingredients for non-mutagenic carcinogenicity even more challenging. A battery of *in vitro* tests is available for certain endpoints but results from different tests may generate contradictory or equivocal results. In this context, the use of *in silico* models and read-across tools provides a useful alternative means to obtain additional

supporting evidence which, when used in conjunction with other lines of evidence, can strengthen the overall weight of evidence for safety assessment. *In silico* approaches can also provide important clues to the mode of action of a chemical to inform *in vitro* testing and the models and structural alerts can enable toxicological assessment of other substances. In this regard, the European SEURAT-1 Cluster went some way to demonstrate a reduction in the reliance on *in vivo* repeated dose toxicity tests and providing a strategy to replace it with read-across, *in silico* and *in vitro* methods. As part of the SEURAT-1 cluster, the COSMOS project specifically aimed to use new alternative tools for safety assessment of cosmetic ingredients.

In keeping with the research aims of the COSMOS project, the overall aim of this research was to evaluate and develop *in silico* models for the human health effects of cosmetic ingredients, focussing on read-across and (Quantitative) Structure-Activity Relationships ((Q)SARs) for carcinogenicity and skin sensitisation. The specific objectives to achieve this aim were:

i. To review the state of the art of grouping approaches, (Q)SARs and available software to predict the toxicity of cosmetic ingredients, including the mechanisms of carcinogenicity and their relationship to existing Adverse Outcome Pathways (AOPs) with an emphasis on non-genotoxic mechanisms.
ii. To identify and evaluate existing data for the carcinogenic potential of the chemicals, as well as repeated dose toxicity, skin sensitisation, dermal absorption and metabolism, assessing quality of both physicochemical and toxicological data.
iii. To assess existing structural alerts for human health effects allowing for the formation of chemical categories, read-across supported by ToxCast data and (Q)SARs supported by data for key events in AOPs.
iv. To study the chemical space of cosmetics ingredients and materials utilising and building on the COSMOS inventory through the analysis of descriptors of molecular structure and physicochemical properties.

v. To develop relevant case studies to provide a proof of concept for cosmetics-related materials focussing on relevant classes of chemicals such as aluminium and the phthalates.

An extensive literature search was performed as part of this thesis and this indicated that a range of *in silico* models, read-across tools and expert systems is available. These are discussed in more detail in Chapters 3 and 5. In addition, certain structural alerts have been identified for some known non-genotoxic mechanisms of carcinogenicity. For example, a small number of structural alerts have been associated with peroxisome proliferation, e.g. substituted phenoxy acids, substituted N-alkyl carboxylic acids, phthalates (or butyl diesters and monoesters), perfluorooctanoic acid and phenoxy herbicides. These are discussed in more detail in Chapter 2. A general comparison between the performance of genotoxic and non-genotoxic carcinogen structural alerts was also conducted in Chapter 2. This comparison showed that the positive predictivity for genotoxic carcinogen structural alerts was more accurate and effective compared to non-genotoxic carcinogen alerts. A detailed analysis of positive predictivity of all available mutagenic structural alerts and profilers within the OECD QSAR Toolbox when compared to experimental mutagenicity data from the CCRIS dataset was conducted in Chapter 3. In Chapter 4, the relationship between scaffolds of a range of diverse compounds and carcinogenicity (both genotoxic and non-genotoxic) was analysed using a dataset of Ames assay data for 10,543 compounds from the SAR genotoxicity database, and carcinogenicity data for 2,870 compounds from the SAR carcinogenicity in the Leadscope® database (Leadscope.com, 2018). Chapter 5 illustrates how essential it is to know the accuracy of the different profilers within the OECD QSAR Toolbox for carcinogenicity, mutagenicity and skin sensitisation in terms of sensitivity, specificity and accuracy, and to investigate possibilities for improvement.

Chapter 2: Assessment of currently available structural alerts for genotoxic and non-genotoxic carcinogens

2.1: Introduction:

Mutagenicity and carcinogenicity are considered amongst the most significant toxicological concerns for human health. As such, they are part of the standard information requirements for regulatory and other risk assessment. To understand the mechanistic basis of these endpoints, Miller *et al*. (1977; 1981) described the electrophilic theory of chemical carcinogenesis which helped rationalise a wide variety of carcinogenic chemicals identified during the 1970s. Miller's theory gave a mechanistic basis for these chemicals to act as mutagens (i.e., in the Ames test). It also stated that various carcinogenic compounds possessed alkylating and electrophilic characteristics. Thus, electrophilic acylating agents are one of the key groups of chemicals that can be considered as direct-acting carcinogens. In addition, Miller *et al*. also reported that there are other carcinogenic chemical compounds, other than acylating agents, such as aromatic amines, which may undergo electrophilic reaction following metabolism. In this way, the (chemical) structural basis of genotoxic carcinogens began to be established.

It is now well known that a significant proportion of the direct acting chemical carcinogens are electrophilic in nature. However, many genotoxic carcinogenic chemicals that are not electrophilic behave as such *in vivo*, as they can react with nucleophilic groups of proteins and nucleic acids present in cells and tissue (Miller and Miller, 1981). Miller's work inspired different researchers to work further in this field. For instance, different strains of genetically engineered *Salmonella typhimurium* have been developed to test specific individual chemical classes, such as alkylating or intercalating chemical carcinogens (Maron and Ames, 1983). The *Salmonella*, or Ames, Test is an *in vitro* model for detecting chemical mutagenicity and consists of different bacterial strains that are susceptible to a large array of DNA damaging agents

(Ames, 1984). The Ames Test was developed from mutant forms of the bacterium *S. typhimurium*, whereby when the bacterial DNA interacts with a potential genotoxic chemical, the change in DNA provides evidence for mutagenicity of the chemical.

Miller's hypothesis supporting the use of *Salmonella* in the Ames Test was relevant at that time in relation to the mechanism of action as carcinogens were thought to be mainly the result of genotoxic interactions (Ashby, Tennant, 1988). In due course, the theory regarding the electrophilic activity of many chemical carcinogens has also been incorporated into a more general theory of chemical carcinogenesis. According to the theories at that time, the initiation of cancer was due either to genetic mutation, or a carcinogen's ability to damage DNA directly (Arcos and Argus, 1995). However, there is yet another type of carcinogen termed "epigenetic" which does not bind covalently to DNA and hence does not damage DNA directly. As a result, epigenetic (non-genotoxic) carcinogens are negative in the most commonly and frequently used assays for mutagenicity (Woo, 2003). As discussed in Chapter 1, there are a number of diverse mechanisms of action of epigenetic carcinogens.

Structural alerts

The term 'structural alert' was defined by Dr John Ashby, who also contributed to the compilation of a list of structural alerts following the electrophilicity theory proposed by Miller (Ashby, 1985; Ashby and Tennant, 1988). Structural alerts are defined as being a definable fragment within a molecule (i.e., a functional group or substructure) representing a structure-activity relationship (i.e., inducing carcinogenic activity). Thus, the potential classes of chemicals that can induce cancer can be identified by structural alerts for carcinogenicity. Depending on their definition, structural alerts for genotoxic carcinogenicity are considered suitable to identify mutagenic compounds, as the main mode of action of this type of carcinogen (i.e., genotoxic carcinogen) is modification and direct interaction with DNA. Structural alerts

for carcinogenicity have been identified from experimental data on animals or from observations on human epidemiological studies.

There are many reasons why the majority of carcinogenic structural alerts were obtained from data derived from rat and mice toxicological studies. These animals are preferred as investigational models because of their relatively low cost and ease of maintenance (compared to other non-rodent assays), short life span and higher susceptibility to tumour induction, as well as the accessibility of characterised strains (Huff *et al*., 1991; Fung *et al*., 1995; Huff and Haseman, 1999). Whilst short-term mutagenicity assays help in the detection of potential genotoxic carcinogens within a much shorter timeframe, non-genotoxic carcinogens cannot be identified easily or exclusively by the use of long-term carcinogenicity studies. These rodent bioassays, in conjunction with bacterial Ames and other *in vitro* tests for mutagenicity, provide an indication of carcinogenicity.

Models based on structural alerts play a major role in predictive toxicology. Software platforms, both commercial (e.g., DEREK Nexus from Lhasa Ltd) and non-commercial (e.g., Oncologic by the US EPA) use structural alerts as the basis to predict mutagenicity/carcinogenicity. Whilst structural alerts generally predict genotoxic carcinogens well, their ability to identify non-genotoxic carcinogens is still in infancy. (Woo, 2003) reported different characteristics of non-genotoxic carcinogens as well as relating them to structural alerts. As mentioned in Chapter 1, non-genotoxic carcinogens have different modes of action and cannot be explained by a clear unifying theory. The main non-genotoxic mechanisms of carcinogenicity can be mainly grouped into the following mechanisms (see Section 1.8 for more details):

1) Peroxisome proliferation, which includes:
    a) Inhibitors of gap junction intercellular communication
    b) DNA methylating agents

2) Inducers of oxidative stress
3) Inducers of hormonal imbalance
4) Agonists and antagonists of aryl hydrocarbon receptor.

Relationship of mechanism of action to structural alerts:

In general, if a single structural alert represents the same or similar chemical class, then it may be assumed to exhibit a similar mode of toxic action. The major chemical groups identified by the structural alerts responsible for direct acting genotoxic carcinogens are sulphur-compounds, epoxides, aziridines, α-haloethers and lactones (Benigni *et al*., 2008). For the purposes of this Chapter, the mode of action of epoxides will be analysed in detail as a representative example.

Epoxides alkylate DNA and this may lead to carcinogenic effects. The strained epoxide ring breaks open easily to form a carbonium ion, which is responsible for the initiation of this alkylation reaction. This, in turn, leads to the substance being able to react with a nucleophilic site, such as DNA, forming 2-hydroxy-2-alkyl adducts (Singer and Grunberg, 1983). Thus, chemicals containing epoxide groups are strongly associated with the induction of mutations in cells and/or cancer induction. The chemical mechanism of the reaction of an epoxide with DNA is shown in Figure 2.1:

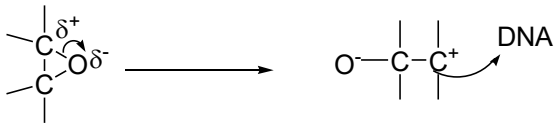


Figure 2.1. Mechanism of epoxide attack on DNA (Benigni *et al.,* 2008).

There are other structural alerts for functional groups relating to genotoxic carcinogens that are not directly acting, but may become genotoxic following metabolic activation. Due to the

complexity of some of the metabolic pathways e.g. involving more than a single metabolic step, a structural alert could point to a range of final toxicological outcomes. For instance, aromatic imines and amines may be metabolically activated to electrophiles and hence have the potential to induce carcinogenicity. A study in mice revealed that the oxidation of aromatic amides and amines formed N- hydroxyarylamines and N-hydroxyarylamides, respectively induced by cytochrome P-450 c (BNF-B) and d (ISF-G). The metabolic conversion of nitroso, nitro, and hydroxylamine derivatives also generates amine groups. Another example of the complexity of metabolic activation is that seven nitroaromatic hydrocarbons are generated through the formation of N-hydroxyarylamine as an intermediate in the presence of cytosolic and microsomal enzymes that act as a catalyst.

The process of the reduction of nitro groups in microsomes can be replicated experimentally in the presence of a cytochrome P-450 complex obtained from rat liver isozymes, namely c (PB-B), d(PB-B), b (PB-B), and e (PB-D). The enzymes responsible for cytosolic nitroreductase activity include DT-diaphorase, alcohol dehydrogenase and enzymes having xanthine and aldehyde groups. The main activation pathway is the nitrogen oxidation and reduction reaction as shown in Figure 2.2. However, there are certain aromatic amines, as well as aromatic nitro compounds, that are converted to electrophilic compounds through a ring oxidation pathway. Unlike other direct electrophilic metabolites, such as hydroxyarylamines, epoxides, and iminoquinones, N-hydroxyarylamides need to go through the esterification process to be capable of reacting with DNA (Benigni, 2005).

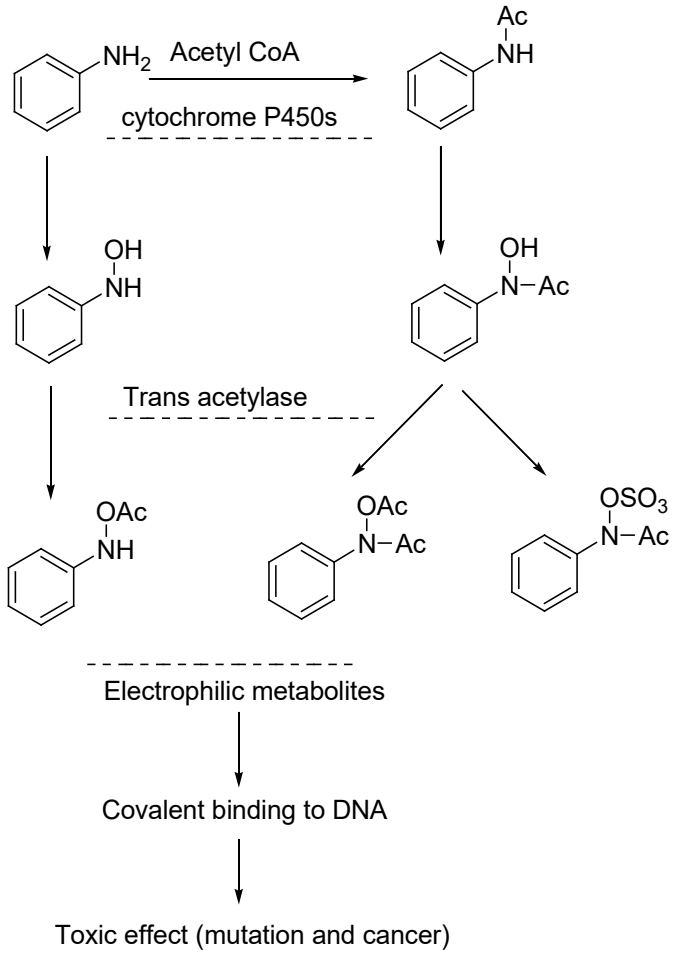


Figure 2.2. The main oxidation pathway of aromatic amines leading to potentially carcinogenic metabolites (Benigni, 2005).

Using more than a single SA is appropriate for some chemical classes as it the mechanism of action of certain groups, such as the aliphatic halogens, is more complicated. As shown in Figure 2.3, the mechanism of action of aliphatic halogens may switch from genotoxic to non-genotoxic pathways depending on the degree of halogenation and the whether the carbon skeleton is a cyclic or linear. The short chain mono-halogenated alkanes, as well as alkenes and dihalogenated alkanes, act directly as alkylating agents (as genotoxic mechanism) either without, or after, conjugation with GSH. Conversely, the mechanism of action of poly-haloalkanes is either a non-genotoxic or a free radical mechanism.

With regard to halogenated cycloalkanes (and cycloalkenes), it is more appropriate to use multiple structural alerts as the mechanism of action of carcinogenicity is still unclear and possibly genotoxic (i.e., alkylation) either directly or after metabolic activation, although non-genotoxic mechanisms have also been proposed. For example, Woo *et al*. (2002) suggested non-genotoxic mechanisms for halogenated cycloalkanes to involve:

a. Hormonal imbalance
b. Degranulation of the rough endoplasmic reticulum
c. Inhibition of other intercellular mechanisms.

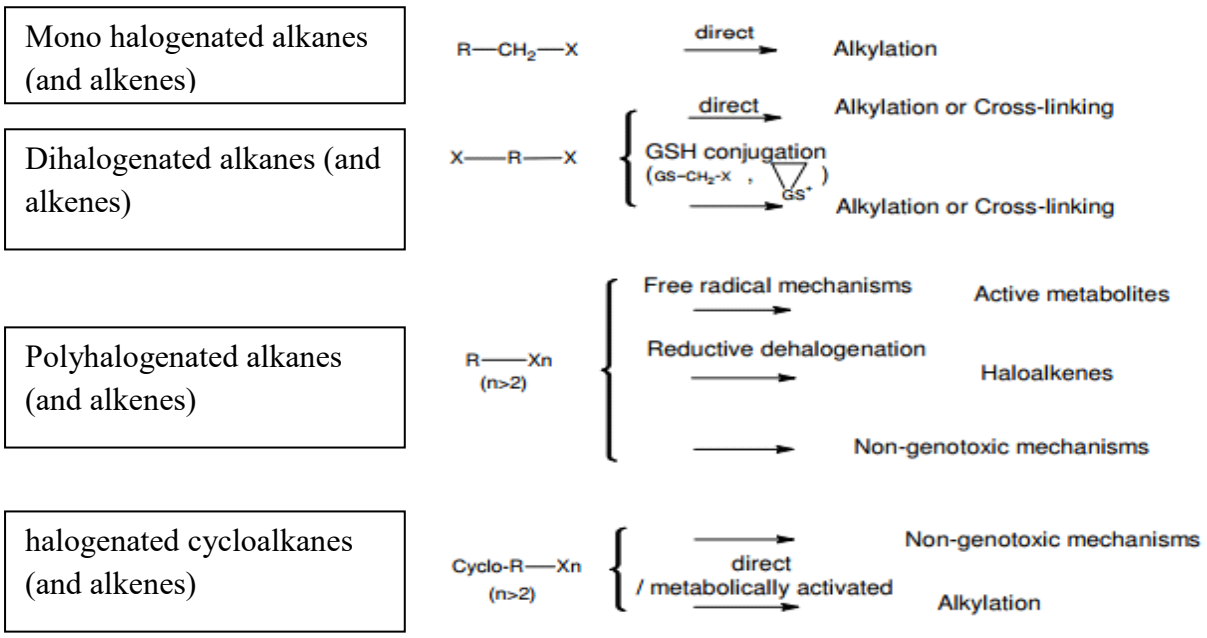


Figure 2.3. The complexity of carcinogenicity mechanism of actions for aliphatic halogens (Benigni, 2005).

In contrast to complex carcinogenicity testing, *in silico* toxicology offers an extremely attractive option in terms of being a rapid and low cost methodology. It also provides the possibility to reduce animals use and make tests more directed and mechanistically based. As

such, structural alerts also provide a means to understand and interpret the mechanisms of genotoxicity and therefore help in the classification of potential carcinogens. The structural alerts published by Benigni *et al.* (2013) and summarised in Tables 2.1 and 2.2 are considered the most advanced list to evaluate both the genotoxic and non-genotoxic carcinogenicity potential of chemicals. These structural alerts have also been implemented as a rule-base system in the Toxtree software and the OECD QSAR Toolbox.

Table 2.1. Currently identified non-genotoxic carcinogen structural alerts (Benigni *et al.*, 2013).

NO	Mechanism of action*	Alert Name
1	HI	Thiocarbonyl
2	HI	Poly halocycloalkane
3	AHR	Halogenated benzenes
4	AHR	Halogenated dibenzodioxines
5	HI	Steroidal oestrogen
6	PP	Substituted phenoxy acids
7	PP	Substituted n-alkylcarboxylic acids
8	PP	Phthalates
9	PP	Perflourooctanoic acid (PFOA)
10	PP	Tri, tetraflouroethylene
11	AHR	Indole-3-carbonyl
12	OXS	Pentachlorophenoles
13	OXS	2-Phenylphenols
14	OXS	Quercetin flavonoid
15	HI	Benzimidazolea
16	HI	Imidazoles ,benzamidazoles
17	HI	Dicarboximides
18	HI	Dimethylpyridinse
19	OXS	Metals
20	HI	Benzsulfonic ether
21	OXS	1,3-Benzdioxole
22	PP	Phenoxy herbicides
23	HI	Alkyl halides

***PP**: peroxisome proliferator, **OXS**: oxidative stress, **HI**: hormonal imbalance, **AHR**: aryl hydrocarbon receptor agonist and antagonist.

Table 2.2. Currently identified genotoxic carcinogen structural alerts (Benigni *et al.*, 2013).

NO	Mechanism of action	Alert Name
1	Direct DNA Alkylation	Alkyl (C5) or benzyl esters of sulphonic or phosphonic acid
2		N-methylol derivatives
3		S- or N- mustards
4		Propiolactones and propiolsulfones
5		Epoxides and arizidines
6		Aliphatic halogens
7		Alkyl nitrites
8		α, β-Unsaturated carbonyls
9		Simple aldehydes
10		Quinones
11		Alkyl and aryl N-nitroso groups
12	Indirect DNA Alkylation	Monohaloakenes
13		Hydrazines
14		Aliphatic azo and azoxys
15		Alkyl carbamate and thiocarbamates
16		Azide and triazene groups
17		Aliphatic N-nitro groups
18		α, β-Unsaturated alkoxy group
19		Pyrrolizidine alkaloids
20		Alkenylbenzenes
21		Steroidal oestrogen (genotoxic and non genotoxic)
22	Direct Acylation	Isocyanate and isothiocyanate groups
23	Amino aryl DNA adduct formation	Aromatic ring N-oxides
24		Aromatic nitroso groups
25		Nitro Aromatics
26		Aromatic amines and hydroxyl amine & its derived esters
27		Aromatic mono and dialkylamino groups
28		Aromatic N-acyl amines
29		Aromatic diazo groups
30	DNA adduct formation by Intercalation	Polycyclic Aromatic Hydrocarbons
31		Heterocyclic Polycyclic Aromatic Hydrocarbons
32		Coumarins and Furocoumarins

Given the utility of structural alerts for all toxic endpoints, and potential benefits in using them for the prediction of carcinogenicity, it is perhaps surprising that there have been no, or few, systematic assessments of their performance, coverage and relevance. This is particularly pertinent to non-genotoxic carcinogens where an assessment of the available structural alerts

could assist in the identification of their strengths, but also clarification of chemical or mechanistic space that is not well covered. The aim, therefore, of this chapter was to assess the currently available structural alerts and *in silico* models for both genotoxic and non-genotoxic carcinogenicity. The analysis focussed on four main mechanisms of non-genotoxic carcinogens: peroxisome proliferation, hormonal imbalance, oxidative stress, and aryl hydrocarbon receptor agonism/antagonism as well as the five main mechanism of action of genotoxic carcinogen: direct DNA alkylation, indirect DNA alkylation, direct acylation, amino aryl DNA adduct formation and DNA adduct formation by intercalation. In this study, the performance of the 23 structural alerts for non-genotoxic carcinogenicity described by Benigni *et al*. (2013), listed in Table 2.1 and as implemented within the Toxtree software, has been assessed by comparison with experimental cancer data compiled in the Carcinogenic Potency Database (CPDB).

2.2: Methods:

2.2.1 Dataset used:

Carcinogenic Potency Database (CPDB)

Data relating to cancer causing chemicals were compiled from the Carcinogenic Potency Database (CPDB), which is freely available from http://toxnet.nlm.nih.gov/cpdb/cpdb.html. This database is a widely used and unique international resource comprising the results of 6,540 chronic, long-term animal carcinogenicity tests on 1,547 chemicals in rats, mice, dogs, hamsters and non-human primates. All important information is included for each experiment to interpret the bioassay, such as strain, species and sex of the test animal along with other details relating to the experimental protocol used, e.g. the route of administration, average daily dose and duration of dosing. Information is also provided on the tumour type, tumour incidence, carcinogenic potency (TD50) and statistical significance of the results. TD50 can be very useful

for comparison and analysis of the relative carcinogenicity of compounds as it provides a standard qualitative measure.

2.2.2: Review process of non-genotoxic structural alerts

The workflow shown in Figure 2.4 outlines the steps in the assessment of the current non-genotoxic structural alerts discussed in this chapter. This workflow is split into two sections: the first section is related to the filtering and extraction of CPDB cancer data. The second section is related to the review process using the rule-based expert system Toxtree version 2.6.13 (downloaded in April 2016) which is freely available from http://toxtree.sourceforge.net. Toxtree predicts different types of toxicological hazard and modes of action by applying decision tree approaches; it can be used for initial hazard assessments (Pavan and Worth, 2008)**.** The review process was conducted after converting the extracted structures from SMILES format to SD/SDF format using the Open Babel programme version 2.3.2 (downloaded in April 2016) which is freely available from https://openbabel.org/docs/dev/Installation/install.html.

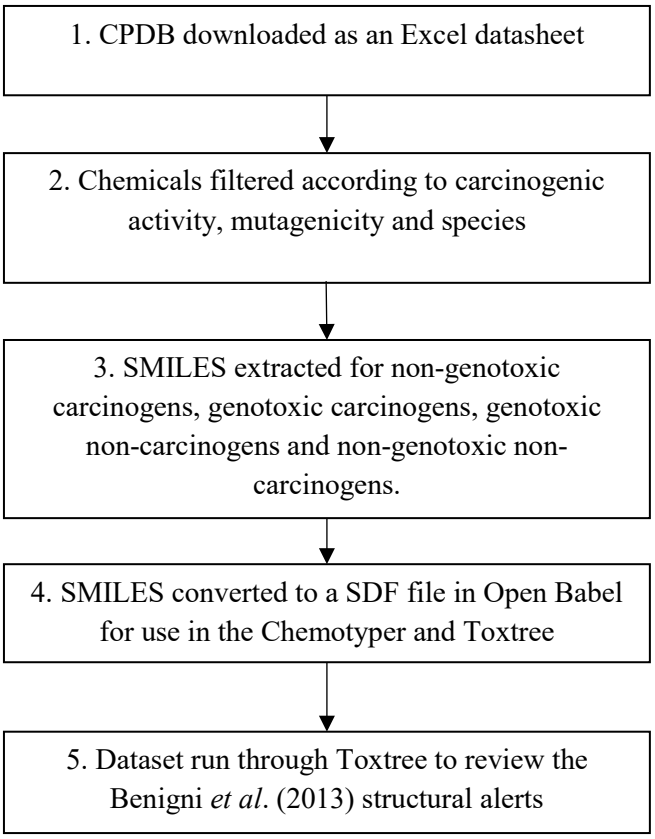


Figure 2.4. Workflow for the process undertaken to assess structural alerts for non-genotoxic carcinogenicity.

The individual steps of the workflow summarised in Figure 2.4 are described below.

Step 1: CPDB downloaded as an Excel datasheet:

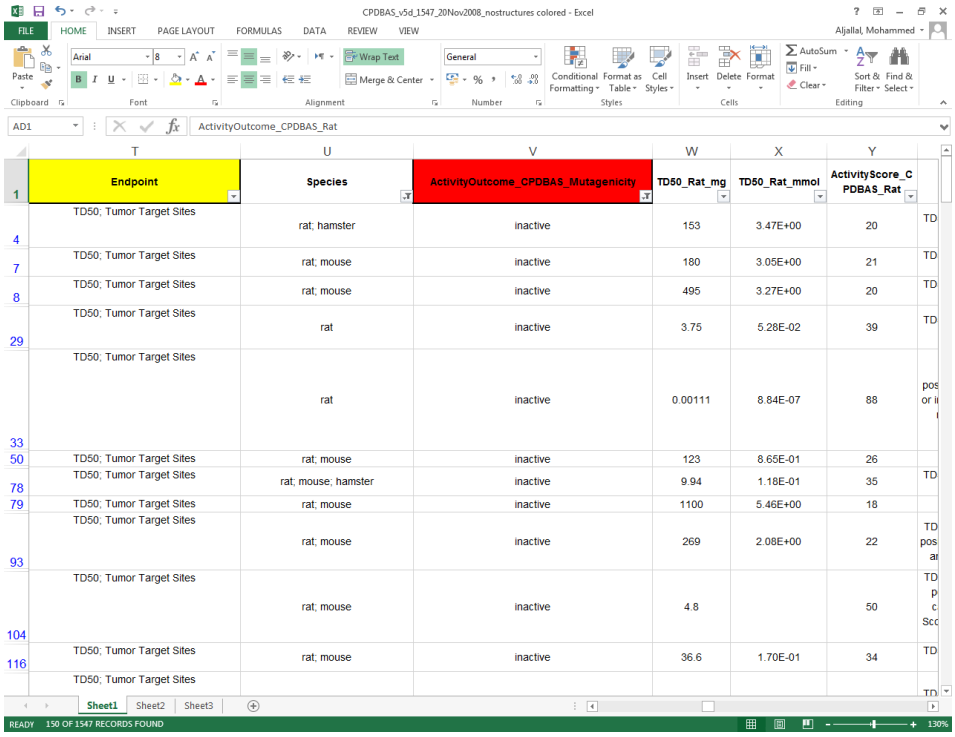
Results were downloaded from the CPDB for the full list of 6,540 experiments on 1,547 chemicals as an Excel spreadsheet. The information downloaded included the full details regarding the diversity of bioassay designs in the CPDB, e.g., dose ranges tested, number of dose groups and the frequency of testing per chemical.

Step 2: Chemicals filtered according carcinogenic activity, mutagenicity and species

The process of filtering the downloaded CPDB data is detailed below.

1. Using the "sort & filter" tool in Excel, the 1,548 chemicals were filtered to select only those chemicals that are negative (inactive) in the Ames test in the column

(ActivityOutcome_CPDBAS_Mutagenicity), as shown in Figure 2.5. This indicates which chemicals have a higher probability of an epigenetic carcinogenicity mechanism.

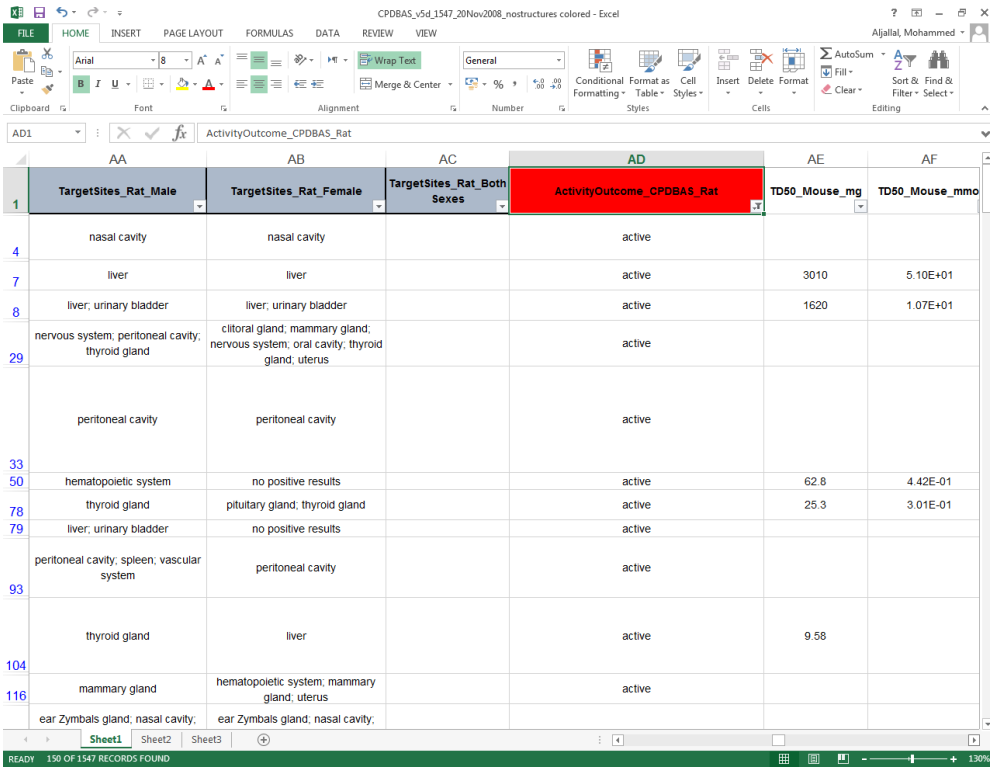


	T	U	V	W	X	Y
1	Endpoint	Species	ActivityOutcome_CPDBAS_Mutagenicity	TD50_Rat_mg	TD50_Rat_mmol	ActivityScore_CPDBAS_Rat
4	TD50; Tumor Target Sites	rat; hamster	inactive	153	3.47E+00	20
7	TD50; Tumor Target Sites	rat; mouse	inactive	180	3.05E+00	21
8	TD50; Tumor Target Sites	rat; mouse	inactive	495	3.27E+00	20
29	TD50; Tumor Target Sites	rat	inactive	3.75	5.28E-02	39
33	TD50; Tumor Target Sites	rat	inactive	0.00111	8.84E-07	88
50	TD50; Tumor Target Sites	rat; mouse	inactive	123	8.65E-01	26
78	TD50; Tumor Target Sites	rat; mouse; hamster	inactive	9.94	1.18E-01	35
79	TD50; Tumor Target Sites	rat; mouse	inactive	1100	5.46E+00	18
93	TD50; Tumor Target Sites	rat; mouse	inactive	269	2.08E+00	22
104	TD50; Tumor Target Sites	rat; mouse	inactive	4.8		50
116	TD50; Tumor Target Sites	rat; mouse	inactive	36.6	1.70E-01	34
	TD50; Tumor Target Sites					

Figure 2.5. Screenshot of the Excel spreadsheet showing filtrated carcinogenic data that are inactive in the Ames test in order to extract non-genotoxic carcinogens from the CPDB.

2. Only data for rats were included in the filtered dataset from the “species” column. Data for other species, such as mice and rhesus and cynomolgus monkeys, were excluded from the dataset, as shown in Figure 2.6.
3. Only “active” chemicals that initiate carcinogenicity were selected from the “ActivityOutcome_CPDBAS_Rat” column, resulting in the selection of 150 non-genotoxic carcinogenic chemicals to the rat.
4. All mixtures and inorganic substances were excluded from the list using the column “STRUCTURE_ChemicalType”.

5. The same process was conducted using the same filtering process as described above to produce three additional groups for comparison. This produced 240 genotoxic carcinogens, 108 genotoxic non-carcinogens and 242 non-genotoxic non-carcinogens.



	AA	AB	AC	AD	AE	AF
1	TargetSites_Rat_Male	TargetSites_Rat_Female	TargetSites_Rat_Both Sexes	ActivityOutcome_CPDBAS_Rat	TD50_Mouse_mg	TD50_Mouse_mmo
4	nasal cavity	nasal cavity		active		
7	liver	liver		active	3010	5.10E+01
8	liver; urinary bladder	liver; urinary bladder		active	1620	1.07E+01
29	nervous system; peritoneal cavity; thyroid gland	clitoral gland; mammary gland; nervous system; oral cavity; thyroid gland; uterus		active		
33	peritoneal cavity	peritoneal cavity		active		
50	hematopoietic system	no positive results		active	62.8	4.42E-01
78	thyroid gland	pituitary gland; thyroid gland		active	25.3	3.01E-01
79	liver; urinary bladder	no positive results		active		
93	peritoneal cavity; spleen; vascular system	peritoneal cavity		active		
104	thyroid gland	liver		active	9.58	
116	mammary gland	hematopoietic system; mammary gland; uterus		active		
	ear Zymbals gland; nasal cavity;	ear Zymbals gland; nasal cavity;				

Figure 2.6. Screenshot of the Excel spreadsheet showing the exclusion of data for species other than the rat to select rat carcinogens only from the activity outcome column.

Step 3: SMILES strings extracted for non-genotoxic carcinogens, genotoxic carcinogens, genotoxic non-carcinogens and non-genotoxic non-carcinogens.

One hundred and fifty non-genotoxic carcinogenic chemical structures, in SMILES strings, were copied from the column "STRUCTURE_SMILES" using the "find & select" tool, selecting only visible cells as shown in Figure 2.7. The same process was performed for the other three groups of compounds. Once selected, SMILES strings were pasted into a new Excel sheet and saved as a txt file.

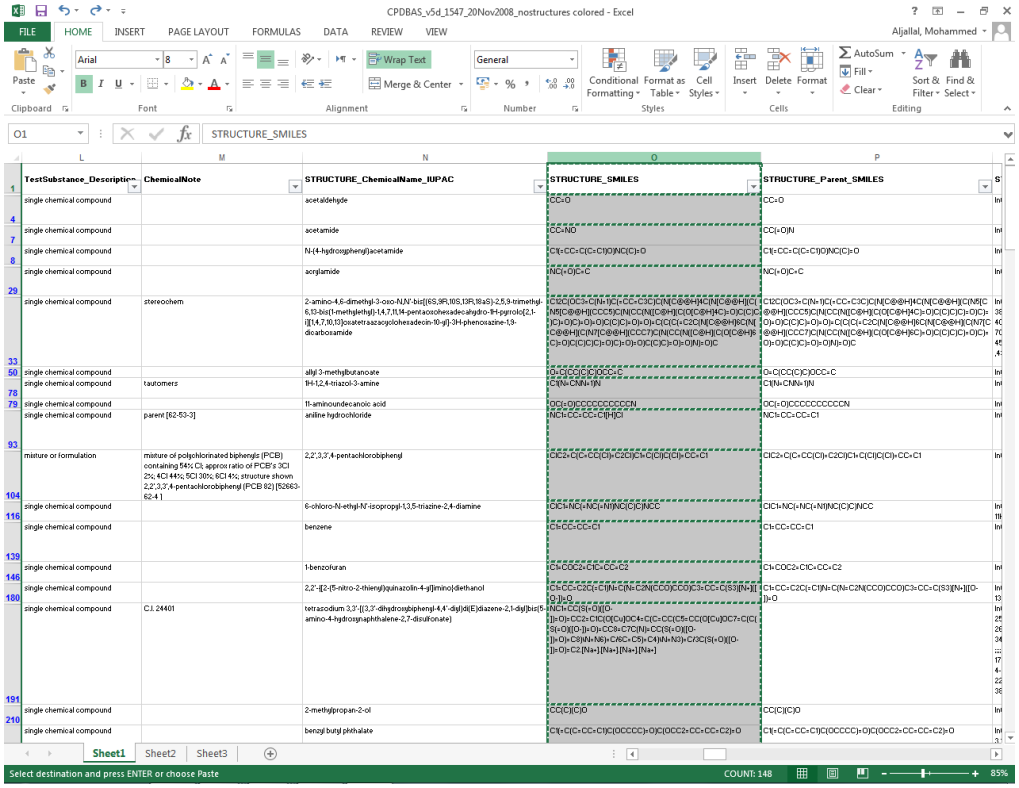


Figure 2.7. SMILES strings copied from the "STRUCTURE_SMILES" column in the CPDB Excel spreadsheet.

Step 4: SMILES converted to a SDF file in Open Babel for use in the Chemotyper and Toxtree.

The most important information about chemicals is easily exchanged using the SDF file format. SDF files include all necessary information about training/test set molecules (i.e., identifiers of all compounds, CAS/ InChI/ name/ formula, 3D structures, experimental and predicted values of target properties/parameters and the values of the molecular descriptors utilised).

In order to convert and store the filtered list of chemical structures, the open source Open Babel programme version 2.3.2 was used. This program is designed to search, convert and store chemical data from molecular modelling. Open Babel version 2.3.2 for Windows is freely available at http://openbabel.org/. Open Babel was used as follows:

1. The SMILES strings of the non-genotoxic carcinogens and the three other groups were saved as a .txt file to act as the input into Open Babel – its content was visualised to confirm it was recorded correctly.
2. In order to save the output as a SDF file, the name and the path of the output file were specified as shown in Figure 2.8.
3. In case of an error occurring for certain chemical structures during the conversion process, Open Babel offers a choice to continue and override to the next structure in the input SMILES list to avoid any delays or error in structure conversion process which was activated before the conversion process.
4. All four resultant SDF files were saved in separate files to be used in Toxtree.

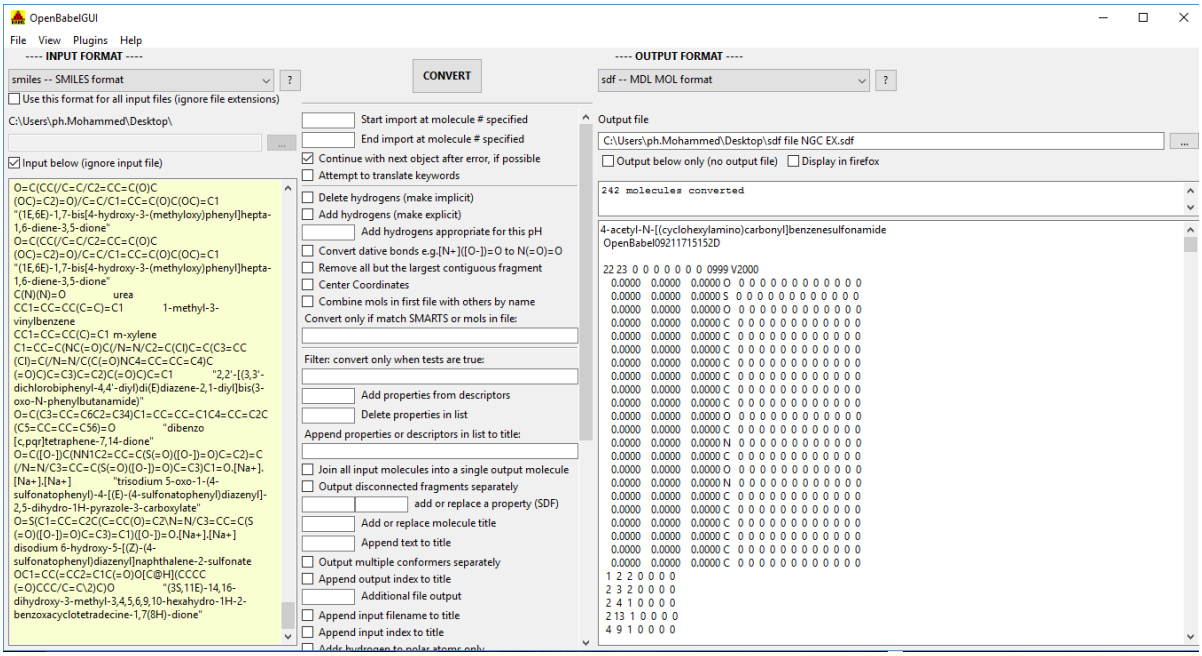


Figure 2.8. Screenshot of the Open Babel software showing the input (SMILES) and output (SDF) format.

Step 5: The data sets were run through Toxtree to review Benigni et al.'s (2013) structural alerts:

Toxtree is a programme developed by IDEAconsult Ltd. (Sofia, Bulgaria) for researchers and other stakeholders (especially in industry) to predict various types of toxic effects using decision trees to place chemicals into appropriate categories. It includes Benigni *et al.*'s (2013) rules for mutagenicity and carcinogenicity. Chemical structures can be entered into Toxtree using SMILES strings, SDF files, and over 110 other chemical file formats. If a structural alert is present in a molecule this is highlighted and can be recorded. The Toxtree software was used as described below.

1. First, it is essential to choose the correct decision tree to predict carcinogenicity and mutagenicity. This was selected by clicking on method from the main tabs and then choosing the desired tree from "select decision tree".
2. As illustrated in Figure 2.9, a list of available decision trees was shown, including that required to use Benigni *et al.*'s (2013) structural alerts, under the name of "carcinogenicity (genotox & nongenotox) by ISS".

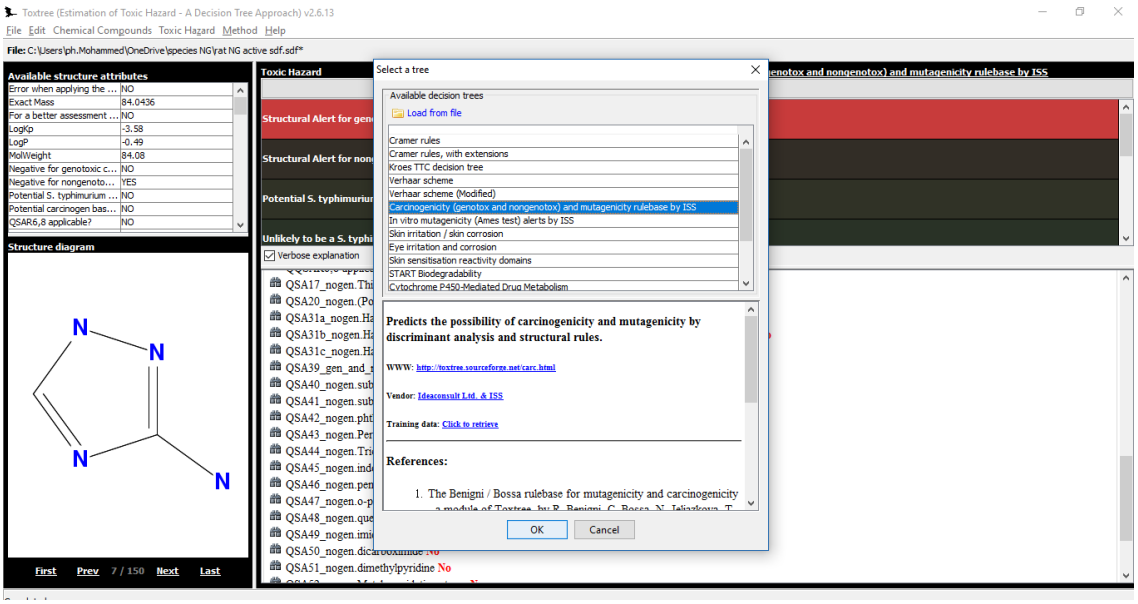


Figure 2.9. The choice of decision tree for "Carcinogenicity prediction by ISS".

3. The 150 chemicals determined to be non-genotoxic to rat were entered into Toxtree as a SDF file and the alerts run on them using the (estimate) tab for each chemical. This process was also applied to the 240 chemicals determined to be genotoxic carcinogen to rat.
4. All structures containing one or more of Benigni *et al.*'s (2013) structural alerts for either genotoxic or non-genotoxic carcinogenicity were highlighted in Toxtree with a brown warning message for non-genotoxic carcinogen and red for genotoxic carcinogen under the estimation tab, as shown in Figure 2.10. The presence of an alert was counted as a positive result.

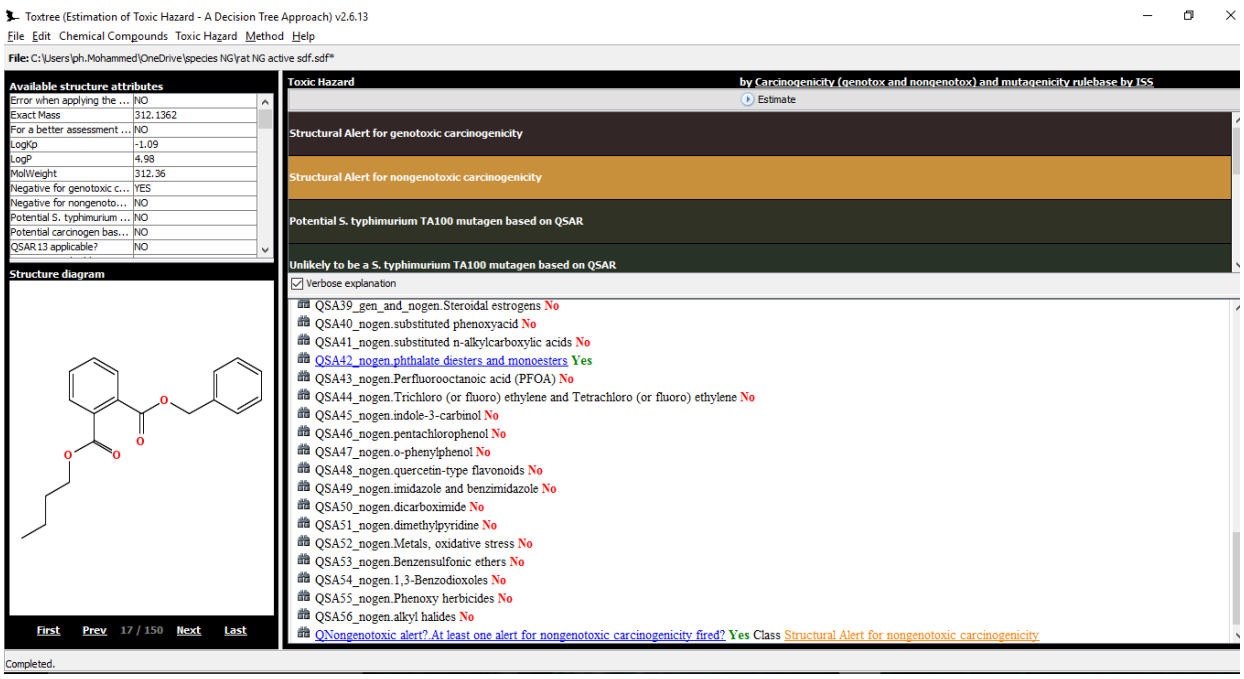


Figure 2.10. Structural alert for the non-genotoxic carcinogenicity for benzyl butyl phthalate.

5. The SDF files for the third and fourth groups of chemical structures (non-carcinogens) were also entered Toxtree to evaluate the negative predictivity of the "carcinogenicity (genotox & nongenotox) by ISS" decision tree.

2.2.3 Statistical analysis

The results of the predictions were analysed in Excel using a four-way contingency table. The performance of 23 non genotoxic carcinogen structural alerts, 32 genotoxic carcinogen structural alerts and the overall performance of ISS carcinogenicity profiler which includes both genotoxic and non-genotoxic structural alerts was assessed against the two groups of substances, 390 carcinogens and 350 non carcinogens. The true positive rate (sensitivity) was calculated for both non genotoxic and genotoxic carcinogens alone and then compared with the true positive rate of the ISS carcinogenicity profiler which includes both. All substances among the 390 carcinogenic group that were correctly predicted either by non-genotoxic or genotoxic carcinogenic structural alerts were counted as true positives, if it failed to predict the carcinogenic substances, then it was counted as a false negative. Among the group of 350 non carcinogenic substances, the true negative rate (specificity) was calculated for both non-genotoxic and genotoxic carcinogenic for structural alerts alone and then calculated for ISS carcinogenicity profiler as a whole. All substances among the group of 350 non carcinogens that were falsely predicted as carcinogenic will be counted as false positive and, if not, were counted as true negative predictions.

2.3. Results and discussion:

The aim of this chapter was to assess the currently available structural alerts for both genotoxic and non-genotoxic carcinogenicity, focussing on the main four mechanisms of action of non-genotoxic carcinogenicity: peroxisome proliferation, hormonal imbalance, oxidative stress, and aryl hydrocarbon receptor agonism/antagonism and the main five mechanisms of action of genotoxic carcinogenicity: direct DNA alkylation, indirect DNA alkylation, direct acylation, amino aryl DNA adduct formation and DNA adduct formation by intercalation. In this Chapter, the performance of 23 structural alerts for non-genotoxic carcinogenicity and 32 structural alerts for genotoxic carcinogenicity as described by Benigni *et al*. (2013) and coded within Toxtree version 2.6.13, was assessed by comparison with experimental data for carcinogenicity which were compiled in, and retrieved from, the CPDB.

Analysis of the CPDB found 390 substances in the CPDB to be experimentally determined to be carcinogenic in rats. Of these 390 substances, 150 were non-genotoxic (i.e., negative in the Ames test). The remaining 240 substances were positive in the Ames test, i.e., rat genotoxic carcinogens. The analysis also found 350 substances to be experimentally determined to be non-carcinogenic in the rat. All 740 substances were assessed using Toxtree version 2.6.13, applying the carcinogenicity rules by ISS. The predictions were compared with the experimental results.

The number of correct predictions for the carcinogenic compounds is reported in Table 2.3. For positive prediction of non-genotoxic carcinogenic substances, only 41 out of the total 150 were correctly assigned as being carcinogenic. Thus, the predictively of Toxtree with regard to the positive identification of non-genotoxic carcinogens was only 27.3%. This is in a sharp contrast to 91% predictivity for genotoxic carcinogenic compounds (223 positively predicted

out of 240) and shows the limitation of the currently available structural alerts in relation to the identification of non-genotoxic carcinogenic chemicals.

Table 2.3. Prediction of genotoxic and non-genotoxic carcinogenicity using the ISS Rulebase in Toxtree v2.6.13.

<table>
<tr><th colspan="2">Name and classification of substances' groups</th><th>Number of substances predicted by the 23 structural alerts as non-genotoxic carcinogen in Toxtree</th><th>Number of substances predicted by the 32 structural alerts as genotoxic carcinogen in Toxtree</th><th colspan="2">ISS carcinogenicity profiler (genotoxic and non-genotoxic)</th></tr>
<tr><td rowspan="2">Carcinogen
390 Substances</td><td>Non-Genotoxic
150 Substance
Genotoxic
240 Substance</td><td rowspan="2">41 True Positive——</td><td rowspan="2">223 True Positive</td><td>264
True
positive</td><td>126
False
Negative</td></tr>
<tr><td rowspan="2">Non-carcinogen
350 Substances</td><td>Genotoxic
108 Substance
Non-Genotoxic
242 Substance</td><td>125
False
positive</td><td>225
True
Negative</td></tr>
<tr><td colspan="2">True positive rate (sensitivity) %</td><td>Positive Predictive Value:

78.8%

Sensitivity among 150 non-genotoxic carcinogens:

27.3%</td><td>Positive Predictive Value:

66.1%

Sensitivity among 240 genotoxic carcinogens:

92%</td><td colspan="2">68%</td></tr>
<tr><td colspan="2">True negative rate (specificity) %</td><td colspan="2"></td><td colspan="2">64%</td></tr>
</table>

2.3.1 Genotoxic carcinogen structural alerts:

The most common structural alerts to predict genotoxic carcinogens were for the aromatic amines and nitro aromatics, with 51 and 40 hits respectively. These substances become carcinogenic through the aminoaryl DNA adduct formation as an indirect acting agent (Benigni, 2005). The structural alerts for compounds that exert their action by alkylating mechanisms were also predictive, as the indirect acting agent alkyl and aryl N-nitroso groups were found in

39 out of 240 genotoxic substances. Direct acting agents (i.e. aliphatic halogens) were found in 20 genotoxic substances. The other structural alerts listed in Table 2.5 were present in different substances; however, they were limited in number compared to aromatic amines and nitro aromatic structural alerts. The alerts were grouped in Table 2.5 based on the main mechanisms of action, as some of the alerts were poorly represented among the experimental carcinogens.

As shown in Table 2.4, seventeen experimentally determined genotoxic carcinogenic substances were not flagged by genotoxic carcinogenic structural alerts out of the total of 240; however, some were obviously carcinogenic, including formaldehyde, selenium sulfide, sodium nitrite and tetra-nitromethane. Nevertheless, there was no common link that could be used to group these structures to derive a new rule.

One of these seventeen carcinogenic substances is selenium sulfide which is used as an anti-dandruff in shampoos. It is believed that selenium sulfide controls dandruff via its anti-*Malassezia* effect, rather than by its antiproliferative effect although it has an effect in reducing cell turnover (Milani *et al*., 2003). *Malassezia* is a genus of fungi that is naturally found on the skin surfaces of many animals, including humans. It has anti-seborrheic properties as well as cytostatic effect on cells of the epidermal and follicular epithelium. Excessive oiliness after use of this agent has been reported in many patients as adverse drug effect (Ranganathan and Mukhopadhyay, 2010).

Selenium sulfide is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from experimental studies in animals. Oral exposure of selenium sulfide caused tumours in two rodent species and at two different tissue sites. Administration of selenium sulfide by stomach tube caused liver cancer (hepatocellular carcinoma) in rats of both sexes and in female mice. In female mice, it also increased the combined incidence of

benign and malignant lung tumours (alveolar/bronchiolar adenoma and carcinoma) (NCI 1980b). When applied topically, selenium sulfide and selsun, an antidandruff shampoo containing 2.5% selenium sulfide, did not cause tumors in mice; however, these studies were considered inconclusive, because the study length was limited to 88 weeks by the animals' early death resulting from amyloidosis (NCI 1980a,c).

Another chemical substance that has been determined experimentally as being a non-genotoxic carcinogen, and not identified by non-genotoxic carcinogenic alerts, was potassium bromate (KBrO3). This is an oxidising agent that has been used as a food additive and in the cosmetic industry. Although adverse effects are not evident in animals fed bread-based diets made from flour treated with KBrO3, the agent is carcinogenic in rats and nephrotoxic in both man and experimental animals when given orally. It has been demonstrated that KBrO3 induces renal cell tumours, mesotheliomas of the peritoneum and follicular cell tumours of the thyroid. In addition, experiments aimed at elucidating the mode of carcinogenic action have revealed that KBrO3 is a complete carcinogen, possessing both initiating and promoting activities for rat renal tumorigenesis. However, the potential seems to be weak in mice and hamsters. Active oxygen radicals generated from KBrO3 were implicated in its toxic and carcinogenic effects, especially because KBrO3 produced 8-hydroxydeoxyguanosine in the rat kidney (Kurokawa *et al*., 1990).

In general genotoxic carcinogen structural alerts showed a high sensitivity rate among large number of carcinogenic substances compared to non-genotoxic alerts.

Table 2.4. Identity of 17 out of 240 experimentally genotoxic carcinogen which were not identified by the ISS genotoxic rule base

Number	Chemical Name IUPAC	SMILES String
1	acrylonitrile	C=CC#N
2	4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one	N/C1=N/C(=O)N(/C=N1)[C@@H]2O[C@H](CO)[C@@H](O)[C@H]2O
3	potassium bromate	Br(=O)(=O)[O-].[K+]
4	buta-1,3-diene	C=CC=C
5	naphthalen-1-yl methylcarbamate	O=C(OC1=C2C(=CC=C1)C=CC=C2)NC
6	trichloro(nitro)methane	ClC([N+](=O)[O-])(Cl)Cl
7	2,6-dimethyl-1,3-dioxan-4-yl acetate	CC1CC(OC(O1)C)OC(=O)C
8	dimethyl phosphonate	O=P(H)(OC)OC
9	formaldehyde	C=O
10	sodium nitrite	O=N[O-].[Na+]
11	1,4-benzoquinone dioxime	ON=C1C=CC(=NO)C=C1
12	selenium sulfide	[Se]=S
13	8-hydroxy-6-(methyloxy)-3a,12c-dihydro-7H-furo[3',2':4,5]furo[2,3-c]xanthen-7-one	O=C1C2=C(C=C3C(=C2OC4=CC=CC(=C14)O)C5C(O3)OC=C5)OC
14	styrene	C=CC1=CC=CC=C1
15	tetranitromethane	O=[N+](C([N+](=O)[O-])([N+](=O)[O-])[N+](=O)[O-])[O-]
16	propane-1,2,3-triyl trioctanoate	O=C(OC(COC(=O)CCCCCCC)COC(=O)CCCCCCC)CCCCCCC
17	zinc bis(dimethyldithiocarbamate)	S=C([S-])N(C)C.[S-]C(N(C)C)=S.[Zn+2]

Table 2.5. Percentage of structural alerts flagged among the group of genotoxic carcinogens organised according to chemical reactivity domain.

Mechanism of Action	Structural Alert	Number of genotoxic carcinogens containing this alert	Total number of genotoxic carcinogen structural alerts by this mechanism	Percentage of the genotoxic carcinogen structural alerts of this mechanism out of total
Alkylating (Direct acting agent)	Alkyl (C5) or benzyl esters of sulphonic or phosphonic acids	4	91	35%
	N-methylol derivatives	0		
	S- or N- mustards	4		
	Propiolactones and propiolsulfones	3		
	Epoxides and arizidines	10		
	Aliphatic halogens	20		
	Alkyl nitrite	1		
	α, β-Unsaturated carbonyls	2		
	Simple aldehydes	1		
	Quinones	7		
	Alkyl and aryl N-nitroso groups	39		
Alkylating (Indirect acting agent)	Monohaloakenes	7	32	13%
	Hydrazines	10		
	Aliphatic azo and azoxy	2		
	Alkyl carbamate and thiocarbamate	4		
	Azide and triazene groups	3		
	Aliphatic N-nitro groups	1		
	α, β-Uunsaturated alkoxy groups	0		
	Pyrrolizidine Alkaloids	3		
	Alkenylbenzenes	2		
	Steroidal oestrogens (genotoxic & non-genotoxic)	0		
Acylating (Direct acting agent)	Isocyanate and isothiocyanate groups	3	3	2%

Amino aryl DNA adduct forming (indirect acting agent)	Aromatic ring N-oxides	0	119	43%
	Aromatic nitroso groups	1		
	Nitro aromatic	40		
	Aromatic amines and hydroxyl amine and their derived esters	51		
	Aromatic mono and dialkylamino groups	7		
	Aromatic N-acyl amines	7		
	Aromatic diazo groups	14		
Intercalating and DNA adduct forming (indirect acting agent)	Polycyclic Aromatic Hydrocarbons	5	19	7%
	Heterocyclic Polycyclic Aromatic Hydrocarbons	11		
	Coumarins and furocoumarins	4		

2.3.2: Non genotoxic structural alerts:

Table 2.6 shows, among the 150 non-genotoxic carcinogens, hormonal balance and oxidative stress structural alerts were more often detected among the total group, with more than 46% and 24% hits respectively. Thiocarbonyl and alkyl halides were the highest detected alerts amongst the hormonal imbalance group, while metals and benzodioxol were detected more amongst the oxidative stress group. Nearly 71% of all non-genotoxic carcinogens were detected by hormonal imbalance or oxidative stress. Aryl hydrocarbon agonist and antagonist and peroxisome proliferator structural alerts were less detectable compared to hormonal imbalance and oxidative stress, as shown in Figure 2.11. The number of structural alerts for aryl hydrocarbon and peroxisome proliferator is still limited and there is a need to do more research to produce new structural alerts based on experimental results for additional chemical substances.

Only 41 non-genotoxic carcinogen substances, out of 150, were identified correctly by these 23 non-genotoxic carcinogen structural alerts. This means that 109 substances experimentally determined as non-genotoxic carcinogen were not identified by these structural alerts. These

109 non-genotoxic carcinogen substances are listed in Table 2.7 in order to allow investigation of why these non-genotoxic carcinogens are not identified and to develop the structural alerts further.

As shown in Table 2.6 (alert number 10, oxidative stress), there are only three non-genotoxic carcinogens containing a structural alert for a metal. These three substances are dicopper:tetrasodium 3,3'-[(3,3'-dihydroxybiphenyl-4,4'-diyl)di(E)diazene-2,1-diyl]bis(5-amino-4-hydroxynaphthalene-2,7-disulfonate), mercury(2+) dichloride and dimethylarsenic acid. The three substances contained copper, mercury and arsenic respectively and thus they were flagged by the structural alert for metals to be non-genotoxic carcinogens. However, after reviewing the list of 109 experimentally non-genotoxic carcinogenic substances that were not identified by the non-genotoxic carcinogen alerts in Table 2.7, there are another three substances containing heavy metals and not identified by the alert for metals. These three substances were cadmium dichloride, lead(2+) diacetate and zinc ethane-1,2-diylbis(dithiocarbamate). This indicates that the structural alert for metals was only predicting copper, mercury and arsenic as non-genotoxic carcinogens but it was failed to predict substances that contain other metals such as lead, cadmium and zinc although that they have known carcinogenic activity with the same oxidative stress mechanism.

The low true positive rate for non-genotoxic carcinogen shows the need to include more structural alerts to give more coverage for this type of carcinogen. Further detailed assessment of the performance of both genotoxic and non-genotoxic carcinogen structural alerts is performed in the next chapters.

Table 2.6. Percentage of non-genotoxic structural alerts flagged among the non-genotoxic carcinogen experimental group of chemicals based their mechanism of action.

No	Mechanism of action	Structural Alert Name	Number of nongenotoxic carcinogens containing this alert	Total number of non-genotoxic carcinogen structural alerts by this mechanism	Percentage of non genotoxic carcinogen structural alerts of this mechanism Out of total
1	Hormonal Imbalance	Thiocarbonyl	6	19	46.3%
2		Poly halocycloalkane	3		
3		Benzimidazole	1		
4		Imidazole,benzamidazole	1		
5		Dicarboximide	1		
6		Dimethylpyridine	0		
7		Benzsulfonic ether	3		
8		Alkyl halide	4		
9		Steroid oestrogen M/N	0		
10	Oxidative Stress	Metals	3	10	24.4%
11		1,3-Benzdioxole	4		
12		Pentachlorophenole	1		
13		2- Phenylphenol	1		
14		Quercetin flavonoid	0		
15	Aryl Hydrocarbon Agonist-Antagonist	Halogenated benzene	3	8	19.5%
16		Halogenated dibenzodioxine	1		
17		Indole-3-carbonyl	0		
18	Peroxisome Proliferator	Phenoxy herbicide	0	4	9.8%
19		Substituted phenoxyacid	1		
20		Substituted N-alkylcarboxylic acid	2		
21		Phthalate	2		
22		Perflourooctanoic acid (PFOA)	0		
23		Tri and tetraflouro ethylene	3		

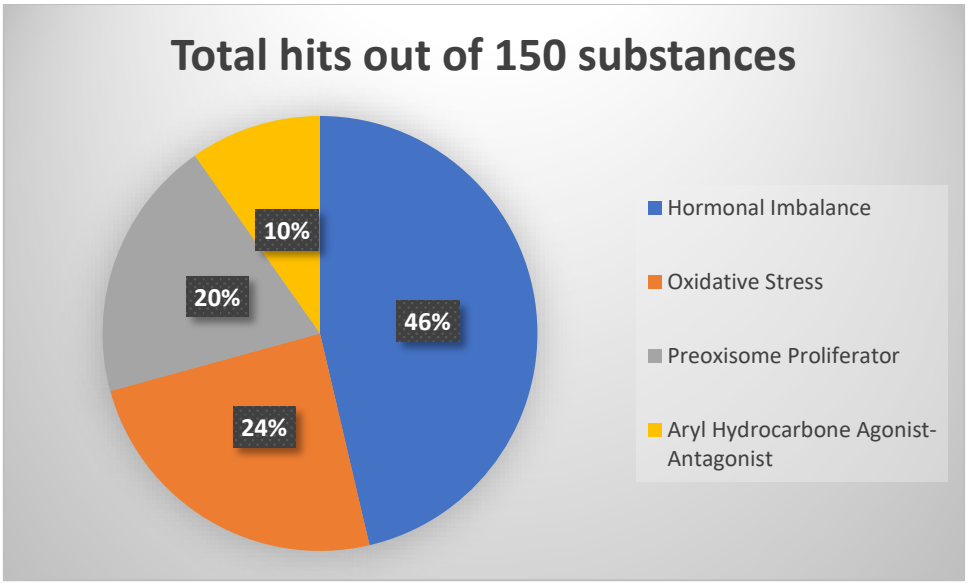


Figure 2.11. Percentage of structural alerts flagged on non-genotoxic carcinogenic chemicals based on their mechanism of action.

Table 2.7. List of 109 out of 150 experimentally non-genotoxic carcinogens which were not identified by the ISS genotoxic rule base structural alerts for non-genotoxic carcinogenicity.

Number	Chemical Name	SMILES String
1	acetaldehyde	CC=O
2	acetamide	CC(=O)N
3	N-(4-hydroxyphenyl)acetamide	C1(=CC=C(C=C1)O)NC(C)=O
4	acrylamide	NC(=O)C=C
5	2-amino-4,6-dimethyl-3-oxo-N,N'-bis[(6S,9R,10S,13R,18aS)-2,5,9-trimethyl-6,13-bis(1-methylethyl)-1,4,7,11,14-pentaoxohexadecahydro-1H-pyrrolo[2,1-i][1,4,7,10,13]oxatetraazacyclohexadecin-10-yl]-3H-phenoxazine-1,9-dicarboxamide	C12C(OC3=C(N=1)C(=CC=C3C)C(N[C@@H]4C(N[C@@H](C(N5[C@@H](CCC5)C(N(CC(N([C@H](C(O[C@H]4C)=O)C(C)C)C)=O)C)=O)=O)=O)C(C)C)=O)=O)=C(C(C(=C2C(N[C@@H]6C(N[C@@H](C(N7[C@@H](CCC7)C(N(CC(N([C@H](C(O[C@H]6C)=O)C(C)C)C)=O)C)=O)=O)C(C)C)=O)=O)N)=O)C
6	allyl 3-methylbutanoate	O=C(CC(C)C)OCC=C
7	1H-1,2,4-triazol-3-amine	C1(N=CNN=1)N
8	11-aminoundecanoic acid	OC(=O)CCCCCCCCCCN
9	aniline hydrochloride	NC1=CC=CC=C1[H]Cl
10	2,2',3,3',4-pentachlorobiphenyl	ClC2=C(C=CC(Cl)=C2Cl)C1=C(Cl)C(Cl)=CC=C1
11	6-chloro-N-ethyl-N'-isopropyl-1,3,5-triazine-2,4-diamine	ClC1=NC(=NC(=N1)NC(C)C)NCC
12	benzene	C1=CC=CC=C1
13	1-benzofuran	C1=COC2=C1C=CC=C2
14	2,2'-{[2-(5-nitro-2-thienyl)quinazolin-4-yl]imino}diethanol	C1=CC=C2C(=C1)N=C(N=C2N(CCO)CCO)C3=CC=C(S3)[N+]([O-])=O
15	2-methylpropan-2-ol	CC(C)(C)O
16	benzyl butyl phthalate	C1(=C(C=CC=C1)C(OCCCC)=O)C(OCC2=CC=CC=C2)=O

17	2-(1,1-dimethylethyl)-4-(methyloxy)phenol	OC1=CC=C(C=C1C(C)(C)C)OC
18	cadmium dichloride	[Cl-].[Cd+2].[Cl-]
19	(2E)-3-(3,4-dihydroxyphenyl)acrylic acid	OC1=C(C=CC(=C1)/C=C/C(=O)O)O
20	[(aminocarbonyl)(nitroso)amino]acetic acid	N(C(=O)N)(N=O)CC(=O)O
21	pyrocatechol	OC1=C(C=CC=C1)O
22	1,2,3,4,6,7,10-heptachlorododecane	ClC(CC(Cl)C(Cl)CCC(Cl)CC)C(Cl)C(Cl)CCl
23	2-chloro-1,1,1-trifluoroethane	C(CCl)(F)(F)F
24	chloro(methoxy)methane	ClCOC
25	3-(4-chlorophenyl)-1,1-dimethylurea	O=C(N(C)C)NC1=CC=C(C=C1)Cl
26	2-chlorobuta-1,3-diene	C=C(Cl)C=C
27	2,4,5,6-tetrachloroisophthalonitrile	ClC1=C(C(=C(C(=C1C#N)Cl)Cl)Cl)C#N
28	(2E)-3-phenylprop-2-en-1-yl 2-aminobenzoate	NC1=C(C=CC=C1)C(=O)OC/C=C/C2=CC=CC=C2
29	(3S,4R)-8-hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3H-isochromene-7-carboxylic acid	CC1=C2C(=CO[C@H]([C@@H]2C)C)C(=C(C1=O)C(=O)O)O
30	4-(2,2-dimethylhydrazino)-4-oxobutanoic acid	O=C(CCC(=O)O)NN(C)C
31	4,4'-sulfonyldianiline	O=S(=O)(C1=CC=C(C=C1)N)C2=CC=C(C=C2)N
32	2,2'-oxydiethanol	OCCOCCO
33	4,4'-(3E)-hex-3-ene-3,4-diyldiphenol	OC2=CC=C(C=C2)/C(CC)=C(CC)/C1=CC=C(O)C=C1
34	chroman-2-one	O=C1OC2=C(C=CC=C2)CC1
35	dimethyl methylphosphonate	CP(=O)(OC)OC
36	dimethyl morpholin-4-ylphosphonate	P(=O)(OC)(OC)N1CCOCC1
37	N,N-dimethylaniline	CN(C1=CC=CC=C1)C
38	1,4-dioxane	C1COCCO1
39	N,N-dimethyl-2-(1-phenyl-1-pyridin-2-ylethoxy)ethanamine succinate	C(CC(=O)O)C(=O)O.C(OCCN(C)C)(C)(C1=CC=CC=C1)C2=CC=CC=N2
40	6',7',10,11-tetramethoxyemetan dihydrochloride	[C@@]12(C3=C(C=C(OC)C(=C3)OC)CCN1C[C@H](CC)[C@H](C2)C[C@@]4(C5=C(C=C(OC)C(=C5)OC)CCN4)[H])[H].[H]Cl.[H]Cl
41	4-[(1R)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol hydrochloride	C1(=C(C=CC(=C1)[C@H](CNC)O)O)O.[H]Cl
42	(17beta)-17-ethynylestra-1(10),2,4-triene-3,17-diol	[H][C@]14[C@@]([C@]3([H])CC[C@@](O)(C#C)[C@](C)3CC4)([H])CCC2=CC(O)=CC=C12
43	S-ethyl-L-homocysteine	N[C@@H](CCSCC)C(=O)O
44	S-ethylhomocysteine	NC(CCSCC)C(=O)O
45	1-(4-ethoxyphenyl)urea	NC(NC1=CC=C(C=C1)OCC)=O
46	ethyl acrylate	O=C(OCC)C=C
47	ethanol	CCO
48	ethylbenzene	CCC1=CC=CC=C1
49	furan	C1=COC=C1
50	2-furylmethanol	C1=C(CO)OC=C1
51	glycine	NCC(O)=O
52	(6aR,11bS)-7,11b-dihydroindeno[2,1-c]chromene-3,4,6a,9,10(6H)-pentol	OC1=C(O)C=C4C(C[C@](COC2=C3C=CC(O)=C2O)([C@@]34[H])O)=C1
53	hexachlorobenzene	ClC1=C(C(=C(C(=C1Cl)Cl)Cl)Cl)Cl
54	N,N,N',N',N'',N''-hexamethylphosphoric triamide	CN(C)P(=O)(N(C)C)N(C)C
55	hydroquinone	OC1=CC=C(C=C1)O
56	2-methylprop-1-ene	CC(C)=C

57	3,5,5-trimethylcyclohex-2-en-1-one	CC1(CC(=CC(=O)C1)C)C
58	isoprene	CC(=C)C=C
59	lead(2+) diacetate	C([O-])(C)=O.[Pb+2].[O-]C(C)=O
60	(4R)-1-methyl-4-(1-methylethenyl)cyclohexene	CC(=C)[C@@H]1CCC(=CC1)C
61	sodium (1E)-3-oxoprop-1-en-1-olate	C(=C/C=O)\[O-].[Na+]
62	1,3,5-triazine-2,4,6-triamine	NC1=NC(=NC(=N1)N)N
63	1,3-benzothiazole-2-thiol	SC1=NC2=C(C=CC=C2)S1
64	N,N-dimethyl-N'-pyridin-2-yl-N'-(2-thienylmethyl)ethane-1,2-diamine hydrochloride	CN(C)CCN(CC2=CC=CS2)C1=NC=CC=C1.Cl
65	4-methoxyphenol	COC1=CC=C(C=C1)O
66	1,1-dimethylethyl methyl ether	CC(OC)(C)C
67	methyl carbamate	NC(=O)OC
68	1-phenylethanol	C1=CC=C(C(O)C)C=C1
69	4-methylbenzene-1,2-diol	OC1=C(C=CC(=C1)C)O
70	4-allyl-1,2-dimethoxybenzene	O(C)c1cc(CC=C)ccc1OC
71	(3R,4R,5R,13aR,13bR)-4,5-dihydroxy-3,4,5-trimethyl-4,5,8,10,12,13,13a,13b-octahydro-2H-[1,6]dioxacycloundecino[2,3,4-gh]pyrrolizine-2,6(3H)-dione	O=C1O[C@@H]3CCN2C\C=C(\COC(=O)[C@](C)(O)[C@](C)(O)[C@H]1C)[C@@H]23
72	1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid	O=C1C2=C(N=C(C=C2)C)N(C=C1C(=O)O)CC
73	naphthalene	C1=C2C(=CC=C1)C=CC=C2
74	2,2',2''-nitrilotriacetic acid	OC(=O)CN(CC(=O)O)CC(=O)O
75	trisodium 2,2',2''-nitrilotriacetate hydrate	N(CC(=O)[O-])(CC(=O)[O-])CC(=O)[O-].[Na+].[Na+].[Na+].O
76	nitrobenzene	O=[N+](C1=CC=CC=C1)[O-]
77	nitromethane	[O-][N+](C)=O
78	2,6-dimethyl-4-nitroso-1-(phenylcarbonyl)piperazine	N1(CC(N(C(C1)C)C(C2C=CC=CC=2)=O)C)N=O
79	N-nitroso-N-phenylaniline	O=NN(C1=CC=CC=C1)C2=CC=CC=C2
80	4-[methyl(nitroso)amino]butanoic acid	O=C(CCCN(C)N=O)O
81	1-methyl-2-nitrobenzene	[N+](=O)([O-])c1ccccc1C
82	1-methyl-4-nitrobenzene	O=N(=O)c1ccc(C)cc1
83	N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl}-L-phenylalanine	O=C(O[C@H](C)C2)C1=C2C(Cl)=CC(C(N[C@@H](CC3=CC=CC=C3)[C@@](O)=O)=O)=C1O
84	17-Hydroxy-2-(hydroxymethylene)-17-methyl-5-alpha-17-beta-androst-3-one	O=C3C[C@@H]4CC[C@@H]1[C@H](CC[C@]2(C)[C@@](C)(O)CC[C@@H]12)[C@@]4(C)C\C3=C\O
85	1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one	CN1N(C2=CC=CC=C2)C(=O)C=C1C
86	3-[(E)-phenyldiazenyl]pyridine-2,6-diamine hydrochloride	NC1=CC=C(/N=N/C2=CC=CC=C2)C(N)=N1.Cl
87	(3β)-cholest-5-en-3-yl {4-[bis(2-chloroethyl)amino]phenyl}acetate	O=C(O[C@@H]5CC([C@@](CC5)(C)[C@]([H])3CC4)=CC[C@@]3([H])[C@@]2([H])[C@@]4(C)[C@]([C@H](C)CCCC(C)C)([H])CC2)CC1=CC=C(N(CCCl)CCCl)C=C1
88	sodium 5-ethyl-4,6-dioxo-5-phenyl-1,4,5,6-tetrahydropyrimidin-2-olate	C1(C2=CC=CC=C2)(C(NC(=NC1=O)[O-])=O)CC.[Na+]
89	3,3-bis(4-hydroxyphenyl)-2-benzofuran-1(3H)-one	O=C1OC(C2=C1C=CC=C2)(C3=CC=C(C=C3)O)C4=CC=C(C=C4)O
90	4-butyl-1,2-diphenylpyrazolidine-3,5-dione	O=C1N(C2=CC=CC=C2)N(C3=CC=CC=C3)C(=O)C1CCCC
91	(11β)-11,17,21-trihydroxypregna-1,4-diene-3,20-dione	[C@]13([C@@](C(=O)CO)(CC[C@H]1[C@@H]2CCC=4[C@@]([C@H]2[C@H](C3)O)(\C=C/C(C=4)=O)C)O)C
92	N-(1-methylethyl)-4-[(2-methylhydrazino)methyl]benzamide	CNNCC1=CC=C(C=C1)C(=O)NC(C)C

93	N-(1-methylethyl)-4-[(2-methylhydrazino)methyl]benzamide hydrochloride	CNNCC1(=CC=C(C=C1)C(=O)NC(C)C).[H]Cl
94	pyridine	N1=CC=CC=C1
95	disodium 3-hydroxy-4-[(E)-(2,4,5-trimethylphenyl)diazenyl]naphthalene-2,7-disulfonate	CC1=CC(C)=C(/N=N/C2=C(C(S([O-])(=O)=O)=CC3=C2C=CC(S([O-])(=O)=O)=C3)O)C=C1C.[Na+].[Na+]
96	trisodium 3-hydroxy-4-[(Z)-(4-sulfonatonaphthalen-1-yl)diazenyl]naphthalene-2,7-disulfonate	C12(C(=CC(=C(C=1/N=N/C3=C4C(=C(C=C3)S(=O)(=O)[O-])C=CC=C4)O)S(=O)(=O)[O-])C=C(C=C2)S(=O)(=O)[O-]).[Na+].[Na+].[Na+]
97	methyl (3β,16β,17α,18β,20α)-11,17-bis(methyloxy)-18-({[3,4,5-tris(methyloxy)phenyl]carbonyl}oxy)yohimban-16-carboxylate	O=C(C4=CC(OC)=C(OC)C(OC)=C4)O[C@@H]1C[C@@]3([H])[C@@](C[C@](N5C3)([H])C2=C(CC5)C(C=C6)=C(C=C6OC)N2)([H])[C@H]([C@](OC)=O)[C@H]1OC
98	tetrahydrofuran	C1CCCO1
99	toluene	CC1=CC=CC=C1
100	2-methylbenzenesulfonamide	CC1=C(C=CC=C1)S(=O)(=O)N
101	tributyl phosphate	CCCCOP(=O)(OCCCC)OCCCC
102	2,4,6-trichlorophenol	OC1=C(C=C(C=C1Cl)Cl)Cl
103	tris(2-chloroethyl) phosphate	O=P(OCCCl)(OCCCl)OCCCl
104	pyrimidine-2,4(1H,3H)-dione	O=C1NC(=O)NC=C1
105	vinyl acetate	CC(=O)OC=C
106	4-vinylcyclohexene	C=CC1CCC=CC1
107	1-vinylpyrrolidin-2-one	O=C1N(C=C)CCC1
108	m-xylene	CC1=CC=CC(C)=C1
109	zinc ethane-1,2-diylbis(dithiocarbamate)	S=C([S-])NCCNC([S-])=S.[Zn+2]

2.4: Conclusions:

The aim of Chapter 2 was to assess the currently available structural alerts for both genotoxic and non-genotoxic carcinogenicity, focussing on the main mechanisms of action of both genotoxic and non-genotoxic carcinogenicity. An existing database of information (i.e. the CPDB) was downloaded and the data curated and cleaned. From this, 240 genotoxic carcinogens and 150 non-genotoxic carcinogens were identified. A well-used rule base for ISS carcinogenicity in Toxtree version 2.6.13 was utilised to investigate the usefulness of the alerts. Out of the 240 genotoxic carcinogens, the true positive rate of the genotoxic carcinogens was 92% with 223 predicted correctly. The majority of genotoxic carcinogens were associated with aromatic amines and nitro aromatics, with 51 and 40 hits respectively. These substances

become carcinogenic through the aminoaryl DNA adduct formation as an indirect acting agent. This high positive predictivity is due to the clear mechanistic information of the molecular initiating event (MIE) which can be used as structural alert and gives a more accurate result. For non-genotoxic carcinogens, the alerts only identified 41 of 150 non genotoxic substances correctly, giving a true positive rate of 27%. The most influential alerts were for hormonal imbalance and oxidative stress mechanism, which accounted for more than 71% of the positive hits, as compared to peroxisome proliferators and aryl hydrocarbon agonists and antagonists with lower hit rates. It was also shown that the structural alert for metals was poorly defined and not able to identify other metals that caused carcinogenicity through oxidative stress e.g. lead, zinc and cadmium. Therefore, better definition of existing alerts, and greater coverage with new alerts is needed. More detailed assessment of individual structural alerts for both genotoxic and non-genotoxic carcinogens will be undertaken in the next chapters.

Chapter 3: Assessment of current profilers and structural alerts for mutagenicity provided in the OECD QSAR Toolbox.

3.1 Introduction

Assessment of the mutagenic potential of the ingredients used in cosmetic products and preparations is one of the priorities of the safety assessment process. Safety assessment is based, in part, on regulatory requirements. For instance, the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation (10th revision, SCCS/1602/18), indicate that mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes may involve a single gene or gene segment, a block of genes or whole chromosomes. Effects on whole chromosomes may be structural and/or numerical. Genotoxicity, on the other hand, is a broader term and refers to processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity (SCCNFP, 2003).

As stated in the SCCS "Notes of Guidance for Testing of Cosmetic Ingredients for their Safety Evaluation" (9th revision, SCCS/1564/15), the safety evaluation procedure refers to the ingredients in the Annexes III, IV, VI and VII of Directive 76/768/EEC as summarised in Table 3.1. The ingredients listed in Annexes III-VII may pose a risk to human health because their use in cosmetic products may lead to high exposure of the consumer because of potentially extensive and routine use over a long period of the time. These Annex ingredients, therefore, require detailed toxicological information, including studies on the mutagenicity potential.

Table 3.1. Ingredients in cosmetic products that require safety evaluation by the Scientific Committee on Cosmetic Product and Nonfood Product (SCCNFB) under Directive 76/768/EEC

Annex III	list of substances which cosmetic products must not contain except subject to restrictions and conditions laid down
Annex IV	list of colouring agents allowed for use in cosmetic products
Annex VI	list of preservatives which cosmetic products may contain
Annex VII	list of UV filters which cosmetic products may contain

As stated in section 1.7, a mutagenic effect can take place via several different mechanisms (Hsu *et al*., 2016). For instance, a compound's reactivity toward DNA can result in the formation of DNA adducts or base deletions, which distort the structure and function of DNA. Non-reactive compounds may also be converted to DNA-reactive metabolites through enzyme-catalysed metabolic activation (Plošnik, Vračko and Dolenc, 2016). DNA distortion can also be caused by intercalation, a process of reversible, non-covalent fixation of a molecule into the DNA (SCCNFP, 2003). For example, compounds with an aromatic polycyclic backbone can intercalate, that is, insert themselves between, or parallel to, base pairs of the DNA double helix, thus form π stacking interactions (Garrett and Grisham, 1995). The distortion of the structure of DNA through DNA reactivity and/or intercalation can disrupt enzymatic DNA repair and replication, which increases the chances of erroneous base replacements or deletions or insertions of base pairs, in other terms mutations (Garret and Grisham, 1995).

In section 2.1, a brief explanation was given about definition and use of the Ames test. The Ames test has become one of the standard tests for mutagenicity determinations as it is relatively simple, fast and inexpensive. Ames tests use a histidine-free medium with an engineered strain of the *Salmonella typhimurium* bacterium that can only proliferate into colonies after certain mutations restore its ability to synthesise histidine (Mortelmans and

Zeiger, 2000). A chemical is considered Ames positive when its addition to the assay causes a significant increase in the number of bacterial colonies with respect to a control experiment. A metabolic activation mixture termed "S9", generally comprising (rat) liver microsomes, can be added to this test to mimic *in vivo* metabolism (Benigni and Bossa, 2008). The term Ames test does not, however, refer to a single unique assay, as evidenced by the different standardised experimental methods, bacterial strains and metabolic activation mixtures that are available (Mortelmans and Zeiger, 2000).

Several factors can limit the reproducibility of the Ames test, such as the purity of the tested chemicals, the variation in the interpretation of dose-response curves, differences in the methodology employed and the materials used (bacterial strains and mixtures for metabolic activation) as well as interference from other toxic side effects, including cytotoxicity (Kazius *et al*., 2006). It has been determined that average inter-laboratory reproducibility for a series of Ames tests is around 85% (Benigni and Bossa, 2011). The Ames test has also been applied to predict rodent carcinogenicity because of the high predictive power of the positive Ames that ranges from 77% to 90% depending on the various factors discussed (Kazius *et al*., 2006). This predictive performance makes it superior to any other *in vitro* genotoxicity assay, all of which have lower performance in terms of predicting genotoxicity (Kazius *et al*., 2006). One of the main databases that contains a large number of chemical records with mutagenicity test results is the Chemical Carcinogenicity Research Information System (CCRIS) database. This database contains Ames test data for approximately 7,000 compounds and mixtures that have been curated and evaluated in terms of their validity. These high-quality data for the Ames test has been reviewed by experts in mutagenesis. The National Cancer Institute (NCI) has developed this database from various studies cited in primary journals, NCI reports and current awareness tools (TOXNET, 2019).

An important research focus of predictive toxicology has been on the identification of the chemicals that are able to bind covalently to DNA (Benigni and Bossa, 2008). Recent legislation such as REACH and the Cosmetics Regulation in the European Union is intended to ensure that all chemicals either manufactured or imported (some of which may be used in cosmetic products) at significant tonnage must have appropriate information relating to safety to human health and environment (EC, 2003, 2006). It is well established that there is a significant ethical responsibility and a high cost when using animal testing to gather the required toxicological information to perform a risk assessment for regulatory purposes (van der Jagt *et al*., 2004). Alternative means for filling the data gaps in the available toxicological information have therefore been sought, including *in silico* models and tools for developing chemical categories (van Leeuwen *et al*., 2009; Enoch and Cronin, 2010).

Based on the assumption that chemicals that have similar structures are likely to have similar toxicological profiles (Enoch *et al*., 2008, 2009a), the chemical category principle can be used to predict a range of toxicological endpoints when populated with suitable data through the so-called process of "read-across". Utilising a common mechanism of action is one of the most powerful methods to group chemicals on the basis of structural/functional similarities, which is the key step in the process of developing chemical categories (EC, 2007; OECD, 2007). In order to group chemicals, the mechanism of action needs to be defined in relation to chemical structure.

Mutagenicity mechanisms involve the formation of a covalent adduct between an exogenous chemical and biological macromolecule such as DNA, RNA or proteins; the covalent interaction may be defined as the MIE. It is important to note that other factors can also determine whether the chemical is mutagenic or not, in addition to those that are defined by an AOP. These factors include any biological repair mechanisms e.g. within the genetic DNA. Therefore, placing a chemical into a mechanistic category, such as those derived from MIEs

for the ability to bind covalently with DNA, or other biological macromolecule, does not necessarily mean that the chemical will be toxic. The read-across of an adverse outcome is, as such, a skilled procedure which involves the compilation of information and expert judgment to form an overall weight of evidence.

The first compilation of covalently reactive structural alerts based on the analysis of mutagenicity data by Ashby and Tennant (1988) defined a board range of reactive structural features responsible for the formation of DNA adducts. Ashby and Tennant (1988) also defined a hypothetical 'super molecule' which was the first attempt to define the potential MIE for genotoxicity (see Figure 3.1). Additional structural alerts for covalent binding to DNA have been suggested by other workers (Benigni and Bossa, 2008; Kazius *et al*., 2005, 2006), and Enoch and Cronin, (2010) who compiled the alerts into a single, mechanistically based, profiler describing the chemistry associated with binding to DNA.

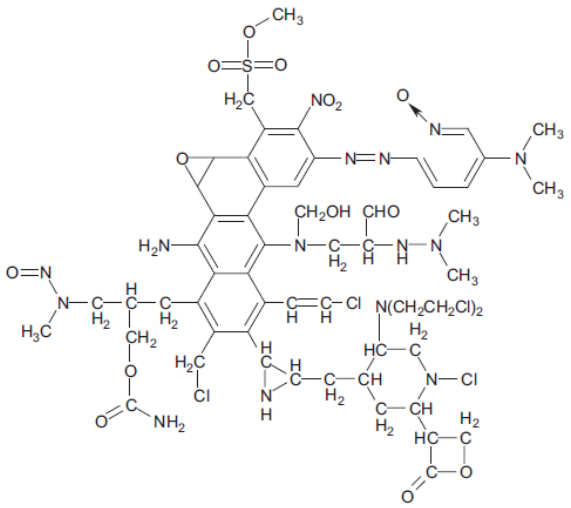


Figure 3.1. Super molecule suggested by Ashby and Tenant (1988) for structurally reactive features that may bind covalently with DNA.

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It is important to know that, aside from structural features, other factors may also contribute to the potential of a compound to be mutagenic and/ or carcinogenic. For example, some cosmetics ingredients may contain one or more structural alerts associated with toxicity; however, the compound may be metabolically inactive. Metabolic inactivity may be caused by the compound's molecular weight, solubility, reactivity, stability and state of matter, or the geometry of the chemical structure, amongst other factors (Plošnik, Vračko and Dolenc, 2016).

Elucidation of the mechanism for electrophilic reactions with biological nucleophiles is founded on basic substitution, conjugation and addition reactions that are characterised by the reaction between electron-deficient and electron-rich moieties. Enoch and Cronin (2010) categorised genotoxic carcinogenic structural alerts according to chemistry into the main known mechanistic domains of organic reaction chemistry. The six main organic chemistry mechanisms relevant to toxicology are Michael addition (MA), acylation (AC), Schiff base formation (SB) and nucleophilic domain reactions (S_N) which include unimolecular aliphatic nucleophilic substitution (S_N1), bimolecular aliphatic nucleophilic substitution (S_N2) and aromatic nucleophilic substitution (S_NAr). The key genotoxic structural alerts linked to chemically mechanistic domains are depicted in Table 3.2. The definition of the chemistry associated with the mechanisms has enabled the grouping of electrophiles depending on their potential to bind covalently with DNA (and hence potential mutagenicity). Some of the aspects of chemistry associated with the mechanisms are illustrated in Table 3.3.

One of the most beneficial computational toxicology applications that has been used by regulators, industry, researchers and many others is OECD QSAR Toolbox (or simply referred to as the 'Toolbox'). This software package was developed by the Organisation for Economic Cooperation and Development (OECD) and has now reached its 10th anniversary (Schultz *et al*., 2018). The Toolbox has number of advantages over the other QSAR prediction

tools including that it is freely available, continuously updated and mainly designed to assess the safety of organic substances (Nicolotti, 2018). The Toolbox was designed to offer a comprehensible and transparent predictions including 'read-across' for the user (Cronin and Madden, 2011).

The Toolbox, and other computational toxicology applications, can identify structural analogues by providing the information on chemicals in standardised, structure-searchable files that are associated with chemical and toxicity data. Toxicity assessment and testing methods are changing and improving through time, which raises the need of computational toxicology software, such as the Toolbox, to integrate new datasets, e.g. the next generation of *in vitro* tests (Nicolotti, 2018).

Fundamentally, grouping substances into chemical categories and using the data from tested chemicals to fill the gaps of untested chemicals was considered as a long-term goal of the Toolbox, thus ensuring its usefulness in regulatory assessment. In order to be useful in a regulatory setting, the Toolbox user must be confident that the predictions coming from this tool are reliable, consistent and correct. This can be achieved by ensuring accuracy in chemical and biological information and, when appropriate, adding statistical assurance. Unlike other QSAR-based software which failed to achieve regular regulatory use, the Toolbox used a unique approach that provided mechanical understanding and high transparency through the category approach and read-across. For other QSARs using descriptors and modelling approaches, putting statistics ahead of chemistry and biology often resulted in "black box" predictions which did not give a mechanical understanding to the user.

The Toolbox predictions are based on the category approach (OECD, 2007). In this approach, one or more chemicals are grouped based on their similarity which is not only defined in terms of their chemical structure and physiochemical properties but also includes similarity in mechanism of interaction with different biomolecular targets (e.g., DNA, protein), as well as

similarity in toxicokinetic and toxicodynamic properties. The available experimental data for one or more members of the category, the source substances, are used to fill the data gap(s) of other unknown chemical substances of the category, the target substances.

The six modules in the Toolbox are based on the category approach that guide the user through a logical workflow (OECD, 2009). These six modules (i.e., Chemical Input, Profiling, Endpoints, Category Definition, Filling Data Gap, and Report) are employed in a sequential workflow as suggested by the Toolbox guidance (Dimitrov *et al*., 2016).

A number of *in silico* profilers are available in the Toolbox for various toxicological endpoints. Of these profilers, the following are relevant to the investigation of mutagenicity in this chapter:

1. DNA binding by OASIS v1.4. This profiler is a mechanistic profiler developed from an analysis of Ames mutagenicity data. It contains 85 structural alerts that have been separated into eight mechanistic domains. Each of the mechanistic domains comprises mechanistic alerts that have been shown to be related to established electrophilic reaction chemistry known to be important in covalent DNA binding. (Mekenyan *et al.* 2004; Serafimova *et al*. 2007).

2. DNA binding by OECD. This profiler is based on structural alerts for the electrophilic reaction chemistry associated with covalent DNA binding (Enoch and Cronin 2010). The profiler is made up of 60 structural alerts that contain electrophilic centres or those that can be metabolically activated to electrophiles.

3. Carcinogenicity (genotoxic and non-genotoxic) alerts by ISS. This profiler is based on a list of 55 structural alerts from the Toxtree software (http://toxtree.sourceforge.net/). Approximately 20 of the alerts are for non-genotoxic carcinogenicity, and the remainder for genotoxic carcinogenicity (mutagenicity).

4. DNA alerts for AMES, MN and CA by OASIS v.1.1. This is a refinement of the DNA binding by OASIS profiler described above. The profiler is based on the 85 structural alerts responsible for interaction of chemicals with DNA extracted from chromosomal aberrations data. There is a slight difference between DNA alerts in the *in vitro* Ames and CA models justified by the different local training set chemicals in both models. The scope of this profiler is to investigate the presence of alerts within the target molecules responsible for interaction with DNA related to chromosomal aberration and micronucleus tests.
5. *In vitro* mutagenicity (Ames test) alerts by ISS. The present list of structural alerts is a subset of the original Toxtree list, obtained by eliminating the structural alerts for non-genotoxic carcinogenicity and is a refinement of the Carcinogenicity (genotoxic and non-genotoxic) alerts by ISS profiler.
6. *In vivo* mutagenicity (Micronucleus) alerts by ISS. This profiler is based on the ToxMic rule-base within the Toxtree software. This rule-base provides a list of 35 structural alerts for a preliminary screening of potential *in vivo* mutagens. These structural alerts are molecular functional groups or substructures that are known to be linked to the induction of effects in the *in vivo* micronucleus assay.

A number of statistical analyses are appropriate to evaluate the predictive performance of the *in silico* profilers against the experimental data. Key amongst these are the Cooper statistics and Mathews Correlation Coefficient. The Cooper statistics (Cooper *et al.,* 1979) are useful to assess the predictions against the experimental values given in the databases, by calculating the sensitivity, specificity, positive predictivity and accuracy of the alert triggers. Sensitivity is defined as the percentage of correctly classified positive predictions among the total number

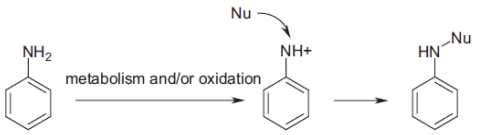
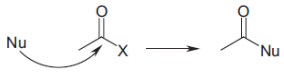
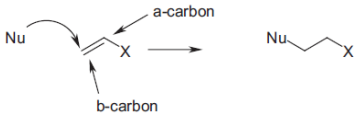
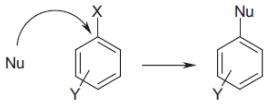
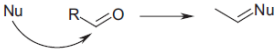
of positive instances. Specificity is the percentage of correct negative predictions compared to the total number of negatives. Accuracy (concordance or "Q") is defined as the total number both positive and negatives correctly predicted among the total number of compounds. The positive predictive value (PPV) or precision is defined as the as the proportion of positives or toxic chemicals that are correctly predicted (see Table 3.4; Pradeep *et al.,* 2016) and can be considered as an estimate of the likelihood that following a positive prediction (i.e., the presence of a structural alert), that the substance will truly be positive (Eriksson *et al*., 2003). The Matthews Correlation Coefficient (MCC) is a weighted value that overcomes any imbalance in the data classes which might lead to over optimistic values of Q (Matthews, 1975). An MCC value of 1 indicates that the model can predict the data classes of unknown compounds perfectly, whilst a MCC value of 0 indicates that the predictions are no better than random guessing, and a MCC value of -1 indicates total disagreement between the predicted data and the actual data.

Since there have been few, or no, attempts to evaluate the statistical performance of the *in silico* profilers, the aim of this chapter was to provide a detailed analysis for positive prediction of each structural alert in six mutagenicity profilers within the OECD QSAR Toolbox against experimental mutagenicity data from the CCRIS dataset. Analysis of the results from this investigation aimed to increase the reliability and accuracy of mutagenicity predictions by these profilers.

Table 3.2. A selection of structural alerts which belong to reactive electrophilic mechanistic domains relating to mutagenicity (Enoch and Cronin, 2010).

Mechanism domain	Structural alert
S_N2	• Alkyl esters of either phosphonic or sulphonic acids • Monohaloalkenes • S- or N- mustards • Propiolactones and propio sulphones • Epoxides and arizidines • Aliphatic halogens • Alkyl nitriles
S_N1	• Aromatic nitro groups • Alkyl hydrazines • Alkyl and aryl N-nitroso groups • Aliphatic N-nitro groups • Aromatic nitroso groups • Aromatic amines and hydroxyl amine • Halogenated polycyclic aromatic hydrocarbons • Halogenated dibenzodioxins
Acylation	• Aromatic diazo groups • Acyl halides
Schiff base formation	• Simple aldehydes • N-methylol derivative
Michael addition	• Quinones • Aromatic N-oxides
S_NAr	• Aromatic mono and dialkylamino groups • Halogenated benzenes

Table 3.3. Mechanisms of covalent binding to cellular nucleophiles (DNA, proteins) *Nu-nucleophilic site of molecule and how this may be translated into usable structural fragments (Enoch and Cronin, 2010)

Type of reaction	illustration
S_N2 reaction	 S_N2: Characteristics: X = halogen or other electronegative leaving group.
S_N1 reaction	 S_N1: Characteristics: ability to form a stabilized positive charge, typically on a carbon or nitrogen
Acylation	 Acylating agents: Characteristics: X = halogen or electronegative leaving group.
Michaels addition	 Michael addition: Characteristics: double or triple bond where X = electron withdrawing substituent (α and β alkene carbon atom as highlighted).
S_NAR	 S_NAr electrophiles: Characteristics: X = halogen or pseudo-halogen. Y = (at least two) NO_2, CN, CHO, CF_3, Halogen.
Schiff base formation	 Schiff base formers: Characteristics: reactive carbonyl species such as aliphatic aldehyde or di-ketones.

3.2 Methods

3.2.1 Dataset used

The main resource to obtain data for this study was the Chemical Carcinogenicity Research Information System (CCRIS) database which was available uploaded in the Leadscope Personal software Version 4.4 (Leadscope.com, 2018). The version of CCRIS used in this study was updated in 2011 and it has not been updated since that time. CCRIS provides historical information from 1985 – 2011 and contains Ames test data for approximately 7,000 compounds and mixtures, the results of which have been curated and evaluated in terms of their validity. The compounds were identified with a CAS registry number and/or chemical name(s). Additional mutagenicity data, although fewer in number, were available from other public toxicity databases, including CPDB, GENETOX, National Toxicology Program Dataset NTP and the genetic activity profile dataset (EPA/ IARC). Compounds whose CCRIS data showed contradictory categorisations with the NTP data were removed from the dataset. In total, a dataset of 8,130 compounds with corresponding molecular structures and toxicity categorisations (3,838 mutagens and 2,861 non-mutagens) was constructed. The chemical structures of the dataset were obtained as in the SDF file format using the Leadscope software so that they may be used in the OECD QSAR Toolbox.

3.2.2 The OECD QSAR Toolbox

For this study, version 4.1 (downloaded in April 2018) of the OECD QSAR Toolbox was used throughout for the profiling process. The Toolbox is freely available and downloaded from qsartoolbox.org.

3.2.3 Data analysis:

The following workflow was implemented in order to assess and evaluate the accuracy of the following six mutagenicity profilers as implemented in the OECD QSAR Toolbox (details on the profilers is given in Section 3.1).

1. DNA binding by OASIS v1.4
2. DNA binding by OECD
3. Carcinogenicity (genotoxic and non-genotoxic) alerts by ISS
4. DNA alerts for AMES, MN and CA by OASIS v.1.1
5. *In vitro* mutagenicity (Ames test) alerts by ISS
6. *In vivo* mutagenicity (Micronucleus) alerts by ISS

The workflow below allowed for detailed analysis of the positive predictivity for each structural alert within each profiler.

Step 1 – Within the Leadscope personal version 4.4 software, the latest (2011) high-quality version of the CCRIS database was selected. The database was searched to identify and extract all experimental results for each substance. A screenshot of this process is shown in Figure 3.2.

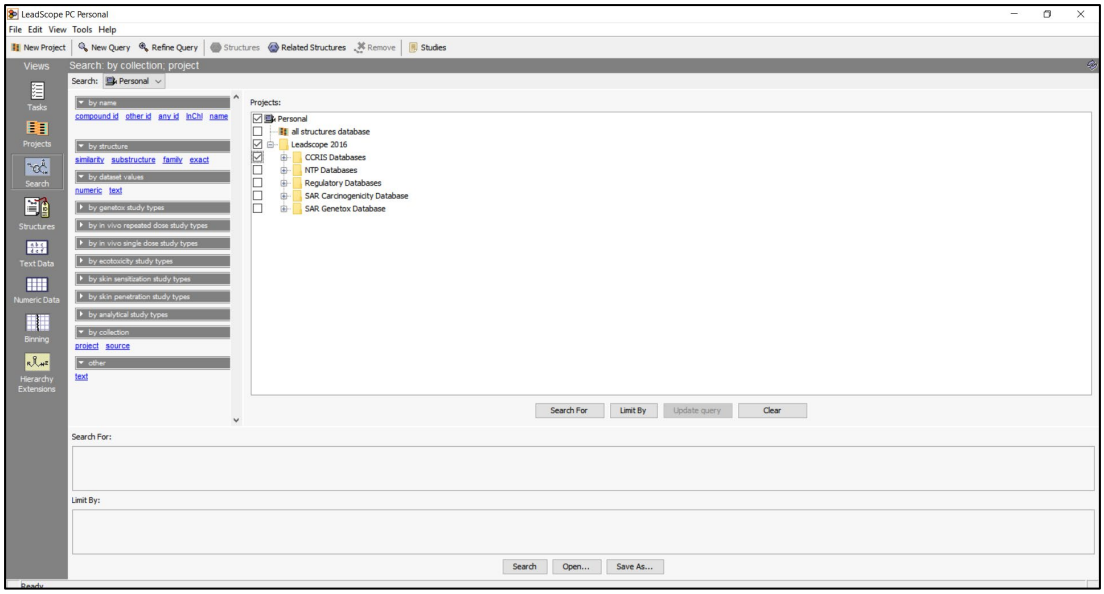


Figure 3.2. Screenshot from Leadscope database with the CCRIS mutagenicity database uploaded.

Step 2 - Bacterial mutagenicity and *Salmonella* experimental results were identified for each chemical substance in the database, the file containing these data was exported as molecular spreadsheet sheet as shown in Figure 3.3.

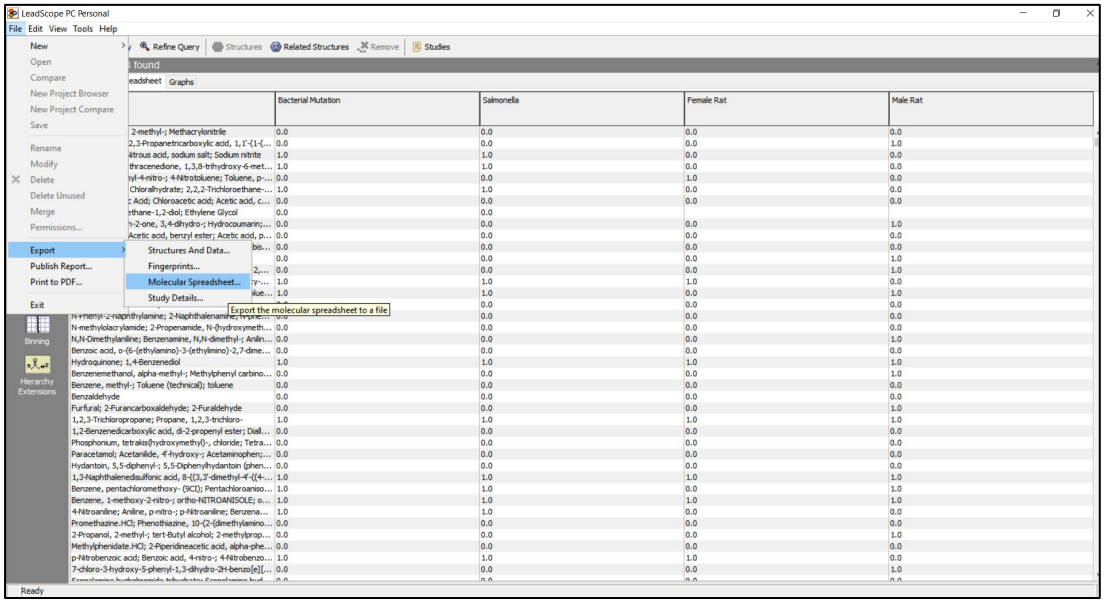


Figure 3.3. Screenshot from the Leadscope software showing the list of substances from the CCRIS database with experimental mutagenicity data.

Step 3 – A SDF version of CCRIS database was profiled through the OECD QSAR Toolbox using the six mutagenic profilers noted above. The results of this profiling were exported as an excel spreadsheet. A screenshot of the profiling in the Toolbox is shown in Figure 3.4.

Step 4 – The data from the Leadscope software and predictions from the OECD QSAR Toolbox were merged into a single spreadsheet so that experimental data and the profiling results for each structural alert triggered could be compared. A screenshot of the spreadsheet is shown in Figure 3.5.

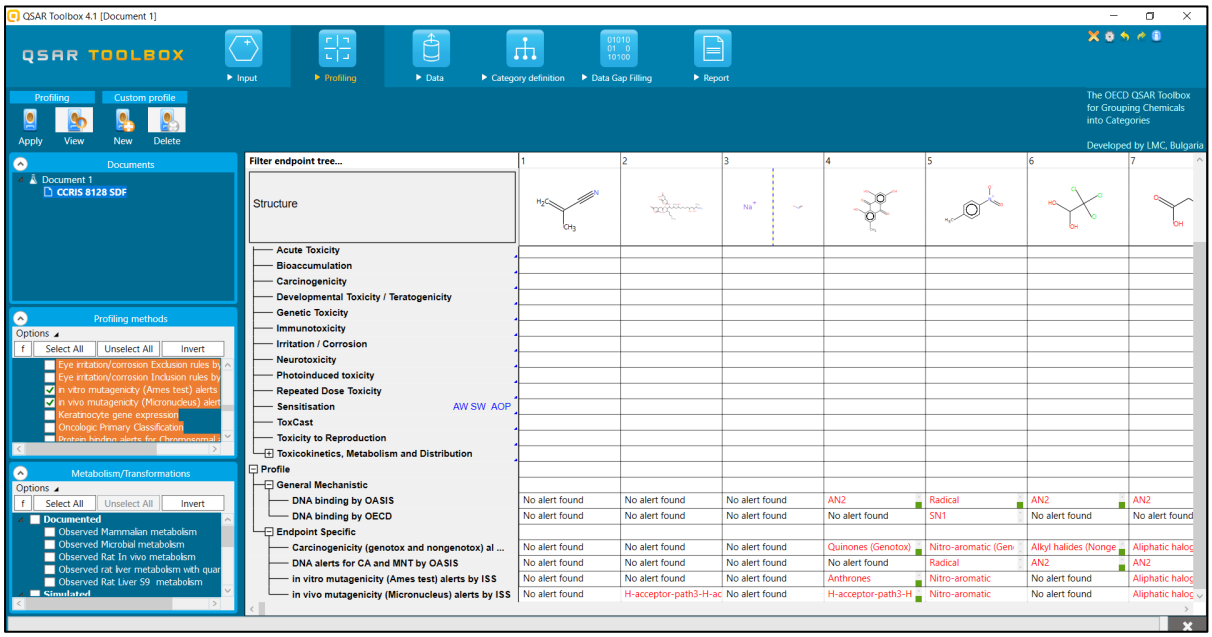


Figure 3.4. Screenshot showing the profiling of a list of 8,210 substances from the CCRIS database.

Step 5 – A separate column for each of the six Toolbox profilers assessed was created in the merged spreadsheet. If the structural alert was triggered, the compound was given a score of 1, if no alerts were triggered, a score of 0 was allocated. The results were compared with the assigned binary activity for mutagenicity from the CCRIS database (positive=1, negative=0).

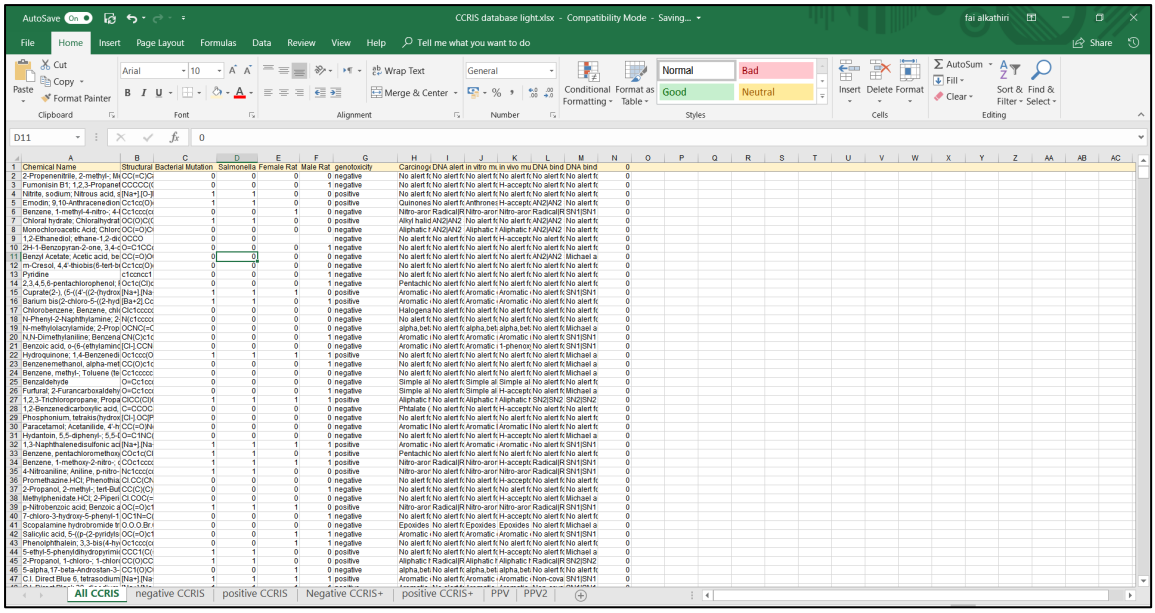


Figure 3.5. Final excel spreadsheet where both profiling and experimental results for each substance from CCRIS database were merged in one single file.

Step 6 - The results from the six profilers within the OECD QSAR Toolbox were assessed statistically against the experimental results value given in CCRIS database. This was performed by calculating sensitivity, specificity, accuracy, precision, negative predictive value, false positive rate, false negative rate, false discovery rate, false omission rate, F1 score, informedness, markedness, and the Mathews correlation coefficient (MCC) of each alert. These statistical parameters are described in Section 3.1 and their definitions provided in Table 3.4.

Table 3.4. 13 statistical assessment parameters of the results from six profilers within the OECD QSAR Toolbox against the experimental results value given in CCRIS database.

Sensitivity (True positive rate)	=	TP/ TP+FN
Specificity (True negative rate)	=	TN/TN+FP
Accuracy	=	(TN+TP)/(TN+FP+FN+TP)
PPV (Positive predictive value) or (precision)	=	TP/ TP+FP
NPV (negative predictive value)	=	TN/TN+FN
FNR (false negative rate) or (miss rate)	=	1- sensitivity
FPR (false positive rate) or (fall out)	=	1- specificity
FDR (false discovery rate)	=	1- PPV
FOP (false omission rate)	=	1- NPV
F1 score	=	2 x (PPV x TPR) / (PPV +TPR)
Informedness (BM)	=	TPR+TNR -1
Markedness (MK)	=	PPV + NPV -1
MCC	=	(TPxTN)-(FPxFN)/√(TP+FN)(TP+FP)(TN+FN)(TN+FP)

Where TP=True positive, TN=True negative, FP=False positive, FN=False negative

Step 7 - A detailed analysis of the positive predictivity value (PPV) for each structural alert within each profiler were conducted. Only substances with one structural alert triggered were assessed, this was to avoid any interference of other structural alerts in cases of substances with multiple structural alerts triggered.

Step 8 - Structural alerts that were triggered in more than 10 substances and showed less than 0.5 positive predictivity were considered to be of limited significance.

3.3: Results and Discussion

The aim of Chapter 3 was to evaluate the performance of six commonly used *in silico* profilers for mutagenicity from the OECD QSAR Toolbox with a view to identifying strongly performing alerts and those in need of more refinement or development.

3.3.1 Data Collection

A dataset of 8,130 compounds with corresponding molecular structures and toxicity categorisations (3,838 mutagens and 2,861 non-mutagens) was constructed. The dataset included data from four mutagenicity tests for each chemical substance, namely bacterial mutation, *Salmonella*, female rat and male rat. No information about metabolism was available for any chemical substance. The major uses for these chemicals varied from drugs (anti-infectives and anti-viral), pesticides (herbicides and plant growth regulators), intermediates (dyes), analytical reagents and solvents. The chemical structures for use in the OECD QSAR Toolbox were obtained as a SDF file from the Leadscope software.

3.3.2 Evaluation of the overall performance of the six in silico profilers for mutagenicity.

The results of the assessment of the profilers against the measured values for mutagenicity are shown in Table 3.**5** and Figure 3.6 respectively.

Table 3.5. Performance statistics for the six mutagenicity profilers provided within the OECD QSAR Toolbox against experimental data.

Profiler	Genotox and nongenotox alerts by ISS	DNA alerts for AMES, MN and CA by OASIS	Ames test alerts by ISS	Micronucleus alerts by ISS	DNA binding by OASIS v.1.4	DNA binding by OECD
CCRIS database						
Sensitivity	0.84	0.52	0.83	0.90	0.71	0.70
Specificity	0.60	0.91	0.69	0.37	0.73	0.62
Accuracy (Q)	0.74	0.69	0.77	0.67	0.72	0.67
Precision (PPV)	0.73	0.88	0.78	0.65	0.77	0.71
Negative predictive value (NPV)	0.74	0.59	0.75	0.74	0.66	0.61
False positive rate (FPR)	0.40	0.09	0.31	0.63	0.27	0.38
False negative rate (FNR)	0.16	0.48	0.17	0.10	0.29	0.30
False omission rate (FOR)	0.26	0.41	0.25	0.26	0.34	0.39
False discovery rate (FDR)	0.27	0.12	0.22	0.35	0.23	0.29
F1 score	0.78	0.65	0.8	0.76	0.74	0.7
Informedness (BM)	0.44	0.43	0.52	0.27	0.44	0.32
Markedness (MK)	0.47	0.47	0.53	0.39	0.43	0.32
MCC	0.46	0.45	0.52	0.33	0.43	0.32

Sensitivity = True positive rate; Specificity = True negative rate; MCC = Matthews Correlation Coefficient;
= MCC below 0.33; = High sensitivity or specificity ; = Low sensitivity or specificity

Table 3.5 shows that the accuracy (Q) (percentage of positives and negatives correctly predicted) of the mutagenicity profilers varies for the profilers from 67% to 77%. Clearly, a profiler with an accuracy of 67% has a significant margin of error that may affect the profiler's ability to predict adequately, whereas 77% is a more acceptable level of prediction that is in line with the experimental error level in the measured data. These accuracy of the profilers reflects the known average interlaboratory reproducibility of Ames tests, which is known to be at least 15%. As such it was concluded that these profilers can be applied to risk assessment processes and can guide the design of chemical libraries for hit and lead optimisation (Kazius *et al*., 2005).

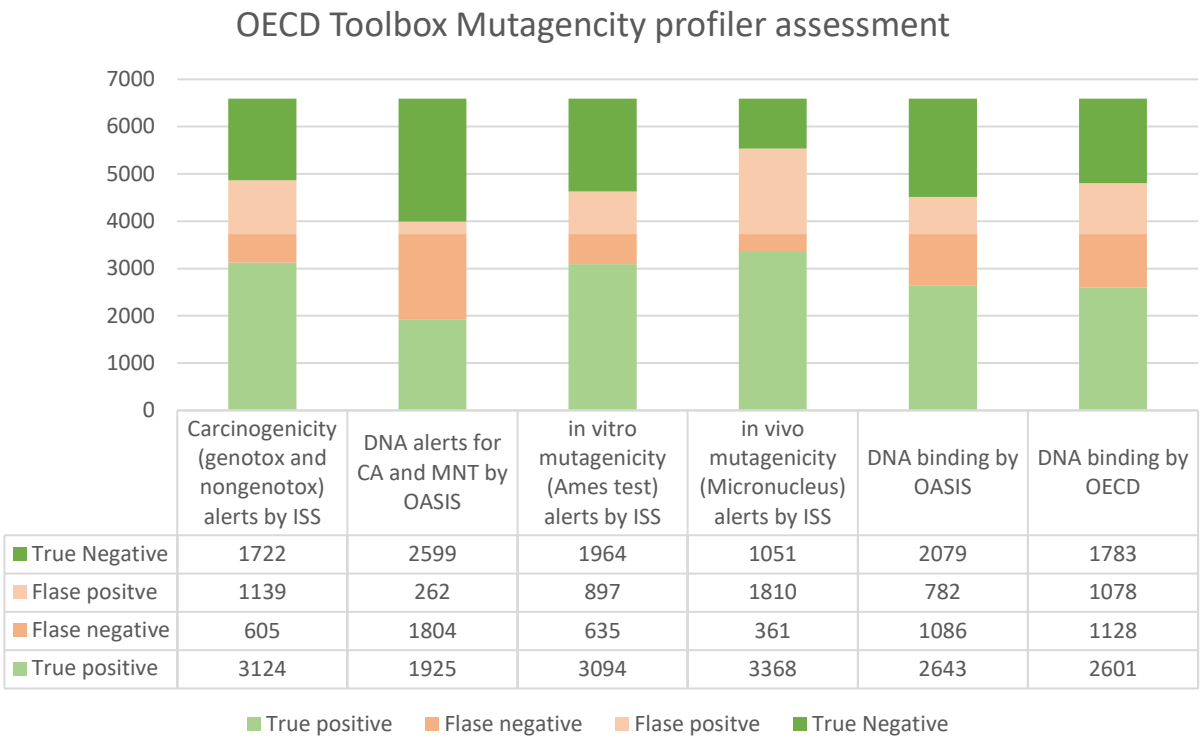
Considering individual profilers in more detail, the true positive rate (sensitivity) was relatively high for Micronucleus alerts with a low specificity (true negative rate) which indicates an over prediction of mutagenicity. In contrast, the true negative rate for DNA-binding alerts for the Ames, MN and CA profiler was high (91%) but with a poor ability to distinguish positive mutagenicity results (52%). The sensitivity rates of the other four profilers ranged from 70% to 84%, which is an acceptable. However, two profilers (genotoxicity and non-genotoxicity alerts (ISS) and OECD DNA binding profilers) failed to adequately predict the non-mutagenic compounds with a true negative rate of only 60%. The positive prediction value (PPV), which measures the ability of the alerts to predict mutagenic compounds, was high (88%) for the "DNA alerts for AMES" profiler, however this profiler showed the lowest Negative predictive value (NPV) of 59%. The PPV and NPV of the other five mutagenicity profilers ranged from 65% -78% and 61%-75% respectively.

The Mathews correlation coefficient (MCC) is more informative than other confusion matrix measures (such as F1 score and accuracy) in evaluating binary classifications. This is because it takes into account the balanced ratios of the four confusion matrix categories (true positives, true negatives, false positives and false negatives). MCC did not exceed 0.5**2** for any of the six profilers for mutagenicity and the lowest MCC value was 0.32 for the "DNA binding profiler by OECD" and 0.33 for "Micronucleus alerts by ISS". The Ames alerts was the only profiler that showed a MCC value greater than 0.5, which indicates that the performance is independent of skewed sample categories. The statistics for individual profilers, and any weak predictive alerts within them, are discussed below.

Whilst good predictivity is desirable, it is should not be expected that any profiler is able to predict all mutagenic compounds. The profilers considered in this analysis are likely to have limited predictive capability for a number of different reasons. Firstly, they are not profilers for mutagenicity in its entirety, but for different aspects (i.e. mechanisms or modes) of it. Secondly,

some or all of the profilers were not designed to be predictive but for grouping allowing for read-across. For a predictive profiler, the desired level of predictivity would be in line with the error level seen in measured data. When this level of predictivity is achieved and demonstrated, these profilers can be considered a more valid substitute for experimental studies. With this in mind, this study seeked to refine those structural alerts that have lower predictivity by analysing which compounds they hit erroneously. Ultimately, such analyses and knowledge will lead to increased reliability and confidence in the predictive ability of these profilers.

155 chemicals, known to be mutagenic experimentally were not identified by any of the six mutagenicity profilers. Of these, 43 chemicals, which represent 30% of these non-identified mutagens, were inorganic chemical substances such as cadmium chloride, manganese dichloride, selenium sulfide, titanium chloride etc. The remaining 112 mutagenic substances were varied organic chemicals that did not have any unifying characteristics for grouping or that could be used to formulate a new rule for a new chemical structural alert. The list of all 155 mutagens that were not identified, including both inorganic and organic chemicals, is available in Appendix I.



	Carcinogenicity (genotox and nongenotox) alerts by ISS	DNA alerts for CA and MNT by OASIS	in vitro mutagenicity (Ames test) alerts by ISS	in vivo mutagenicity (Micronucleus) alerts by ISS	DNA binding by OASIS	DNA binding by OECD
True Negative	1722	2599	1964	1051	2079	1783
Flase positve	1139	262	897	1810	782	1078
Flase negative	605	1804	635	361	1086	1128
True positive	3124	1925	3094	3368	2643	2601

Figure 3.6. Column chart of the sensitivity and specificity rates for each of the six mutagenicity profilers in the OECD QSAR Toolbox when compared against experimental results taken from the CCRIS database.

3.3. Evaluation of individual structural alerts within the six in silico profilers for mutagenicity

Further detailed analysis was conducted on each structural alert within the six mutagenicity profilers. The purpose here was to identify structural alerts that are over-predictive i.e. when the precision or PPV is lower than 0.5. These over-predictive structural alerts were excluded to increase the sensitivity and overall accuracy of the profiler and are listed in Tables 3.6 - 3.11 respectively. The results for the individual profilers are described in more detail below and a final list of 28 structural alerts with low predictivity, obtained from the six mutagenicity profilers, is summarised in Table 3.12.

3.3.1 Genotox and Nongenotox alerts by ISS profiler:

The sensitivity and accuracy of this profiler were fairly acceptable (see Table 3.6) with a 84% true positive rate and 74% accuracy. The analysis of positive predictivity of the alerts within this profiler revealed that eight alerts showed low predictivity for positive mutagens; five were non-genotoxic carcinogenicity (1,3-benzodioxoles, benzenesulfonic ethers, halogenated benzene, substituted n-alkylcarboxylic acids and thiocarbonyl) and three were genotoxic carcinogenicity structural alerts (alkenylbenzenes, α,β-unsaturated carbonyls and simple aldehydes). As shown in Table 3.6, the alert for α,β-unsaturated carbonyls was the highest triggered alert being found in 187 substances with 102 false positives and hence a low PPV of 45%. The alert for substituted n-alkylcarboxylic acids had the lowest PPV value (12%) with 23 false positive result out of 26 substances triggered. Halogenated benzenes and simple aldehydes alerts were flagged in 82 and 74 substances respectively, but they were over predictive amongst negative substances with PPVs of 23% and 38% respectively. This indicates that they may be not suitable to be used to predict mutagenic substances. The remaining four alerts with low PPV ranged in their predictivity from 23% for benzenesulfonic ethers to 42% for 1,3-benzodioxoles. However, Table 3.6 shows some of the alerts in the

profiler showed high positive predictivity values for many positive mutagens including the alert for nitroso-aromatic with 82% of 395 substances and that for Polycyclic Aromatic Hydrocarbons with 91% among 271 mutagenic substances.

Table 3.6. Positive prediction analysis (PPV) for each structural alert within the "Carcinogenicity (genotox and nongenotoxic) alerts by ISS" profiler.

alert		TOTAL	TP	FP	PPV
Non genotoxic carcinogen structural alert	(Poly) Halogenated Cycloalkanes	14	2	12	0.14
	1,3-Benzodioxoles	12	5	7	0.42
	Alkyl halides	17	13	4	0.76
	Benzenesulfonic ethers, methylation	13	3	10	0.23
	Halogenated benzenes	82	19	63	0.23
	Halogenated dibenzodioxins	4	1	3	0.25
	Imidazoles, benzimidazoles	37	17	20	0.46
	Indole-3-carbinols	1	1	0	1.00
	Metals, oxidative stress	51	27	24	0.53
	o-Phenylphenols	13	6	7	0.46
	Pentachloro phenols	2	1	1	0.50
	Quercetin type flavonoids	5	3	2	0.60
	Substituted n-alkylcarboxylic acids	26	3	23	0.12
	Thiocarbonyls	17	6	11	0.35
	Trichloro (or fluoro) ethylenes and Tetrachloro (or fluoro) ethylenes (Nongenotox)	10	9	1	0.90
genotoxic carcinogen structural	Acyl halides	17	12	5	0.71
	Aliphatic azo and azoxys	21	20	1	0.95
	Aliphatic halogens	214	162	52	0.76
	Aliphatic N-nitro groups	13	11	2	0.85
	Alkenylbenzenes	17	4	13	0.24
	Alkyl (C<5) or benzyl ester of sulphonic or phosphonic acid	49	31	18	0.63
	Alkyl and aryl N-nitroso groups	96	83	13	0.86

	Alkyl carbamate and thiocarbamates	17	8	9	0.47
	Alkyl nitrites	7	7	0	1.00
	α,β-Unsaturated aliphatic alkoxy groups	16	14	2	0.88
	α,β-Unsaturated carbonyls	187	85	102	0.45
	Aromatic diazos	74	41	33	0.55
	Aromatic mono-and dialkylamines	52	31	21	0.60
	Aromatic N-acyl amines	42	23	19	0.55
	Aromatic nitroso groups	23	23	0	1.00
	Aromatic ring N-oxides	9	3	6	0.33
	Azide and triazene groups	52	51	1	0.98
	Coumarins and Furocoumarins	22	16	6	0.73
	Epoxides and aziridines	235	186	49	0.79
	Heterocyclic Polycyclic Aromatic Hydrocarbons	98	82	16	0.84
	Hydrazines	50	34	16	0.68
	Isocyanate and isothiocyanate groups	3	3	0	1.00
	Monohaloalkenes	10	6	4	0.60
	Nitro-aromatics (Genotox)	395	322	73	0.82
	Polycyclic Aromatic Hydrocarbons	271	246	25	0.91
	Primary aromatic amine,hydroxyl amine and its derived esters	311	211	100	0.68
	Propiolactones or propiosultones	5	4	1	0.80
	Pyrrolizidine alkaloids	4	4	0	1.00
	Quinones	111	82	29	0.74
	S or N mustards	19	15	4	0.79
	Simple aldehydes	74	28	46	0.38
	TOTAL PPV	2818	1964	854	0.70
	GENOTOXIC CARCINOGEN PPV	2514	1848	666	0.74
	NON GENOTOXIC CARCINOGEN PPV	304	116	188	0.38

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

3.3.2 DNA alerts for AMES, MN and CA by OASIS profiler:

In contrast to other profilers, the DNA alerts for Ames profiler showed low true positive (sensitivity) and high true negative rates (see Table 3.7). This indicates that there is a lack of alerts that can predict mutagens and those that are available are unspecific and have a high number of false negatives (48%).

Table 3.7. Positive predictivity analysis for each structural alert among the "DNA alerts for AMES, MN and CA by OASIS" profiler.

DNA alerts for CA and MNT by OASIS	TOTAL	TP	FP	PPV
AN2|AN2 >> Schiff base formation|AN2 >> Schiff base formation >> Dicarbonyl compounds	15	11	4	0.73
Non-covalent interaction|Non-covalent interaction >> DNA intercalation|Non-covalent interaction >> DNA intercalation >> DNA Intercalators with Carboxamide and Aminoalkylamine Side Chain	3	2	1	0.67
Non-covalent interaction|Non-covalent interaction >> DNA intercalation|Non-covalent interaction >> DNA intercalation >> Quinolone Derivatives	17	10	7	0.59
SN2|SN2 >> Alkylation, direct acting epoxides and related|SN2 >> Alkylation, direct acting epoxides and related >> Epoxides and Aziridines	181	166	15	0.92
SN2|SN2 >> Alkylation|SN2 >> Alkylation >> Alkylphosphates, Alkylthiophosphates and Alkylphosphonates	47	19	28	0.40
SN2|SN2 >> Coordination with nucleoside bases|SN2 >> Coordination with nucleoside bases >> Short-Chain Alkyltin and Alkylgermanium Halides	7	1	6	0.14

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

The only alert that showed low PPV was the (SN2| Alkylphosphates, Alkylthiophosphates and Alkylphosphonates) alert which was incorrectly predicted 28 mutagenic substances out of 47 substances with 40% PPV. On the other hand, as illustrated in Table 3.7, the alert for epoxides and aziridines (SN2|SN2 >> Alkylation, direct acting epoxides and related|SN2 >> Alkylation,

direct acting epoxides and related >> Epoxides and Aziridines) was accurate as it correctly detected 166 mutagenic substances out of 181 with 91% PPV. DNA alkylation of epoxides is caused by the epoxidation reaction when three-membered epoxide ring opened with nucleophilic DNA centre via substituted nucleophilic reaction SN2 (Figure 3.7). Epoxidation is a common ring-opening reaction leading to DNA alkylation (Sawatari, 2001). Importantly, reactive epoxides can be produced after the metabolism of a range of alkenes, resulting in DNA reactivity. Overall, the predictive power of this profiler showed that there should be a high confidence in a hit from this profiler for mutagenicity.

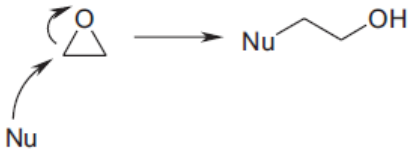


Figure 3.7. Epoxide ring opening reaction (Enoch and Cronin, 2010)

3.3.3 In vitro mutagenicity (Ames test) alerts by ISS profiler:

In the ISS profiler the true positive and true negative rates were reasonably good at 83% and 69% respectively. Compared to the other profilers for mutagenicity, the Ames test alerts had the highest MCC value, which was greater than 0.5 which is a good indicator for predicting both positive and negative mutagens. The positive prediction of mutagenicity for the structural alerts in this profiler was acceptable (78%) and well balanced with the negative prediction value (75%). Four structural alerts were over-predictive and showed false positive results in more than 60% of triggered substances. Three of these four alerts with low PPV values were analogous to poorly performing alerts in the genotoxic and non-genotoxic by ISS profiler. These four alerts were alkenyl benzenes, α,β-unsaturated carbonyls, aromatic ring N-oxide and

simple aldehydes. The highest error rate was for the α,β−unsaturated carbonyl alerts with only 44% positive predicated substances among 192 substances as shown in Table 3.8.

Table 3.8. PPV analysis for each structural alert among "*in vitro* mutagenicity (Ames test) alerts by ISS" profiler.

In vitro mutagenicity (Ames test) alerts by ISS	TOTAL	TP	FP	PPV
Acyl halides	22	16	6	0.73
Aliphatic halogens	224	166	58	0.74
Aliphatic N-nitro groups	12	10	2	0.83
Alkenylbenzenes	21	3	18	0.14
Alkyl (C<5) or benzyl ester of sulphonic or phosphonic acids	55	36	19	0.65
Alkyl and aryl N-nitroso groups	98	84	14	0.86
Alkyl carbamate and thiocarbamates	20	10	10	0.50
Alkyl hydroperoxides	10	9	1	0.90
α,β-Unsaturated aliphatic alkoxy groups	2	0	2	0.00
Alkyl nitrites	7	7	0	1.00
α,β-Unsaturated carbonyls	192	84	108	0.44
Anthrones	3	1	2	0.33
Aromatic diazos	78	44	34	0.56
Aromatic mono-and dialkylamines	53	32	21	0.60
Aromatic N-acyl amines	45	22	23	0.49
Aromatic ring N-oxides	10	4	6	0.40
Azide and triazene groups	55	54	1	0.98
Coumarins and Furocoumarins	22	16	6	0.73
Epoxides and aziridines	219	165	54	0.75
Flavonoids	5	3	2	0.60
Heterocyclic Polycyclic Aromatic Hydrocarbons	107	88	19	0.82
Hydrazines	50	33	17	0.66
Hydroxamic acid derivatives	9	8	1	0.89
Monohaloalkenes	10	6	4	0.60
N-aryl-N-acetoxyacetaamides	4	3	1	0.75
Nitro-aromatics	427	347	80	0.81
Polycyclic Aromatic Hydrocarbons	287	258	29	0.90
Primary aromatic amine,hydroxyl amine and its derived esters	340	227	113	0.67
Propiolactones or propiosultones	5	4	1	0.80
Quinones	35	18	17	0.51
S or N mustards	24	17	7	0.71
Simple aldehydes	80	29	51	0.36
Steroidal estrogens	5	0	5	0.00
Xanthones, Thioxanthones, Acridones	31	24	7	0.77

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

In contrast, other structural alerts in this profiler showed high and accurate precision (PPV) e.g. the alerts for nitro-aromatics, Polycyclic Aromatic Hydrocarbons as shown in Table 3.8. The mechanism of mutagenicity of compounds containing aromatic amine, nitro, nitroso or hydroxylamine moieties can be explained by partially overlapping metabolic activation pathways (Cronin, 2010). Although an aromatic nitro group requires enzymatic reduction (catalysed by both cytosolic and microsomal enzymes) to form an aromatic hydroxylamine intermediate, the analogous reduction of an aromatic nitroso group is probably non-enzymatic. An aromatic amine moiety, on the other hand, requires enzymatic oxidation to form the same aromatic hydroxylamine intermediate. Subsequent activation of aromatic hydroxylamine intermediates by O-acetylation, O-sulfation, or O-protonation is suggested to form electrophilic intermediates that covalently bind to DNA as shown in Figure 3.8 (Enoch and Cronin, 2010). This shows that the performance of the structural alerts is better when the chemical relationship that explains the mutagenicity is clear and well known.

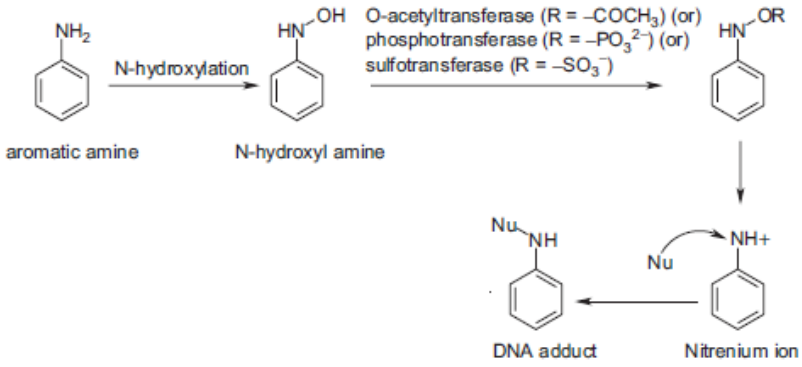


Figure 3.8. Metabolic conversion of aniline to the electrophilic nitrenium ion (Enoch and Cronin, 2010).

3.3.4 In vivo mutagenicity (Micronucleus) alerts by ISS:

The results of the analysis of the "*in vivo* mutagenicity (Micronucleus) alerts by ISS" profiler are provided in Table 3.9 and show an imbalance between true positive rate (sensitivity) 90% and true negative rate (specificity) 37%. Five alerts in this profiler are over-predictive, namely H-acceptor-path3-H-acceptor, 1-phenoxy-benzene, α,β-unsaturated carbonyls, aromatic N-acyl amine and simple aldehydes. The majority of false positive results were from the H-acceptor-path3-H-acceptor alert among non-mutagenic substances. This alert was triggered in 1114 substances and incorrectly predicts 791 non-mutagenic substances as mutagenic with a PPV of only 29%. This also explains the high value of true positive rate (sensitivity) caused by this alert. This alert explores the possibility that a chemical interacts with DNA and/or proteins *via* non-covalent binding, such as DNA intercalation or groove-binding (Snyder *et al*. 2006) which may not be relevant to Ames test data, however, it suggests that this alert should go under detailed further investigation using Micronucleus data (this was undertaken in Chapter 5). Clearly, this alert significantly affects the performance of the profiler's ability to predict the mutagenicity, as the profiler has the lowest MCC value (0.33) of the six profilers considered. This indicates removing this alert from the profiler would improve the overall performance of the profiler. The unsaturated carbonyl and simple aldehyde alerts also showed low positive predictivity, 47% and 18% respectively, confirming the similar conclusion from the genotoxic and nongenotoxic profilers and Ames test by ISS profiler. The alert for "Simple aldehydes" is based on the theory that all compounds containing an aldehydic group can potentially undergo Schiff base formation with a primary amine. They are to be considered potentially genotoxic, as demonstrated by their ability to react *in vivo* with nucleobases, without metabolic activation, forming adducts, interbase cross-links (both intra and inter-strand) and DNA-protein crosslinks. The carbon chain length of aliphatic aldehydes and, in general, molecular size can strongly modulate the formation of every type of cross-link and even the accessibility of the DNA

nucleobases. DNA-protein crosslinks have been reported as the primary DNA damage induced by formaldehyde (Speit *et al.* 2007). Thus, the overall analysis has indicated that the alert for simple aldehdyes, whilst valid mechanistically, requires further and detailed definition to enable it for use. On the other hand, alerts for primary aromatic amines, nitro aromatics, epoxides and aziridines had high mutagenicity predictivity with PPV ranging from 75% to 90%.

Table 3.9. Positive predictivity analysis for each single structural alert within the *in vivo* mutagenicity (Micronucleus) alerts by ISS profiler.

in vivo mutagenicity (Micronucleus) alerts by ISS	TOTAL	TP	FP	PPV
H-acceptor-path3-H-acceptors	1114	323	791	0.29
1,3-dialkoxy-benzenes	2	1	1	0.50
1-phenoxy-benzenes	14	3	11	0.21
Acyl halides	21	15	6	0.71
Aliphatic azo and azoxys	9	9	0	1.00
Aliphatic halogens	173	131	42	0.76
Aliphatic N-nitro groups	5	4	1	0.80
Alkyl (C<5) or benzyl ester of sulphonic or phosphonic acids	47	33	14	0.70
Alkyl and aryl N-nitroso groups	30	27	3	0.90
Alkyl carbamates and thiocarbamates	12	7	5	0.58
Alkyl nitrites	7	7	0	1.00
α,β-unsaturated aliphatic alkoxy groups	7	5	2	0.71
α,β-unsaturated carbonyls	60	28	32	0.47
Aromatic diazos	8	6	2	0.75
Aromatic mono- and dialkylamines	35	19	16	0.54
Aromatic N-acyl amines	26	10	16	0.38
Aromatic nitroso groups	17	17	0	1.00
Aromatic ring N-oxides	6	2	4	0.33
Azide and triazene groups	22	21	1	0.95
Carbodiimides	2	1	1	0.50
Coumarins and Furocoumarins	5	1	4	0.20
Epoxides and aziridines	140	103	37	0.74
Heterocyclic Polycyclic Aromatic Hydrocarbons	71	62	9	0.87
Hydrazines	15	12	3	0.80
Isocyanate and isothiocyanate groups	3	3	0	1.00
Monohaloalkenes	6	6	0	1.00
Nitro-aromatics	218	174	44	0.80
Polycyclic Aromatic Hydrocarbons	247	221	26	0.89
Primary aromatic amine, hydroxyl amine and its derived esters	174	123	51	0.71
Propiolactones or propiosultones	5	4	1	0.80
S or N mustards	15	11	4	0.73
Simple aldehydes	33	6	27	0.18

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

3.3.5 DNA binding by OASIS v.1.4 Profiler:

The DNA binding by OASIS profiler showed reasonably acceptable overall sensitivity and specificity values, 71% and 73% respectively. Accuracy was also good (72%), but MCC was still low at 0.43. Table 3.10 shows three alerts had a low PPV (19%, 43% and 30% respectively), namely: carboxamides and aminoalkylamines via non covalent interaction, thiols via a radical mechanism and alkylphosphates, alkylthiophosphates and alkylphosphonates via alkylation. The structural alert for alkylphosphates was triggered in 69 substances with only 21 true positives predicted. This alert is based on the theory that some of the organophosphorus fragments are known to be strongly electrophilic (mainly phosphonic and phosphoric acid derivatives). Two chemo-toxicological mechanisms have been suggested: phosphorylation and alkylation of the biological macromolecules. Compared to the carbon atom of the alkyl group, the phosphorus atom is more electron-deficient and susceptible to attack by nucleophiles (Braun *et al*., 1982). This mechanism may be not relevant to the Ames test which could explain the low predictivity of this alert. Other alerts in this profiler, such as those for epoxides and aziridines showed high PPVs for a large proportion of substances (92% of 181 substances were identified correctly).

Table 3.10. Positive predictivity analysis (PPV) for each structural alert with the DNA binding by OASIS profiler.

DNA binding by OASIS	TOTAL	TP	FP	PPV
AN2|AN2 >> Michael-type conjugate addition to activated alkene derivatives|AN2 >> Michael-type conjugate addition to activated alkene derivatives >> Alpha-Beta Conjugated Alkene Derivatives with Geminal Electron-Withdrawing Groups	2	0	2	0
AN2|AN2 >> Schiff base formation|AN2 >> Schiff base formation >> Dicarbonyl compounds	17	12	5	0.71
Non-covalent interaction|Non-covalent interaction >> DNA intercalation|Non-covalent interaction >> DNA intercalation >> DNA Intercalators with Carboxamide and Aminoalkylamine Side Chain	32	6	26	0.19
Non-covalent interaction|Non-covalent interaction >> DNA intercalation|Non-covalent interaction >> DNA intercalation >> Quinolone Derivatives	23	15	8	0.65
Radical|Radical >> Radical mechanism by ROS formation (indirect) or direct radical attack on DNA|Radical >> Radical mechanism by ROS formation (indirect) or direct radical attack on DNA >> Organic Peroxy Compounds	34	28	6	0.82
Radical|Radical >> Radical mechanism via ROS formation (indirect)|Radical >> Radical mechanism via ROS formation (indirect) >> Anthrones	3	1	2	0.33
Radical|Radical >> Radical mechanism via ROS formation (indirect)|Radical >> Radical mechanism via ROS formation (indirect) >> Thiols	23	10	13	0.43
SN2|SN2 >> Alkylation, direct acting epoxides and related|SN2 >> Alkylation, direct acting epoxides and related >> Epoxides and Aziridines	280	223	57	0.80
SN2|SN2 >> Alkylation|SN2 >> Alkylation >> Alkylphosphates, Alkylthiophosphates and Alkylphosphonates	69	21	48	0.30
SN2|SN2 >> Coordination with nucleoside bases|SN2 >> Coordination with nucleoside bases >> Short-Chain Alkyltin and Alkylgermanium Halides	7	1	6	0.14
SN2|SN2 >> Direct acting epoxides formed after metabolic activation|SN2 >> Direct acting epoxides formed after metabolic activation >> Quinoline Derivatives|SN2 >> SN2 at an activated carbon atom|SN2 >> SN2 at an activated carbon atom >> Quinoline Derivatives	85	47	38	0.55
SN2|SN2 >> Direct acylation involving a leaving group|SN2 >> Direct acylation involving a leaving group >> Acyl Halides	20	16	4	0.80
SN2|SN2 >> DNA alkylation|SN2 >> DNA alkylation >> Vicinal Dihaloalkanes|SN2 >> Internal SN2 reaction with aziridinium and/or cyclic sulfonium ion formation (enzymatic)|SN2 >> Internal SN2 reaction with aziridinium and/or cyclic sulfonium ion formation (enzymatic) >> Vicinal Dihaloalkanes	34	19	15	0.56
SN2|SN2 >> SN2 at sulfur atom|SN2 >> SN2 at sulfur atom >> Sulfonyl Halides	2	0	2	0.00

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

3.3.6 DNA binding by OECD profiler:

The true negative rate (specificity) for the "DNA binding by OECD" profiler was, to some extent, less than acceptable at 62%. The poorer performance was also reflected in the MCC value of 0.32 which was the second lowest value amongst the six profilers. Six alerts in this profiler showed low PPV including one through the acylation mechanism (1,1-dihaloalkanes), three alerts for the Michael addition mechanism (furan, α,β–unsaturated ester, α,β-unsaturated ketone), one Schiff base former mechanism (mono aldehyde) and two alerts for unimolecular aliphatic nucleophilic substitution S_N1 reactions (aromatic phenyl urea and aliphatic tertiary amines). Of the six alerts with low PPV, that for aliphatic tertiary amines was the most triggered hitting 218 substances. However, this alert falsely predicted 149 positives out of 218 with only 30% PPV, as shown in Table 3.11. The alert for aliphatic tertiary amines was described in this profiler as relating to a mechanism of action whereby P450 metabolism converts the aliphatic tertiary amine to a reactive iminium species. This has been suggested as a potential pathway to DNA adducts via an S_N1 mechanism, the reaction shown in Figure 3.9 (Kalgutkar and Soglia, 2005). The relatively unspecific nature of the chemical relationship supporting this structural alert seems to be broad, this results in many false positive predictions and eventually lower accuracy of the overall profiler.

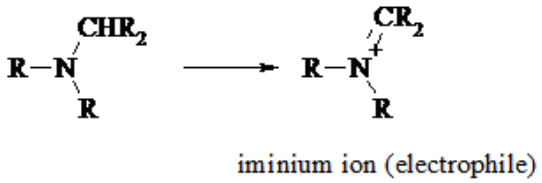


Figure 3.9. DNA adduct formation mechanism of aliphatic tertiary amine (Kalgutkar and Soglia, 2005)

Table 3.11. Positive predictivity analysis for DNA binding by OECD profiler

DNA binding by OECD	TOTAL	TP	FP	PPV
Acylation|Acylation >> Direct Addition of an Acyl Halide|Acylation >> Direct Addition of an Acyl Halide >> Acyl halide	18	16	2	0.9
Acylation|Acylation >> Isocyanates and Isothiocyanates|Acylation >> Isocyanates and Isothiocyanates >> Isocyanates	11	7	4	0.6
Acylation|Acylation >> Isocyanates and Isothiocyanates|Acylation >> Isocyanates and Isothiocyanates >> Isothiocyanates	2	2	0	1.0
Acylation|Acylation >> P450 Mediated Activation to Acyl Halides|Acylation >> P450 Mediated Activation to Acyl Halides >> 1,1-Dihaloalkanes	16	4	12	0.3
Acylation|Acylation >> P450 Mediated Activation to Isocyanates or Isothiocyanates|Acylation >> P450 Mediated Activation to Isocyanates or Isothiocyanates >> Formamides	7	5	2	0.7
Michael addition|Michael addition >> P450 Mediated Activation of Heterocyclic Ring Systems|Michael addition >> P450 Mediated Activation of Heterocyclic Ring Systems >> Furans	20	7	13	0.4
Michael addition|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> 5-alkoxyindoles	4	1	3	0.3
Michael addition|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Alkyl phenols	41	23	18	0.6
Michael addition|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Arenes	119	55	64	0.5
Michael addition|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Hydroquinones	87	42	45	0.5
Michael addition|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Methylenedioxyphenyl	8	2	6	0.3
Michael addition|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals|Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Polycyclic (PAHs) and heterocyclic (HACs) aromatic hydrocarbons-Michael addition	51	47	4	0.9

Michael addition|Michael addition >> Polarised Alkenes-Michael addition|Michael addition >> Polarised Alkenes-Michael addition >> Alpha, beta- unsaturated aldehydes	23	15	8	0.7
Michael addition|Michael addition >> Polarised Alkenes-Michael addition|Michael addition >> Polarised Alkenes-Michael addition >> Alpha, beta- unsaturated amides	4	1	3	0.3
Michael addition|Michael addition >> Polarised Alkenes-Michael addition|Michael addition >> Polarised Alkenes-Michael addition >> Alpha, beta- unsaturated esters	55	9	46	0.2
Michael addition|Michael addition >> Polarised Alkenes-Michael addition|Michael addition >> Polarised Alkenes-Michael addition >> Alpha, beta- unsaturated ketones	24	9	15	0.4
Michael addition|Michael addition >> Polarised Azo Compounds|Michael addition >> Polarised Azo Compounds >> Azocarbonamides	1	1	0	1.0
Michael addition|Michael addition >> Quinones and Quinone-type Chemicals|Michael addition >> Quinones and Quinone-type Chemicals >> Quinones	16	14	2	0.9
Schiff base formers|Schiff base formers >> Chemicals Activated by P450 to Glyoxal |Schiff base formers >> Chemicals Activated by P450 to Glyoxal >> Ethanolamines (including morpholine)|Schiff base formers >> Chemicals Activated by P450 to Glyoxal >> Ethylenediamines (including piperazine)	1	1	0	1.0
Schiff base formers|Schiff base formers >> Chemicals Activated by P450 to Glyoxal |Schiff base formers >> Chemicals Activated by P450 to Glyoxal >> Ethanolamines (including morpholine)	4	0	4	0.0
Schiff base formers|Schiff base formers >> Direct Acting Schiff Base Formers|Schiff base formers >> Direct Acting Schiff Base Formers >> Alpha-beta-dicarbonyl	10	7	3	0.7
Schiff base formers|Schiff base formers >> Direct Acting Schiff Base Formers|Schiff base formers >> Direct Acting Schiff Base Formers >> Mono aldehydes	35	15	20	0.4
SN1|SN1 >> Carbenium Ion Formation|SN1 >> Carbenium Ion Formation >> Aliphatic N-Nitro	11	10	1	0.9
SN1|SN1 >> Carbenium Ion Formation|SN1 >> Carbenium Ion Formation >> Allyl benzenes	6	3	3	0.5
SN1|SN1 >> Carbenium Ion Formation|SN1 >> Carbenium Ion Formation >> Polycyclic (PAHs) and heterocyclic (HACs) aromatic hydrocarbons-SN1	110	103	7	0.9
SN1|SN1 >> Iminium Ion Formation|SN1 >> Iminium Ion Formation >> Aliphatic tertiary amines	218	69	149	0.3
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Aromatic azo	30	14	16	0.5

SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Aromatic N-hydroxylamines	27	24	3	0.9
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Aromatic nitro	481	396	85	0.8
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Aromatic phenylureas	10	2	8	0.2
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Aromatic nitroso	21	21	0	1.0
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Primary (unsaturated) heterocyclic amine	66	53	13	0.8
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Primary aromatic amine	265	189	76	0.7
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Secondary (unsaturated) heterocyclic amine	11	1	10	0.1
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Secondary aromatic amine	34	16	18	0.5
SN1|SN1 >> Nitrenium Ion formation|SN1 >> Nitrenium Ion formation >> Tertiary aromatic amine	59	36	23	0.6
SN2|SN2 >> Direct Acting Epoxides and related|SN2 >> Direct Acting Epoxides and related >> Aziridines	36	35	1	1.0
SN2|SN2 >> Direct Acting Epoxides and related|SN2 >> Direct Acting Epoxides and related >> Epoxides	184	144	40	0.8
SN2|SN2 >> Direct Acting Epoxides and related|SN2 >> Direct Acting Epoxides and related >> Sulfuranes	3	1	2	0.3
SN2|SN2 >> Episulfonium Ion Formation|SN2 >> Episulfonium Ion Formation >> 1,2-Dihaloalkanes	27	23	4	0.9
SN2|SN2 >> Epoxidation of Aliphatic Alkenes|SN2 >> Epoxidation of Aliphatic Alkenes >> Halogenated polarised alkenes	14	8	6	0.6
SN2|SN2 >> SN2 at an sp3 Carbon atom|SN2 >> SN2 at an sp3 Carbon atom >> Aliphatic halides	103	80	23	0.8
SN2|SN2 >> SN2 at an sp3 Carbon atom|SN2 >> SN2 at an sp3 Carbon atom >> Phosphonic esters	5	0	5	0.0

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

3.4. Conclusion

This chapter provides detailed analysis of the positive predictions of each structural alert in six mutagenicity profilers within the OECD QSAR Toolbox, as compared to experimental mutagenicity data from the CCRIS dataset. The analysis of the results from this investigation aimed to increase the reliability and accuracy of mutagenicity predictions by these profilers. All six mutagenicity profilers showed reasonably acceptable overall sensitivity and specificity ranging from the excellent predictive performance of the "Ames test alert by ISS" profiler to an acceptable predictive performance with the remaining five mutagenicity profilers. The OECD QSAR Toolbox mutagenicity profilers play an important role in read-across from the expermintal data to predict mutagenicity for untested compounds. Knowing and investigating the accuracy and senstivity of these profilers will highlight which strucutral alerts need to be kept or ignored, to improve the overall preformance of the profilers.

As this study has shown, 28 strucutural alerts were found to be too inaccurate to be used as an indicator for mutagenicity and need more refinment and evaluation, these are summarised in Table 3.12. The alert "Hacceptor-path3-Hacceptor" in the micronucleus profiler was the highest triggered alert amongst the total 28 alerts for 1114 subsatances. The positive predicitvity of this alert was too low (30%) and removing this alert may improve the overall preformence of the profiler. Similarly, aliphatic tertiary amines in the DNA binding profiler by OECD was the second highest alert to be triggered among the 28 alert list. This alert was triggered in 219 substances and correctly predicts only 30% of the substances, which raises the need to remove it to improve the overall accuracy of the profiler in addition to the other low PPV alerts suggested in each profiler in Table 3.12.

Table 3.12. A list of 28 structural alerts showing low mutagenicity predictivity among the six mutagenicity profilers within the OECD QSAR Toolbox.

ALERT	TOTAL	TP	FP	PPV
genotox and nongenotox alerts by ISS				
1,3-Benzodioxoles	12	5	7	0.42
Benzenesulfonic ethers, methylation	13	3	10	0.23
Halogenated benzenes	82	19	63	0.23
Substituted n-alkylcarboxylic acids	26	3	23	0.12
Thiocarbonyls	17	6	11	0.35
Alkenylbenzenes	17	4	13	0.24
α,β-unsaturated carbonyls	187	85	102	0.45
Simple aldehydes	74	28	46	0.38
DNA alerts for AMES, MN and CA by OASIS				
SN2| Alkylphosphates, Alkylthiophosphates and Alkylphosphonates	47	19	28	0.40
in vitro mutagenicity (Ames test) alerts by ISS				
Alkenylbenzenes	21	3	18	0.14
alpha,beta-unsaturated carbonyls	192	84	108	0.44
Aromatic ring N-oxides	10	4	6	0.40
Simple aldehydes	33	6	27	0.18
in vivo mutagenicity (Micronucleus) alerts by ISS				
H-acceptor-path3-H-acceptors	1114	323	791	0.29
1-phenoxy-benzenes	14	3	11	0.21
alpha,beta-unsaturated carbonyls	60	28	32	0.47
Aromatic N-acyl amines	26	10	16	0.38
Simple aldehydes	33	6	27	0.18
DNA binding by OASIS v.1.1				
Non-covalent interaction| DNA Intercalators with Carboxamide and Aminoalkylamine Side Chain	32	6	26	0.19
Radical|Radical >> Radical mechanism via ROS formation (indirect)| Thiols	23	10	13	0.43
SN2|SN2 >> Alkylation| Alkylphosphates, Alkylthiophosphates and Alkylphosphonates	69	21	48	0.30
DNA binding by OECD				
Acylation >> P450 Mediated Activation to Acyl Halides| 1,1-Dihaloalkanes	16	4	12	0.3
Michael addition| >> P450 Mediated Activation of Heterocyclic Ring Systems| Furans	20	7	13	0.4
Michael addition| >> Polarised Alkenes| Alpha, beta- unsaturated esters	55	9	46	0.2
Michael addition| >> Polarised Alkenes-| Alpha, beta- unsaturated ketones	24	9	15	0.4
Schiff base formers >> Direct Acting Schiff Base >> Mono aldehydes	35	15	20	0.4
SN1|SN1 >> Iminium Ion Formation| Aliphatic tertiary amines	218	69	149	0.3
SN1|SN1 >> Nitrenium Ion formation| Aromatic phenylureas	10	2	8	0.2

Total = number of substances flagged by alert , TP= True positive (mutagens identified positive by alert) , FP= False positive (non-mutagens identified positive by alert) , PPV= Positive Predictivity value

Overall Chapter 3 confirms the need, and value derived from, the study of the suitability and merits of each of the alerts within the profilers in the OECD QSAR Toolbox. This is equally applicable to other *in silico* toxicity platforms e.g. knowledge-based expert systems for predictive toxicology. These analysis are important to identify possibilities to improve the performance of the profilers, as well as to provide estimates of confidence in the alerts. This will, by implication, also improve the reliability of chemical read-across and grouping/categorisation for classification, labelling and risk assessment.

Chapter 4: Identification of the core structural features of genotoxic and non-genotoxic carcinogens in cosmetic ingredients using scaffold analysis.

4.1. Introduction:

As described in Chapter 1, safety of cosmetics in Europe is regulated under the Cosmetic Regulation (EC) No 1223/2009. The Regulation prohibits the use of any carcinogenic, mutagenic and reproductive toxicant (CMR) substances in cosmetic products. The CMR classifications can be summarised as follows (see Section 1.4 for more details):

Carcinogenic
- Cat. 1A: Known to have carcinogenic potential for humans
- Cat. 1B: Presumed to have carcinogenic potential for humans
- Cat. 2: Suspected human carcinogen

Mutagenic
- Cat. 1A: Substance known to induce heritable mutations in the germ cells of humans
- Cat. 1B: Substance to be regarded as if it induces heritable mutations in the germ cells of humans
- Cat. 2: Substance which causes concern for humans owing to the possibility that it may induce heritable mutations in the germ cells of humans

Reproductive toxicants
- Cat. 1A: Known human reproductive toxicant
- Cat. 1B: Presumed human reproductive toxicant
- Cat. 2: Suspected human reproductive toxicant

.As a consequence of the regulatory pressures and ethical considerations discussed in Chapter 1, the use of alternative testing methods, such as computational toxicology and QSAR modelling, have considerably increased for the assessment of toxic hazards (Nendza *et al*., 2013; Plošnik, Zupan and Vračko, 2015). At the same time, the incidence of cancer has increased by 70% during the last two decades and this has put cancer as the second leading cause of mortality worldwide (Stewart and Wild 2017). The use of any carcinogenic chemical ingredient in a cosmetic product is considered as of high concern as all personal products provide direct exposure to the consumer and a route of emission to the environment. In order

to develop *in silico* models, a mechanistic basis is strongly encouraged. As discussed the preceding Chapters 1 and 2, two mechanisms that can initiate cancer – these are based around genotoxic and non-genotoxic pathways and these need to be considered for modelling purposes.

In this chapter, the word "scaffold" is used primarily to describe the core structure of compounds. This chapter focused mainly on the identification of the core structural "scaffold" through which the potential of other (untested) carcinogenic chemical agents may be assessed. This chapter encompassed chemical substances with either genotoxic or non-genotoxic carcinogenic mechanisms of action. In accordance with the European Cosmetic Regulation, the emphasis of this study was to explore QSARs as an alternative (non-animal) method for assessment of the potential carcinogenicity for the chemicals that are intended to be used as cosmetic ingredients. The QSAR field has seen much progress in the past couple of decades, and a number of high quality *in silico* models, expert systems and read-across tools based on both Ames test and experimental rat carcinogenicity test data are now available (Mostrag-Szlichtyng *et al.,* 2010). In addition to the predictivity (from chemical structure) of the *in silico* models and tools, they also provide very useful information in regard to mechanistic pathways or structure-activity relationships (Cronin and Madden, 2011).

Various structural alerts have also been derived for mutagenicity/carcinogenicity. For example, the Benigni-Bossa rules are utilised by various expert systems i.e. Derek Nexus and Toxtree to predict the carcinogenicity or mutagenicity of unknown chemicals (Benigni, 2008). On the other hand, QSAR based approaches illustrate the relationship between the carcinogenicity of the chemicals and physicochemical properties of the molecules such as lipophilicity, electron density, etc by linear or non-linear models (Schultz *et al*., 2006). One of the main examples of programmes that use the QSAR approach is Leadscope Model Applier (LSMA) which has been

used in this study to generate the main structural scaffolds for grouping of the chemicals according to mutagenic/carcinogenic potential.

A major limitation in using QSAR or structural alerts to predict carcinogenicity is that neither of the approaches (structural alerts or QSAR) can link a core structural "scaffold" with the ability of a chemical substance to be carcinogenic. Structural alerts mainly depict the outcomes of an evaluation linked to functional group(s), whereas QSAR approaches derive estimates from a series of similar (analogous) chemicals in their properties and their activity (Kho *et al*., 2005). As a result, neither of the approaches can precisely flag the carcinogenic potency when it arises from a certain core structural feature (scaffold). Different carcinogenic chemicals may, however, share one or more structural features (i.e. the scaffold) contained within the functional groups or side chains and the scaffold analysis approach was therefore investigated in this Chapter to seek clues to find a link between a compound's chemical structure and potential carcinogenicity. For this, the relationship between scaffolds of a range of diverse compounds and carcinogenicity (both genotoxic and non-genotoxic) was analysed using a dataset of Ames assay for 10,543 compounds from the SAR genotoxicity database, and carcinogenicity data for 2,870 compounds from the SAR carcinogenicity database both within the Leadscope® database (Leadscope.com, 2018). The scaffold generation feature in Leadscope® was utilised to generate hierarchical relationships of scaffolds between these compounds and the activities. Through analysis of scaffold relationships, lists of the scaffolds with potential mutagenicity or carcinogenicity were established. These scaffolds can be used as the basis for predicting the carcinogenic potency of new chemicals planned to be used in cosmetic product which helps to prevent using potentially carcinogenic chemicals. The knowledge of scaffolds implicated in carcinogenic effects may also make it possible to redesign some of the compounds by replacing them with non-carcinogenic scaffolds.

4.2. Materials and Methods:

4.2.1: Leadscope SAR carcinogenicity and genotoxicity databases:

Leadscope SAR carcinogenicity and genotoxicity databases are high-quality data resources that can be used to build a predictive model and for the 'read-across' of data for other chemicals to determine their potential to be carcinogenic or mutagenic. Both databases contain summarised data for *in vitro* and *in vivo* cancer and mutagenicity endpoints along with chemical structures. These databases are used by the Leadscope program to build predictive models. To ensure high-quality data for SAR, analyses of various salt forms of chemical compounds and their respective toxicity data have also been carried out to derive an overall endpoint for the active portion of the chemical. Several sources of experimental test results have been included in these databases, such as from FDA, NTP, CCRIS, CPDB and other primary sources. All chemical structures have been provided in SAR in the neutral and, if appropriate, tested form and confirmed for accuracy.

The SAR carcinogenicity database contains 1,948 SAR structures. The database includes 3,598 compounds with 11,538 test results and provides carcinogenicity study endpoints for male and female rats (1,774 and 1,725 compounds respectively) and male and female mice (1,640 and 1,675 compounds respectively). The SAR genotoxicity database provides compound-level calls for 46 genetic toxicity endpoints. These include 32 bacterial mutagenicity endpoints, 4 *in vitro* mammalian, 5 *in vitro* chromosomal aberration and 6 *in vivo* micronucleus results. An overview of the datasets is presented in Table 4.1.

Table 4.1. An overview of the number of compounds and the endpoints total results for both SAR genetox and SAR carcinogenicity databases. * = (Number of tests)

SAR genotoxic database (10543)*	Mutagenic (positive)	Non-mutagenic (negative)
Bacterial mutation	**4530**	**4173**
Salmonella	**4235**	**4180**
In vivo micronucleus	**274**	**912**
SAR carcinogenic database (2870)*	carcinogenic (positive)	Non-carcinogenic (negative)
Male rat	**745**	**869**
Female rat	**686**	**892**

4.2.2 Scaffold generation feature in Leadscope:

The scaffold analysis feature for a large dataset of bioactivity values in the Leadscope personal Ver 4.4 programme was used in this study to generate hierarchical structuring and visualisation of the main carcinogenic scaffolds covering both genotoxic and non-genotoxic mechanisms. This feature was also used to navigate and explore the chemical space of different complex structures in both the SAR carcinogenicity and SAR genotoxicity databases. The carcinogenic scaffolds were extracted by removing all side chains except the linking double bonds and exocyclic groups to generate chemically meaningful compound scaffolds. Using the scaffold generating parameters, the level of quality of the extracted scaffolds from the database was selected by choosing the minimum compounds per scaffold and the minimum atoms per scaffold. Choosing higher numbers yields lower number of scaffolds but of higher quality. In this study, the criteria used were that a carcinogenic scaffold has to cover at least 10 compounds and therefore the minimum compounds number per one scaffold was set to 10, and the same number was set for the minimum number of atoms per scaffold.

The scaffold generation feature in Leadscope has another advantage in that it arranges scaffolds to form a tree of "virtual scaffolds" that are constructed *in silico*. This tree is built in hierarchical arrangement of parent and child scaffolds (see Figure 4.1). The smaller size scaffold (parent), which covers a larger number of compounds, yields bigger sized (child) scaffolds that cover fewer compounds but have more specificity in terms of activity. Child scaffolds that share the same substructure of parent scaffold are usually termed as sibling scaffolds since they are all linked to one parent scaffold.

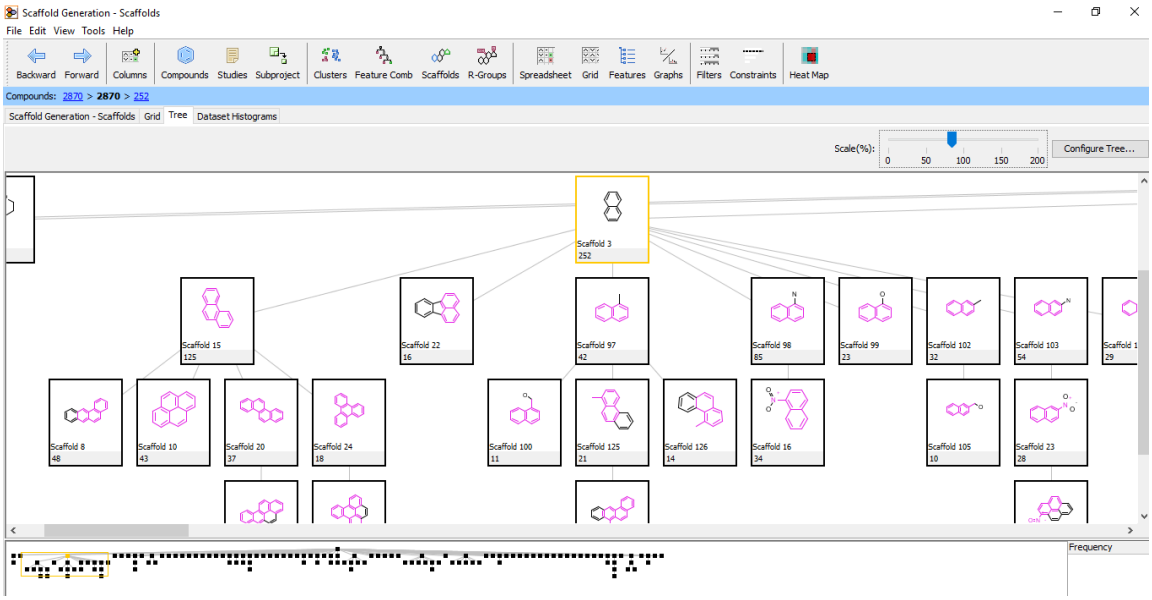


Figure 4.1. A screenshot of the Leadscope program which shows the virtual scaffolds tree in hierarchical arrangement of parent and child carcinogenic scaffolds.

In this study, the scaffold generation feature in Leadscope was used to construct a scaffold tree of both carcinogenic and mutagenic scaffolds extracted from the SAR carcinogenicity and genotoxicity databases. This approach also helped to illustrate any relationship(s) between carcinogenicity and mutagenicity scaffolds since visual analysis of the structural relationship between parent and child scaffolds was easier using the hierarchical arrangement tree.

4.2.3 Cut-offs for Selecting Carcinogenic and Mutagenic Scaffolds:

Carcinogenicity and mutagenicity values were assigned to each scaffold within a scaffold tree. In the case of the SAR carcinogenicity database, this value was defined as the ratio of carcinogenic compounds to the total compounds that are contained in that scaffold. While in the SAR genotoxicity database, this value was defined as the ratio of mutagenic compounds to the total compounds that are contained in that scaffold. Cut-off values were then specified in order to select the representative carcinogenicity or mutagenicity scaffold. If the value of mutagenicity or carcinogenicity of any scaffold was equal or greater than the cutoff value, it was considered as a representative active scaffold (carcinogenicity or mutagenicity) whereas the scaffolds less than the cut-off value were defined as non-active (non-carcinogenic or non-mutagenic). In addition, each scaffold had to cover at least 10 compounds to be selected to the scaffold group. The main goal of adjusting the cut-off value was to select the minimum number of carcinogenicity and mutagenicity scaffolds that would cover the largest possible number of carcinogenic and mutagenic compounds. The ratio of activity (carcinogenicity or mutagenicity) (C1/S), where C1 represent total active compounds (carcinogenic or mutagenic) and S represent the number of active compounds that contain this scaffold, was adjusted to be 0.7 based on selection criteria discussed above. All the values between 0.7-0.3 were considered equivalent while values below 0.3 were considered as non-active scaffolds (non-carcinogenicity or non-mutagenicity).

On the other hand, the non-active compounds' ratio was also adjusted to select the minimum number of non-active (non-carcinogenicity or non-mutagenicity) scaffolds that would cover maximum number of non-active compounds. The ratio of non-activity (C2/S), where C2 represent the total non-active compounds (non-carcinogenic or non-mutagenic) and S represent the number of non-active compounds that contain this scaffold, was adjusted to be 0.7 based on selection criteria discussed above, which is equal to 0.3 in ratio of activity.

All scaffolds that were equal or more than the activity ratio (0.7) in carcinogenicity and equal or more than activity ratio (0.7) in mutagenicity and covered more than 10 structures were classified as genotoxic carcinogenicity scaffolds. On the other hand, all scaffolds that are more than or equal to activity ratio (0.7) in carcinogenicity and less than or equal to non-activity ratio (0.3) in mutagenicity were classified as non-genotoxic carcinogenicity scaffolds.

4.3. Results and Discussion:

The aim of the study presented in this chapter was to investigate the relationship between structural scaffolds and carcinogenicity or mutagenicity for diverse chemical substances using experimental data from the SAR carcinogenicity and genotoxicity database. A scaffold is defined as "fixed part of a molecule, on which functional group or other side chain can be substituted or changed" (Hsu *et al*., 2016). The experimental toxicity data on which this analysis was based included the results of both genotoxic and non-genotoxic carcinogenicity studies.

Through application of stringent selection criteria, 17 carcinogenicity scaffolds (C/S scores greater than or equal to 0.7), 21 mutagenicity scaffolds (C/S scores greater than or equal to 0.7), and 7 non-mutagenicity scaffolds (C/S scores lower than or equal to 0.3) were identified. Each scaffold has two values: score-1 for carcinogenicity obtained from the SAR carcinogenicity database and score-2 for mutagenicity obtained from the SAR genotoxicity database. Some scaffolds showed a high carcinogenicity score with the absence of a mutagenicity scaffold(s) obtained from SAR genotoxicity database confirming that the mode of action was non-genotoxic carcinogen.

To determine the main structural features for carcinogenicity the structure-activity relationships for the 17 carcinogenic scaffolds were examined. Any structural similarity or common features in the carcinogenic scaffolds were grouped together and a scaffold tree was

build. Of the 17 carcinogenic scaffolds, 12 scaffolds shared the same structural feature(s) and were categorised into three groups 1) phenanthrenes, 2) 9H- fluorene and 3) nitronaphthalene. The other five carcinogenic scaffolds were listed as a separate (fourth) group.

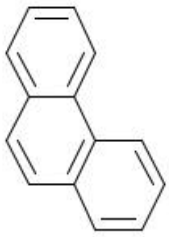
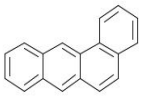
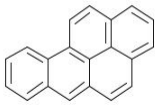
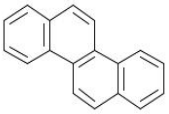
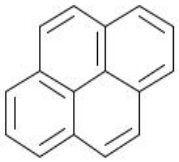
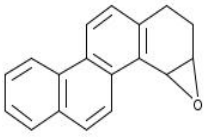
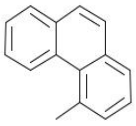
All children scaffolds listed under these three main groups met the selection criteria - i.e. they had carcinogenicity scores equal or greater than 0.7, and covered more than 10 compounds. The fourth group, which contained five parent scaffolds with no children scaffolds, comprised anthracene-9,10-dione, benzidine, 1-methylnaphthalene, estradiol and 5-methylbenzodioxole. The statistics concerning the carcinogenicity value and the number of compounds covered by the scaffold are shown in Table 4.1. The analysis demonstrated that it is highly probable that any chemical structure containing one of these carcinogenic scaffolds would induce carcinogenicity regardless of the attached functional group or side chain to the scaffold.

In the following section, the structural feature(s) of all of the carcinogenicity scaffolds are discussed in detail and their classification based on mode of action (genotoxic or non-genotoxic) is presented at the end of the chapter.

4.3.1 Major carcinogenicity scaffold (1): Phenanthrene:

In the SAR carcinogenicity database, more than 70% of compounds containing phenanthrene as the main core structure were carcinogenic. 86% of these compounds (in 108/125) were tested carcinogenic in female rat. Phenanthrene was, therefore, considered one of the major carcinogenic scaffolds. The scaffold tree for phenanthrene consists of six child scaffolds (shown in Table 4.2). These were: (2) tetraphene, (3) benzo[pqr] tetraphene, (4) chrysene, (5) 3a,5a-dihydropyrene, (6) 1,2,2a,3a,tetrahydrochryseno[3,4-b]oxirene and (7) methyl-phenantherene. All of the child scaffolds were considered as major carcinogenic scaffolds because all compounds containing these scaffolds were carcinogenic with a carcinogenicity value more than 0.7. From the total of 48 compounds containing tetraphene, 100%, were carcinogenic in both female and male rats. For the other compounds containing scaffolds the carcinogenicity value were as follows: benzo[pqr] tetraphene (100%, 16/16) , chrysene (100%, 37/37), 3a,5a dihydropyrene (100%, 43/43), tetrahydrochryseno oxirene (100%, 11/11) and methyl phenantherene (100%, 12/12).

Table 4.2. First major carcinogenic scaffolds (phenanthrene) with six child scaffolds identified from the SAR carcinogenicity database (includes both genotoxic and nongenotoxic mechanisms) that are equal or more than 0.7 c/s ratio.*F: female rat M: male rat.

<table>
<tr><td colspan="4">Phenanthrene
Frequency: 125 C/S: M:0.7, F:0.86
  </td></tr>
<tr><td>Frequency: 48
C/S: M:1, F:1</td><td>Frequency: 16
C/S: M:1 , F:1</td><td>Frequency: 37
C/S: M:1 , F:1</td><td>Frequency: 43
C/S : M:1 , F:0.7</td></tr>
<tr><td> 
tetraphene</td><td> 
benzo[pqr] tetraphene</td><td> 
chrysene</td><td> 
3a,5a dihydropyrene</td></tr>
<tr><td colspan="2">Frequency: 11
C/S : M:1 , F:1</td><td colspan="2">Frequency: 12
C/S : M:1 , F:1</td></tr>
<tr><td colspan="2"> 
1,2,2a,3a,tetrahydrochryseno[3,4-b]oxirene</td><td colspan="2"> 
4 methylphenanthene</td></tr>
</table>

The differences between the parent scaffold of phenantherene and its children scaffolds were: three of the children scaffold, tetraphene, chrysene and 3a,5a dihydropyrene, have an additional benzene ring compared to the parent scaffold. The fourth child scaffold, benzo[pqr]tetraphene, has additional two benzene rings, the fifth child scaffold, tetrahydrochryseno oxirene, has an additional oxirene group, and the sixth child scaffold methyl phenantherene had an additional methyl group compared to the parent scaffold.

Phenanthrene and chrysene have been found in some cosmetic ingredients such wood tar (SCCNFP, 2003). They have also been used by ship-builders and sailors for a long period of time as wood preservative because they have disinfectant properties (SCCNFP, 2003). A typical wood tar preparation contains polyaromatic hydrocarbons (PAHs) such as phenanthrene and chrysene that are classified as highly genotoxic compounds and classified as Category 3 mutagens in the EU i.e. substances that cause concern for human health owing to possible mutagenic effects (EU, 2002). According to the EU Scientific Committee on Cosmetic and Non-Food Products (SCCNFP), using wood tar in cosmetic products can pose a high risk to the consumer health because of the highly genotoxic carcinogen compounds such as phenanthrene, chrysene and other polycyclic aromatic hydrocarbons (PAHs). It has also been found that wood tar and its preparations could present a high risk of skin cancer in humans because they may form a DNA adduct in the skin. In addition, wood tar and its preparations may induce benign and malignant tumours in mouse skin (SCCNFP, 2003).

However, wood tar preparation is also used as a psoriasis treatment and different types of wood tar, based on different sources, may show differing genotoxicity activity (SCCNFP, 2003). For example, juniper tar (cade oil) was found to form DNA adduct by covalent binding in both human and mouse skin. This was found both in human biopsy samples from psoriasis treated patients with juniper tar, and in skin and lung cells of mice treated with juniper tar. On the other

hand, pine tar resin has not been found to be genotoxic in the Ames test and did not induce any mutations in *S. typhimurium* (SCCNFP, 2003).

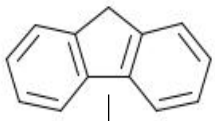
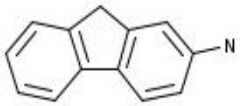
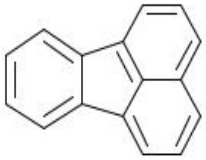
4.3.2 Major carcinogenicity scaffold (2): 9H-Fluorene

This study considered 9H-fluorene as the second major carcinogenicity scaffold on the basis of analysis of SAR carcinogenicity database as more than 70% of fluorene containing compounds, from a total of 22 compounds, were positive in the carcinogenicity test. The 9H-fluorene parent scaffold consists of two carcinogenic child scaffolds that were found in more than 10 compounds and were carcinogenic in more than 70% of these compounds. In Table 4.3, the structural relationship between the parent 9H-fluorene scaffold and the two carcinogenic children scaffold is shown. The first of those two carcinogenicity child scaffolds, 9H-fluorene-2amine was present in 16 compounds and 100% of these compounds were carcinogenic. It can be observed that 9H-fluorene-2-amine has an added amino group compared to the parent scaffold 9H-fluorene. Interestingly, the addition of an amino group led to 25% increase of carcinogenicity compared to the parent scaffold 9H-fluorene. 100% of compounds containing the other carcinogenicity child scaffold, fluoranthene, in 16 compounds were carcinogenic. Fluoranthene has an added benzene ring compared to the parent scaffold 9H-fluorene. The addition of a benzene group increases the carcinogenicity rate by 30% compared to 70% in parent scaffold 9H-fluorene. Fluorene containing compounds, such as like coal tar, are prohibited to be used in cosmetic products according to European regulation (EC) No 1223/2009.

The Chronic Exposure of 9H-fluorene in rats was evaluated . A group of 18 female Buffalo strain rats, 0.9 months of age, were fed 0.05% fluorene in the diet for 18 months (total average intake, 2553 mg/rat), and surviving animals were killed at 20.1 months; the average age /at/ autopsy was 19 months. Tumours reported were one uterine carcinosarcoma, one uterine fibrosarcoma, one granulocytic leukemia, and four pituitary adenomas (IARC, 1983). In a

control group of 18 rats, 3.5 months of age, fed a basal diet for an average of 15.5 months, one uterine adenocarcinoma, two uterine fibro-epithelial polyps, five adrenal cortical adenomas, six pituitary adenomas, and one inguinal region fibroma were reported (IARC, 1983).

Table 4.3. Second major carcinogenic scaffolds (9H-Fluorene) with two child scaffolds identified from the SAR carcinogenicity database (includes both genotoxic and nongenotoxic mechanisms) that are equal or more than 0.7 c/s ratio.*F: female rat M: male rat.

<table>
<tr><td colspan="2">9H-Fluorene
Frequency: 22C/S : M:0.7F:0.7
  </td></tr>
<tr><td>Frequency: 13
C/S : M:1 , F:1</td><td>Frequency: 16
C/S: M:1, F:1</td></tr>
<tr><td> 
9H-fluorene- 2amine</td><td> 
Fluoranthene</td></tr>
</table>

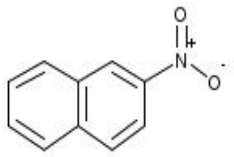
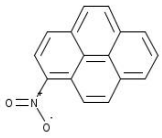
4.3.3 Major carcinogenicity scaffold (3): Nitronaphthalene:

The third major carcinogenicity scaffold was 2-nitronaphthalene, 80% of the total 28 compounds with the nitronaphthalene core structure were carcinogenic. This high probability of carcinogenicity of any compound with nitronaphthalene as its core structure means it should be avoided in any cosmetic product intended to be manufactured. 2- Nitronaphthalene is a by-product of the commercial preparation of 1-nitronaphthalene which is synthesised by the action of a mixture of nitric and sulfuric acids on finely ground naphthalene (Verschueren, 1985). 1,6-Dinitopyrene and 5-nitro-1,2-dihydroacenthylene are two examples of structures containing the 2-nitronaphthalene scaffold as part of their structure. These two compounds were positive in carcinogenicity tests in both male and female rats.

Carcinogenic aromatic amines and nitro compounds are metabolised to "activated" intermediates generally believed to be responsible for producing tissue alterations (Miller and Miller, 1969). N-Oxidation of l- and 2-naphthylamine and nitro reduction of l- and 2-nitronaphthalene may lead to identical N-oxy intermediates which have been shown to be carcinogenic and/or mutagenic in several *in vitro* and *in vivo* studies (Crabtree *et al*., 1991).

The nitronaphthalene scaffold consists of one carcinogenicity child scaffold, namely nitropyrene (see Table 4.4). Nitropyrenes were present in 13 compounds and have a carcinogenicity rate of 0.8 in female rats. The nitropyrene has additional two benzene rings attached to the parent nitronaphthalene scaffold. The additional two benzene rings in child nitropyrene scaffold did not change the carcinogenicity level compared to the parent nitronaphthalene scaffold since the carcinogenicity level were still 80% in female rat.

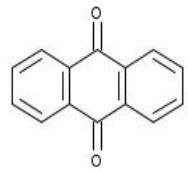
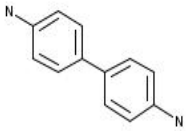
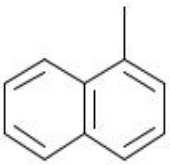
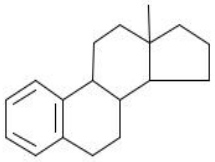
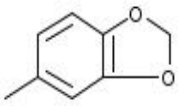
Table 4.4. Major carcinogenic scaffold (nitrophthalene) with one child scaffold identified from the SAR carcinogenicity database (includes both genotoxic and nongenotoxic mechanisms) that are equal or more than 0.7 c/s ratio.*F: female rat M: male rat.

Nitronaphthalene Frequency: **28**C/S : M:0.8, F:0.6 
Frequency: **13** C/S: M:0.**5**, F:0.**8**
 Nitropyrene

4.3.4 Major carcinogenicity scaffold (4): five groups without child scaffolds:

The fourth major carcinogenicity group consisted of five carcinogenic scaffolds with no child scaffold (see Table 4.5). These five scaffolds were anthraquinone, benzidine, 1-methyl naphthalene, estradiol and methylbenzodioxole.

Table 4.5. Main 5 carcinogenic scaffolds with no child scaffold identified from the SAR carcinogenicity database (includes both genotoxic and nongenotoxic mechanisms) that are equal or more than 0.7 C/S ratio.*F: female rat M: male rat.

<table>
<tr><th colspan="4">Other carcinogenic scaffolds with no child scaffolds</th></tr>
<tr><td>Frequency: 16
C/S: M:0.7, F:0.8</td><td>Frequency: 24
C/S: M:0.7, F:0.7</td><td>Frequency: 42
C/S: M:0.8, F:1</td><td>Frequency: 18
C/S: M:0.8, F:0.8</td></tr>
<tr><td> 
Anthraquinone</td><td> 
benzidine</td><td> 
1-methyl naphthalene</td><td> 
(Estradiol)
3methyl-decahydro-cyclopenta-phenanthrene</td></tr>
<tr><td colspan="4">Frequency: 10
C/S: M: 0.8, F:1</td></tr>
<tr><td colspan="4"> 
5-methylbenzodioxole</td></tr>
</table>

4.3.4.1: Anthraquinone carcinogenic scaffold :

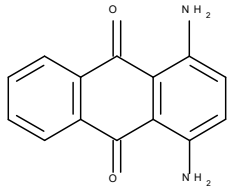
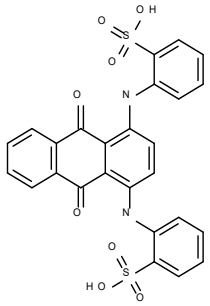
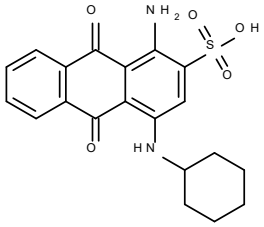
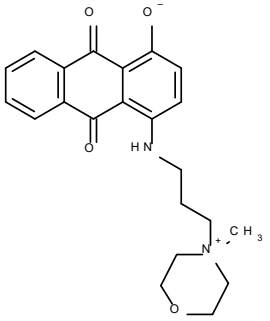
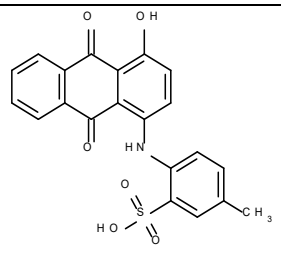
The anthraquinone scaffold was present in 16 compounds as part of their structure with a carcinogenicity rate of 70% and 80% in male and female rat respectively. 1-Amino-2-methylanthraquinone (C.I disperse orange 11) and (C.I. disperse blue 1) are two examples of

compounds that contain the anthraquinone scaffold in SAR carcinogenicity database as the main core structure and were positive in carcinogenicity tests in both male and female rats. In cosmetic products, anthraquinone hair dyes are used mainly within semi-permanent and temporary hair dyes. Acid blue 62 is one example of a chemical with the anthraquinone moiety used as a temporary hair dye that can cause a wide range of toxicities including increase in kidney weight, increased aminotransferase and decrease body weight (SCCP, 2005). Table 4.6 lists the most used hair dyes that contain anthraquinone as a core structure along with a list of side effects in male and female rat that have been noted in the SCCP Opinions.

The International Agency for Research on Cancer (IARC, 2013) stated that anthraquinone increased the incidence of both benign and malignant neoplasms in rat in a 2 year study. In female rats treated with anthraquinone the tumours were noted as renal tubular adenoma of kidney, hepatocellular adenoma, and urinary bladder papilloma of transitional epithelial cell. In experimental animal tests, kidney and urinary bladder tumours and hepatoblastoma are rare.

The metabolism of anthraquinone has been investigated mainly in rat and there are insufficient data in humans (IARC, 2013). The absorption of anthraquinone occurred completely after oral administration and distributed systemically with no sign of accumulation in any specific organ. The most important metabolites of anthraquinone in rodents relevant to mechanistic considerations for anthraquinone (parent molecule) are 1-and 2-hydroxyanthraquinones.

Table 4.6. The main anthraquinone hair dyes and SCCS opinions on carcinogenicity and the maximum allowed limit in hair dyes. ↑ = elevation (increase in level), ↓ = (lowering), ♀ = female , ♂ = male

	Structure	Compound Name	Adverse effects used to derive LO(A)EL within SCC(NF)P and SCCS opinions
1		Disperse Violet 1	↑ Centrilobular/Midzonal hepatocyte hypertrophy↑ Triglycerides (♀)↑ Cholesterol↓ Motor activity Based on the information provided, the SCCS is of the opinion that the use of Disperse Violet 1 in semi-permanent hair dye formulations at a maximum concentration of 0.5% does not pose a risk to the health of the consumer, apart from its moderate skin sensitising potential.
2		Acid Green 25	↑ Kidney weight Acid Green 25 is proposed for use in semi-permanent hair dye formulations as a direct dye at a maximum concentration of 0.3% in the finished cosmetic product. The SCCS is of the opinion that the use of Acid Green 25 as a non-oxidative hair dye with a maximum on head concentration of 0.3% does not pose a risk to the health of the consumer.
3		Acid Blue 62	↑ Kidney weight ↑ Liver weight↑ Ptyalism↑ Tubular nephrosis↑ Centrilobular hepatocyte hypertrophy↑ Blood Urea↑ Albumin↑ Cholesterol↑ AAT↓ Body weight↓ Glucose The SCCS opinion is not available
4		Hydroxyanthraquinone Aminopropyl Methyl Morpholinium Methosulfate	↓ Absolute thymus weight (♀)↓ Body weight (♂)↓ Relative thymus weight The SCCS is of the opinion that the use of Hydroxyanthraquinone aminopropyl methyl 26 morpholinium methosulfate with a maximum concentration of 0.5% in non-oxidative hair 27 dye formulations does not pose a risk to the health of the consumer, apart from its 28 sensitising potential.
5		Acid Violet 43	↑ PT ↑ APTT The SCCP is of the opinion that the information submitted is inadequate to assess the safe use of the substance as a hair dye.

In mutagenicity tests, anthraquinone itself showed conflicting results. In early studies, neither anthraquinone nor its metabolites showed any genotoxicity in the *Salmonella* mutagenicity test (Salamone *et al*., 1979). I However, in later studies, anthraquinone was shown to be mutagenic in the *Salmonella* bacterial mutagenicity test (Zeiger *et al*., 1988). The conflicting results in negative studies were due to the variable amounts of contaminants resulting from the production method, the contaminants included 9-nitroanthracene, anthrone and phenanthrene and it was concluded that they were non mutagenic or weakly mutagenic (NTP, 2005). On the other hand, the major anthraquinone urinary metabolite (2-hydroxyanthraquinone) was clearly mutagenic, and another major metabolite, 1-hydroxyanthraquinone, was carcinogenic in rats (Mori *et al*., 1990).

The mechanism by which anthraquinone can cause carcinogenicity is not clearly recognised. However, in male and female rats treated with anthraquinone, it has been noted in kidney and urinary bladder that there was an increased cell proliferation and cytotoxicity. An accumulation of hyaline droplets, which is indicator for nephropathy, in male rat kidney has been observed, with less severe effects in female rats (IARC working group, 2013).

There is some recent evidence that genotoxicity may play a role in the mechanism of action for anthraquinone-induced cancer (NTP, 2005).

4.3.4.2: Benzidine carcinogenicity scaffold:

The second non-child scaffold carcinogenicity within the fourth group was benzidine (see Figure 4.2) that was present in 24 compounds. The carcinogenicity rate for benzidine was 70% in both male and female rats.

Benzidine is known to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in humans (NTP, 2016a). All benzidine based azo dyes, 4,4'-diarylazobiphenyl

dyes, with the exception of those specified elsewhere in Annex I to Directive 67/548/EEC, are prohibited to be used in cosmetic product in Europe.

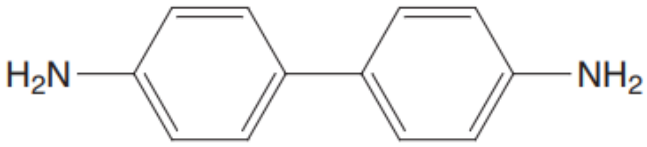


Figure 4.2. Chemical structure of benzidine

A strong relationship between the occupational exposure to benzidine and urinary bladder cancer has been reported (IARC, 2010). The association has been reported by different research workers in different labs, as well as by a number of epidemiological studies that include both case reports and cohort studies (IARC, 2010). Additionally, epidemiological studies have shown that when measures were taken to reduce exposure to benzidine in the workplace, there was a decrease in the number of cases of bladder cancer amongst the workers (IARC, 1972, 1982, 1987, 2010).

Although there are few studies on exposure to benzidine in occupational environments where workers were not exposed simultaneously to other types of chemicals, some studies indicate that increases in the percentage of urinary bladder cancer are associated with increased length of exposure to benzidine (IARC, 1982). There is limited and inconclusive evidence from other studies on the possibility of benzidine causing other types of cancer in tissues other than urinary bladder, such as cancers of liver, kidney, oral cavity, central nervous system**,** oesophagus, larynx, stomach and gall bladder (Choudhary, 1996). Benzidine was listed and reviewed by IARC as a carcinogen in the first annual report of the agency (IARC, 2010).

At the level of laboratory experiments on animals, there is sufficient evidence that there is a link between cancer and exposure to benzidine. The type of cancer is different depending on

the type of animal and the method of laboratory injection. For example, injection of animals with benzidine under the skin causes liver cancer in mice and Zymbal-gland tumours in rats. When the animals were exposed orally to benzidine, female rats were diagnosed with mammary gland cancer, liver cancer in rats and urinary bladder cancer in dogs. Rats injected with benzidine into the peritoneum membrane were diagnosed with Zymbal-gland tumours and mammary gland cancer (NTP, 2016a).

The mechanism of benzidine to cause cancer has been studied in detail. It includes formation of electrophilic compounds that bind through covalent bond to DNA after the metabolism of benzidine by cytochrome P450 (via N oxidation) (Choudhary, 1996). Benzidine also caused DNA mutations in various experimental test system both *in vivo* and *in vitro*. The exception to this was for cultured rodent cells where benzidine gave conflicting results, it causes DNA mutation in bacteria, plants, yeast, cultured human and rodents exposed *in vivo*. The damage caused by benzidine to DNA includes DNA strand break, micronucleus formation, chromosomal aberration and mitotic gene conversion (in yeast). In humans, according to the National Toxicology Program (NTP 2016a), the chromosomal aberration rate in white blood cells in workers that were exposed to benzidine or dyes that contain benzidine as main constituent was relatively higher than those who were not exposed.

4.3.4.3: Methyl naphthalene carcinogenicity scaffold:

The third non-child carcinogenicity scaffold was methyl naphthalene. Methyl naphthalene was present in 40 compounds as part of the structure. The carcinogenicity rate for methyl naphthalene was 80% and 100% in male and female rat respectively. The National Toxicology Program (NTP, 2000) concluded that there was clear evidence of carcinogenic activity of 1-methylnaphthalene in male and female F344/N rats based on increased incidences of respiratory epithelial adenoma and olfactory epithelial neuroblastoma of the nose. Nearly all rats in all exposure groups showed nonneoplastic nasal lesions in both olfactory and respiratory

epithelia, including atypical hyperplasia in olfactory epithelium, hyaline degeneration in olfactory and respiratory epithelia, and Bowman's gland hyperplasia (NTP, 2000).

4.3.4.4: Estradiol carcinogenicity scaffold :

The fourth non-child carcinogenicity scaffold was estradiol. Estradiol was present in 18 compounds for which the carcinogenicity rate was 80% in both male and female rats.

Studies have shown that the natural estradiol hormone, known as 17β-estradiol (E_2), can induce tumours in humans, including breast and uterine cancer (Patisaul and Jefferson, 2010). The risks of breast and uterine cancer are increased by either endogenous elevation of estradiol level, or by external sources that increase the estradiol level, such as oestrogen medication or cosmetic products that contain oestrogen as an ingredient or contaminant. Tumour induction by estradiol has been shown in different organs of rats, mice and hamsters (Liehr, 2000).

Compounds that mimic the estradiol hormone in both steroidal and non-steroidal forms have been found in different cosmetic products such as creams and shampoos (Kurzer and Xu, 1997). Most of these oestrogens are phytoestrogens derived from plants such as soya. Phytoestrogens have certain health benefits in terms of lowering the risk of heart disease, lowering the menopausal symptoms and osteoporosis in women. However, many of these compounds are also considered as endocrine disruptors and could be a possible source of adverse health effects. For those people who are using oestrogen containing compounds for long periods of time, or in case of children, these compounds may increase the risk of certain cancers due to increase in the endogenous oestrogens than the normal levels (Patisaul and Jefferson, 2010).

In September 1993, the US FDA issued a decision that all hormone containing products used on the skin or topically, and sold over the counter (OTC) without a prescription, are not fully safe and effective (Accessdata.fda.gov, 1993).

The estradiol mechanism of causing carcinogenicity is considered to be a non-genotoxic mechanism (epigenetic) as these compounds show negative results in mutagenicity tests in bacterial cells i.e. the Ames test and mammalian cell assays. It has been proposed that there is a dual role of oestrogen to induce carcinogenicity which includes the stimulation of cell proliferation and induction of genetic damage as a pro-carcinogen (Feigelson and Henderson, 1996). Oestrogen is metabolically converted by catalysation of 4-hydroxylase (CYP1B1) to 4-hydroxyestradiol and then further activated to yield a reactive intermediates semiquinone/quinone that can initiate tumours. All these substances, oestrogen, 4-hydroylestradiol and quinone induce DNA damage by a free radical mediated mechanism, directly and indirectly, and this has been shown both in *in vitro* or *in vivo* cell systems (Li and Li, 1990). Other chromosomal and genetic damage can also be caused by oestrogen which includes chromosomal aberration, microsatellite instability, gene amplification and aneuploidy in both *in vitro* and *in vivo* cell systems (Jackson, Chen and Loeb, 1998; Tsutsui and Barrett, 1997; Liehr, 2000).

Estradiol is classified as carcinogen based on biological studies in animal and epidemiological studies in humans. In a small group of animals, it was noticed that the tumours were induced after using pharmacological dose of estradiol. In humans, the risk of breast and uterine cancer was increased due to the elevation of circulating estradiol level caused either by elevation of endogenous production or by therapeutic medication (IARC,1999).

4.3.4.5: Methylbenzodioxole carcinogenicity scaffold :

The fifth non-child carcinogenicity scaffold was methyl benzodioxole. Methyl benzodioxole was present in 10 compounds with a carcinogenicity rate of 80% and 100% in male and female rat respectively. Examples of structures containing methyl benzodioxole scaffold are safrole and isosafrole (as shown in Figure 4.3).

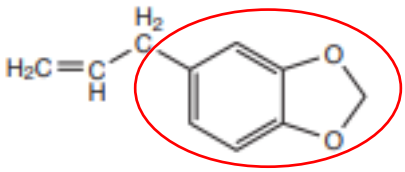


Figure 4.3. Isosafrole structure is an example of a positive carcinogenic compound in rat that contains methylbenzodioxole as the core structure

Safrole has been used in cosmetics such as soaps and perfumes, it also has been used as flavoring agent in drug manufacturing and as preservative in mucilages. In the food industry, oil of sassafras has been used as flavouring agent in some beverages such as root beer (IARC 1972, 1976; HSDB 2009). In 1960, the US FDA banned any use of safrole in food and listed it as a carcinogen in the Second Annual Report on Carcinogens. Liver tumours are reported to be caused by safrole by two different methods of administration in two rodent species. The administration of safrole through diet caused liver cancer in male mice and both rat sexes. Oral gavage of safrole followed by dietary exposure was shown to cause liver cancer in mice of both sexes. Infant male mice were shown to have liver cancer when injected with safrole subcutaneously (NTP, 2016b).

4.3.5: Major genotoxic and non-genotoxic scaffolds:

A molecular scaffold analysis was carried out to classify all 17 previously identified carcinogenicity scaffolds as to whether they are genotoxic or non-genotoxic using the structures within the Leadscope SAR Genetox Database. The Leadscope SAR Genetox Database comprises the results for 10,543 mutagenicity tests. In addition, the SAR Genetox Database provides mutagenicity test results for 46 genetic toxicity endpoints, which includes 32 bacterial mutagenicity, 4 *in vitro* mammalian, 5 *in vitro* chromosomal aberration and 6 *in vivo* micronucleus endpoints. The scaffold analysis of the SAR Genetox Database identified in 28 main mutagenicity scaffolds and 7 main non-mutagenicity scaffolds. The resulting scaffolds met the cut-off criteria - i.e. the C/S ratio greater than or equal to 0.7 and present in at least 10 compounds. For scaffolds associated with mutagenicity, the mutagenicity ratio criterion was set to be more than or equal to 0.7, whilst for non-mutagenicity scaffolds the mutagenicity ratio not to exceed 0.3 (in other word the non-mutagenicity ratio should be more than or equal to 0.7) as shown in Tables 4.7 and 4.8. The selection from those mutagenicity and non-mutagenicity scaffolds was only for those scaffolds that are cross-referenced with the previous 17 carcinogenicity scaffolds to confirm whether the carcinogenicity scaffolds were related to mutagenicity or non-mutagenicity pathways. If a scaffold (mutagenicity or non-mutagenicity) resulting from the SAR Genetox Database did not match one of the previous 17 carcinogenic scaffolds, then it was not added to the final list of scaffolds (genotoxic carcinogenic and non-genotoxic carcinogenic scaffolds). For those carcinogenic scaffolds that are not cross-referenced with mutagenic scaffolds, a further manual scaffold search within the SAR Genetox database experimental results was conducted (shown in Table 4.7). The cut-off in Leadscope was set at 70% as to whether it was related to genotoxicity or non-genotoxicity. The mutagenicity scaffold also had to cover at least 10 compounds, the minimum compounds number per one scaffold was assigned to 10 and the same number was assigned for the

minimum number of atoms per scaffold.

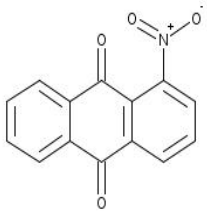
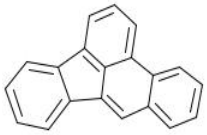
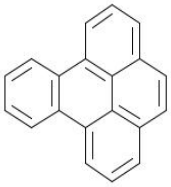
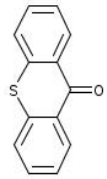
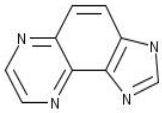
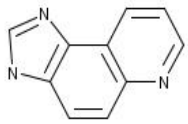
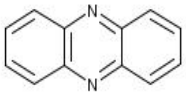
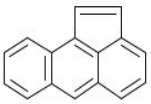
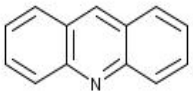
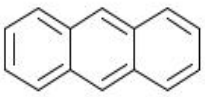
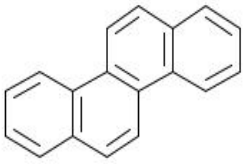
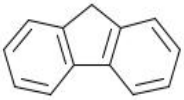
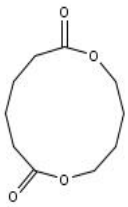
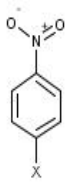
Of the 28 scaffolds associated with mutagenicity, five matched with carcinogenicity scaffolds group automatically by Leadscope. These were chrysene, 9H-fluorene, anthraquinone, 2-nitronaphthalene and phenanthrene. The mutagenicity C/S scores for these five scafolds were 0.96 out of 124 compounds for chrysene, 0.85 out of 72 compounds for 9H-fluorne, 0.86 out of 159 compounds for anthracene-9,10-dione, 0.96 out of 132 compounds for 2-nitronaphthalene and 0.95 out of 579 compounds for phenenethrene. This implied that these were scaffolds related to genotoxic carcinogenicity. The five mutagenic scaffolds that matched carcinogenicity scaffolds are highlited in blue in Table 4.7. The analysis showed 18 mutagenicity scaffolds did not overlap with carcinogenicity scaffolds but were highly significant as mutagenicity scaffolds. Conversely, the scaffold analysis for the absence of mutagenicity resulted in only seven non-mutagenic scaffolds. Only one carcinogenicity scaffold matched with this group, which was for estradiol, and that was also negative in the mutagenicity tests for all of 21 related compounds with a mutagenicity score of 0 as shown in Table 4.8. The previous scaffold analysis was performed in the Leadscope software in automatic mode which can miss some of the muagenicity scaffolds that may not be present in large enough number of compounds. Therefore, a manual check for mutagenicity scaffolds in the expermintal mutagenicity results was performed to further ensure that there was no missing scaffold that was not detected. The manual check resulted in six additional mutagenicity scaffolds that matched with the 17 carcinogenic scaffold list. These scaffolds are highlighted in yellow in Table 4.7. The scaffolds are 1-methyl naphthalene, tetraphene, 3a,5a-dihydropyrene, methylphenanthene, benzidine and a new additional mutagenic carcinogenicity scaffold - diethylaniline. The mutagenicty score for these scaffolds were 0.84 out of 992 compounds for 1-methylnaphthalene, 0.93 out of 139 compounds for tetraphene, 0.71 out of

21 compounds for 3a,5a-dihydropyrene, 0.87 out of 64 compounds for methylphenanthene, 0.93 out of 87 compounds for benzidine and 0.7 out of 100 compounds for diethylaniline.

4.4 : Conclusions:

The main findings and conclusions of this study showed that a total 17 molecular scaffolds associated with carcinogenicity could be identified from the available databases (Tables 4.2, 4.3, 4.4 and 4.5). Of these, 11 were genotoxic carcinogenicity related and included structures such as anthraquinone, phenanthrene, benzidine, 2- nitronaphthalene, 9H-fluorene, chrysene, diethylaniline, 1-methylnaphthalene, tetraphene, 3a,5a-dihydropyrene and methylphenanthrene. The single non-genotoxic carcinogenicity related scaffold that could be identified through this study was estradiol, whilst other six carcinogenicity scaffolds did not have supporting experimental data for mutagenicity. All of the children carcinogenicity scaffolds that were derived from the three major groups of parent carcinogenicity scaffolds were also related to carcinogenicity. The parent carcinogenic scaffolds were phenanthrene, 9H fluorene, 2-nitronaphthalene. The fourth scaffolds had no child scaffold. These findings will be useful in identifying the presence of any carcinogenicity related scaffold in a cosmetic ingredient or drug candidate compound and will provide the opportunity to replace them with a safer moiety at early stages of the lead optimisation and further development. A new scaffold based profiler which includes these 17 carcinogenic scaffolds as a starting point is suggested. This new suggested profiler should be in the form of molecular scaffolds since it is supported by animal data.

Table 4.7. The most significant 28 mutagenic scaffolds from the Leadscope SAR Genetox database (=scaffolds matched carcinogenic list and its mutagenicity score obtained by Automatic generation from the software), = scaffolds matched carcinogenic list and its mutagenicity score obtained by manual search through the SAR genotoxicity database)

Frequency: 10 C/S: Mutagen:1	Frequency: 16 C/S: Mutagen:1	Frequency: 23 C/S: Mutagen:1	Frequency: 11 C/S: Mutagen:1
 1-nitroanthracene 9,10-dione	 Dihydrobenzoacephenanthrylene	 Dihydrobenzo pyrene	 9H-thioxy-9-one
Frequency: 25 C/S: Mutagen:1	Frequency: 10 C/S: Mutagen:1	Frequency: 27 C/S: Mutagen:1	Frequency: 38 C/S: Mutagen:1
 imidazoquinoxaline	 imidazoquoline	 phenazine	 aceanthrylene
Frequency: 38 C/S: Mutagen:1	Frequency: 152 C/S: Mutagen:1	Frequency: 266 C/S: Mutagen: 0.9	Frequency: 124 C/S: Mutagen: 0.96
 acenaphthylene	 Acridine	 anthracene	 Chrysene
Frequency: 72 C/S: Mutagen:0.85	Frequency: 38 C/S: Mutagen:1	Frequency: 38 C/S: Mutagen:1	Frequency: 1129 C/S: Mutagen:0.8
 9H-fluorene	 1,4 dioxacycloudecane-5,11dione	 nitrobenzene	 Naphthalene

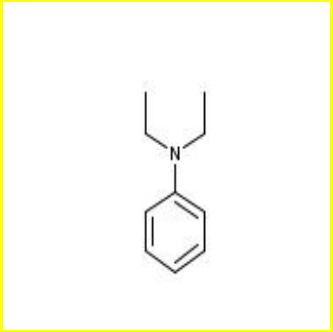
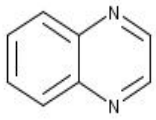
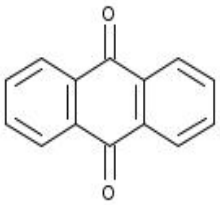
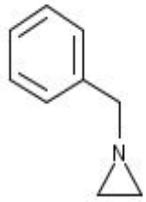
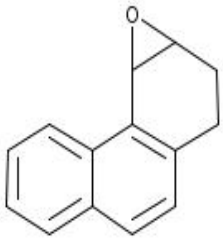
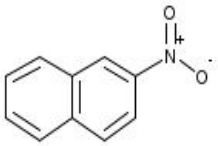
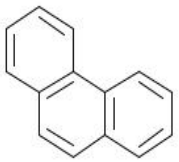
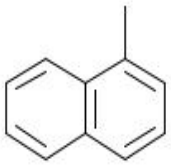
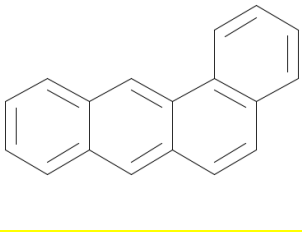
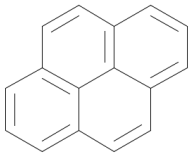
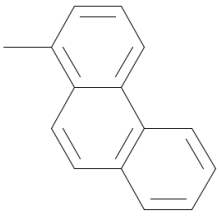
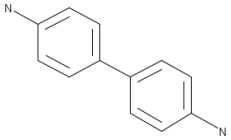
Frequency: 100 C/S: Mutagen:0.7 *C/S: Carcinogen: 0.7 of 17 substance	Frequency: 38 C/S: Mutagen:1	Frequency: 159 C/S: Mutagen:0.86	Frequency: 38 C/S: Mutagen:1
 diethylaniline	 quinoxaline	 anthraquinone	 1-benzylaziridine
Frequency: 159 C/S: Mutagen:0.86	Frequency: 132 C/S: Mutagen:0.96	Frequency: 579 C/S: Mutagen:0.95	Frequency: 992 C/S: Mutagen:0.85
 tetrahydophenanthrooxirene	 2-nitronaphthalene	 phenanthrene	 1-methyl naphthalene
Frequency: 139 C/S: Mutagen:0.93	Frequency: 21 C/S: Mutagen:0.71	Frequency: 64 C/S: Mutagen:0.87	Frequency: 87 C/S: Mutagen:0.93
 tetraphene	 3a,5a dihydropyrene	 methylphenanthene	 benzidine

Table 4.8. The most 7 significant non-mutagenic scaffolds from SAR Genetox database: (=scaffolds matched carcinogenic list and its mutagenicity score obtained by Automatic generation from the software)

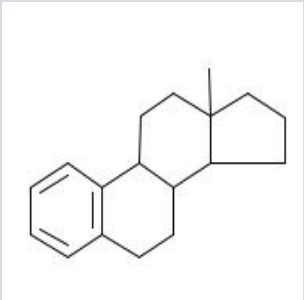
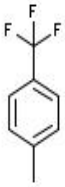
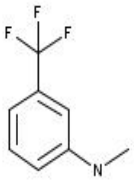
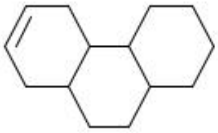
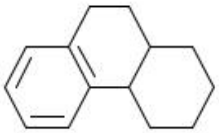
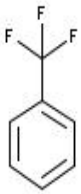
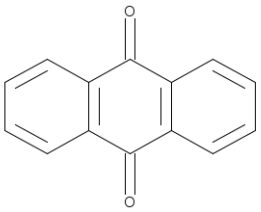
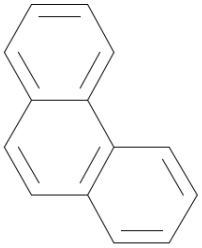
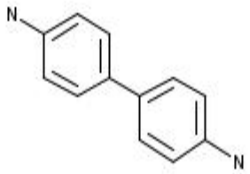
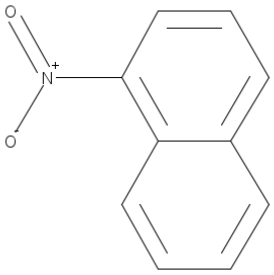
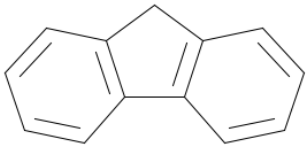
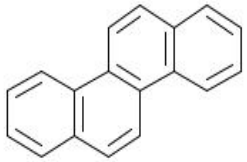
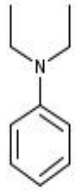
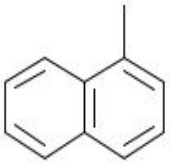
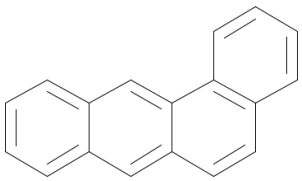
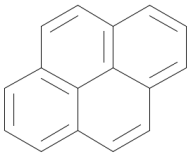
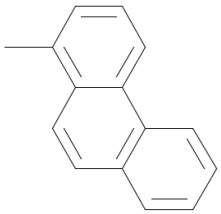
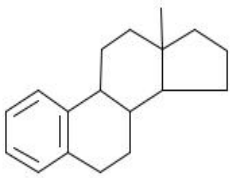
Frequency: 21 C/S: Mutagen: 0	Frequency: 38 C/S: Mutagen:0.1	Frequency: 12 C/S: Mutagen:0.1	Frequency: 38 C/S: Mutagen:0.86
 Estradiol) 3methyl-decahydro-cyclopenta-phenanthrene	 dimethylhexanediamine	 1-methyl-4(trifluromethyl)benzene	 N-methyl-3-(trifluromethyl) aniline
Frequency: 10 C/S: Mutagen:0.1	Frequency: 41 C/S: Mutagen:0.09	Frequency: 68 C/S: Mutagen:0.1	
 dodecahydrophenanthrene	 octahydophenanthrene	 Trifluromethyl benzene	

Table 4.9. The molecular scaffolds of the major genotoxic and non-genotoxic carcinogens groups

The scaffold structures of major genotoxic carcinogens scaffold groups		
 Anthraquinone	 phenanthrene	 benzidine
 2-nitronaphthalene	 9H-fluorene	 Chrysene
 diethylaniline	 1-methyl naphthalene	 tetraphene

 3a,5a-dihydropyrene	 methylphenanthene
The scaffold structures of major non-genotoxic carcinogens scaffold groups	
 Estradiol	

Chapter 5: Assessment of the Profilers Provided in the OECD QSAR Toolbox for Category Formation of Carcinogenic Chemicals

5.1 Introduction

In the previous chapters, several methods for searching and evaluating structural alerts for non-genotoxic carcinogens were reviewed. This chapter will focus on the performance of the profilers provided in the OECD QSAR Toolbox for category formation on the basis of the mutagenic, carcinogenic and skin sensitisation potential of chemical substances. In this regard, the use of *in silico* methods based on read-across (i.e. from the properties and effects of a group of structurally and/or functionally similar substances to an untested compound) will be discussed.

Under most regulatory frameworks, tests for carcinogenicity are only required when there is either a positive *in vitro* mutagenicity/gentoxicity test, or there are indications of carcinogenic effects from long-term *in vivo* tests. The ban on animal testing under the EU Cosmetic Regulation, and the absence of appropriate alternative methods, make it is likely that the potential carcinogenic effects of non-genotoxic carcinogens will not be the identified in the current risk assessment scheme. This is where *in silico* methods, including (Q)SAR modelling and read-across, can play a major role in relation to grouping/categorisation of chemicals to identify potential NGCs. The concept behind the grouping approach is based on the notion that 'similar substances usually have similar effects'. Based on the number of substances used for *read-across*, different terms are used to describe the process - e.g. a *category approach* is used when a large number of substances within a group has been used for *read-across*, whereas an *analogue approach* is used where limited number (usually one-to-one) of substances have been used (EChA, 2017). The grouping approach aims to identify substances that are similar in terms of chemical structure, and/or effects with (where possible) high quality data for a given physiochemical, toxicological, or environmental endpoint. At the physicochemical level, the

factors considered for grouping could be the similarities in chemical structure, functional group(s); (metabolic) degradation profiles, or other parameters such as log P, protein binding, etc. When sufficient 'similarity' criteria are met in a set of chemical substances that follow a regular pattern, it can be considered a 'category' of substances.

The European Chemicals “REACH” Regulation encourages the use of grouping and categorisation of chemicals for classification, risk assessment and labelling purposes. Some of the data gaps in this regard can be filled using read-across, which aims to interpolate experimental data from tests conducted on a one or more 'similar' substances termed as reference substances(s) or source substances(s) to other untested substances termed as target substances(s). The data for the endpoint in question for the target substance(s) are predicted using the experimental data for the same endpoint of the source substance(s). Since each endpoint has a different set of complexities, e.g. with regard to the biological target site or other key parameter(s), it is essential to consider read-across on an endpoint-by-endpoint basis (EChA, 2017).

There is a growing body of knowledge regarding how to undertake and report read-across assessment, with many learnings taken from well-established case studies (Ball *et al.* 2016; Schultz and Cronin 2017). There are several strong themes that run through the use of read-across for data gap filling. For instance, scientific justification and documentation for read-across is fundamentally required to strengthen its use in chemical grouping/categorisation. The structural or other similarities between the target and the source substances need to be described clearly to justify the read-across. Explanation of the rationale for the prediction of target substance properties/effects from the data on source substance(s) also needs to be provided for each specific endpoint. For a valid read-across for regulatory purposes, it is essential to provide accurate information on the substance identity and chemical composition (including impurity profile) to assist the evaluation of the similarities within the group of substances. The activity

or toxicity of substances may also differ for different forms or phases of substances (e.g. different valence, crystalline or particulate forms), and this requires evidence to show that the substance used in the read-across are representative of the structure/activity aspects used a basis for grouping, categorisation and risk assessment. In the first place, each substance used in read-across needs to have a specific chemical identity, as well as sufficient characterisation data in relation to purity/ impurity profiles to allow derivation of a meaningful read-across.

Furthermore, similarity(ies) between target and source substances are not necessarily represented by structural similarity alone. Other aspects of chemistry (and biology) also need to be assessed to build a robust read-across case. Such aspects include the presence of common functional groups, similarities in the core structure, bonding patterns, structural alerts for a particular activity, stereoisomerism, potential difference that may arise from steric hindrance or specific reactivity, etc (Cronin, 2013). It is also essential for a read-across argument that differences in structure and properties are defined and understood.

Certain physicochemical properties of both target and source substances that are relevant to the endpoint (e.g. log P, molecular weight, vapour pressure) also need to be clearly understood as they may play an important role in the read-across of a specific toxicological endpoint (EChA, 2013; Cronin, 2013). Chemical read-across is generally consolidated using data from all available sources, including from the scientific literature and predictive QSAR models. Information on the mode of action can be established from the mechanistic data or other methods (e.g. data from omics methods). In order to rationalise the recording of properties and effects, templates have been created for their storage that can act as a guide for developers as well as allowing for the easy assessment of a read-across (Schultz *et al.* 2015).

It needs to be highlighted that read-across is not only used for positive prediction of toxicity. The approach can also be used to predict the absence of toxicity, however a negative prediction may require more proof to support the level of confidence as compared to positive predictions.

The proof is required to ensure that the absence of toxicity of the source substances implies the absence of toxicity of the target substance(s). The reduction of uncertainties to allow for the prediction of "no or low" toxicity needs to be considered and addressed to avoid the potential underestimation of the positive toxicity of target substances. The use of read-across, along with other *in silico* methods, such as SAR/QSAR modelling, is also increasingly used in a "weight of evidence" approach that may further incorporate information from Adverse Outcome Pathways (AOPs). Thus, all elements relating to interaction of a chemical with the exposed biological system are included in the analysis. Schultz *et al.* (2017) demonstrated that increased evidence was required for the read-across of the toxicity of compounds – in this case n-alkanols – that were considered to be of low toxicity and utilising various sources of information could reduce uncertainty.

As the use of read-across has become more mainstream, the definition of 'similarity' has evolved to include physicochemical, structural, mechanistic and/or metabolic similarity (Schultz and Cronin, 2017). In order for read-across to be valid, a robust category of analogues must be derived from the available datasets. A valuable tool for achieving this is the OECD QSAR Toolbox, which is a freely available multifunctional platform that allows the users to make informed decisions about toxicity predictions for a range of (eco)toxicological endpoints (Schultz *et al.,* 2018). As part of the process of identifying a set of analogues for read-across, the Toolbox allows the user to apply structural alerts as in the form of computational "profilers". For many relevant toxicological endpoints, there are one or more profilers that are designed to aid this process – these may be based around chemistry e.g. covalent binding to DNA or be mechanistically or toxicologically derived. The target compound is first subjected to profiling, and then the profile(s) is used to screen for compounds in the databases with the same or similar mechanistic, toxicological and/or structural profiles. The analogues found this way are reduced

to those that have measured values for the specific endpoint(s) of interest and therefore provide a basis prediction of the endpoint value of the target compound.

The OECD QSAR Toolbox is widely used platform for chemical grouping/categorisation and estimation of chemical toxicity by *in silico* methods and read-across. Despite the increasing reliance of risk assessors on the Toolbox, attempts have only recently started to assess the reliability and limitations of the profilers provided in the system (Devillers *et al*., 2011; Mombelli, 2012; Yordanova *et al*., 2019). As part of the current study, it was felt that such an assessment of the profilers was necessary to understand the usefulness and limitations of their use in screening databases for analogous compounds that can be used subsequently for read-across or development of (Q)SAR models. The aim of this chapter, therefore, was to investigate the OECD QSAR Toolbox profilers for mutagenicity, carcinogenicity and skin sensitisation potential of chemical substances, in the context of how reliably they report both positive and negative compounds contained within the databases for these endpoints.

5.2: Materials and Methods

5.2.1 The OECD QSAR Toolbox

The OECD QSAR Toolbox (referred to herein as the "Toolbox") is a software application intended to be used to fill gaps in toxicity and ecotoxicity data needed for to assess the hazards of chemicals. The Toolbox incorporates databases on chemical data (e.g. properties), experimental toxicological and ecotoxicological data, estimated values from a large range of QSAR tools, together with incorporated QSAR models, built within an informatics chassis designed for regulatory application. The Toolbox therefore allows the user to perform a number of functions (OECD 2008):

- Identification of analogues for a chemical, retrieval of experimental results available for those analogues and data gap filling by read-across or trend analysis;
- Categorisation of large inventories of chemicals according to mechanisms or modes of action;
- Filling of data gaps for a chemical by using appropriate model(s) from the collection of QSAR models;
- Evaluation of the robustness of a potential analogue for read-across;
- Evaluation of the appropriateness of a (Q)SAR model for filling a data gap for a particular target chemical; and
- The capability of building QSAR models.

For this study version 3.1 of the Toolbox was used throughout. The version of the Toolbox used had been augmented with a number of extra publicly available databases (as detailed below).

5.2.2. Profilers

The following profilers in the Toolbox (version 3.1) were applied to the datasets described in Section 5.2.3.

5.2.2.1 Mutagenicity profilers

5.2.2.1.1. DNA binding by OASIS v1.1. This profiler is a mechanistic profiler developed from an analysis of Ames mutagenicity data. It contains a number of structural alerts that have been shown to be related to established electrophilic reaction chemistry known to be important in covalent DNA binding (Mekenyan *et al.* 2004; Serafimova *et al*. 2007).

5.2.2.1.2 DNA binding by OECD. This profiler is based on structural alerts for the electrophilic reaction chemistry associated with covalent DNA binding (Enoch and Cronin 2010). The profiler returns a range of structural alerts that contain electrophilic centres or those that can be metabolically activated to electrophiles.

5.2.2.1.3 Carcinogenicity (genotox and nongenotox) alerts by ISS. This profiler is based on a list of 55 structural alerts from the Toxtree software (http://toxtree.sourceforge.net/). About 20 of the alerts are for non-genotoxic carcinogenicity, and the remainder for genotoxic carcinogenicity (mutagenicity).

5.2.2.1.4 DNA alerts for AMES, MN and CA by OASIS v.1.1 is a refinement of 2.2.1.1 above.

5.2.2.1.5 *In vitro* mutagenicity (Ames test) alerts by ISS. The list of structural alerts is a subset of the original Toxtree list, obtained by eliminating the structural alerts for non-genotoxic carcinogenicity and is a refinement of 2.2.1.3 above.

5.2.2.1.6 *In vivo* mutagenicity (Micronucleus) alerts by ISS. This profiler is based on the ToxMic rule-base of the software Toxtree. This rule-base provides a list of 35 structural alerts (SAs) for a preliminary screening of potentially *in vivo* mutagens. These SAs are molecular functional groups or substructures that are known to be linked to the induction of effects in the *in vivo* micronucleus assay.

5.2.2.2 Carcinogenicity profilers

5.2.2.2.1. DNA binding by OASIS v1.1. As above.

5.2.2.2.2. DNA binding by OECD. As above.

5.2.2.2.3 Carcinogenicity (genotox and nongenotox) alerts by ISS. The SAs for carcinogenicity are molecular functional groups or substructures known to be linked to the carcinogenic activity of chemicals. As one or more SAs embedded in a molecular structure are recognised, the system flags the potential carcinogenicity of the chemical.

5.2.2.2.4. OncoLogic Primary Classifier. This profiler consists of molecular definitions derived by the Toolbox developers to mimic the structural criteria of chemical classes of potential carcinogens covered by the U.S. Environmental Protection Agency's OncoLogic™ Cancer Expert System for Predicting the Carcinogenicity Potential (www.epa.gov/oppt/sf/pubs/oncologic.htm).

5.2.2.3 Skin Sensitisation profilers

5.2.2.3.1 Protein binding by OASIS.

These profilers have been developed to indicative of skin sensitisation potential and consist of 85 structural alerts relating to 11 reactions, or chemical interactions, which are known to be associated with skin sensitisers.

5.2.2.3.2. Protein Binding by OECD

The protein binding by OECD profiler contains 16 mechanistic alerts covering 52 structural alerts. These data are supported by mechanistic chemistry and references to the scientific literature (the meta data). They represent a parallel approach to those of the OASIS profiler and capture mechanistic features of target compounds.

5.2.2.3.3 Protein binding potency

This profiler is developed on the basis of empirical data for thiol reactivity expressed by the *in chemico* RC50 value. All the chemicals have two common electrophilic mechanisms of interaction with GSH – interaction via S_N2 and interaction via Michael addition (MA) mechanism. The profiler contains 49 MA and 46 S_N2 categories

5.2.2.3.4 Keratinocyte gene expression

This profiler is built in relation to the database derived from the KeratinoSens assay, which examines the potential for chemicals to induce the expression of a luciferase reporter gene under control of a single copy of the ARE element of the human AKR1C2 gene stably inserted into immortalised human keratinocytes. Relevance to skin sensitisation is inferred from the

relationship of Keap1-Nrf2-ARE regulatory pathway and its detection of electrophilic chemicals to sensitisation. The profiler contains 22 categories.

5.2.2.3.5 Protein binding alerts for skin sensitisation by OASIS

This profiler seems to be much the same as the one at 2.2.3.1 above though there are some minor differences.

5.2.2.3.6 DPRA Lysine peptide depletion

This profiler is built on the basis of data derived from Direct Peptide Reactivity Assay (DPRA). The DPRA is a reactivity assay which evaluates the ability of chemicals to react with proteins. Model synthetic peptides containing either lysine or cysteine are used. The remaining concentration of cysteine- or lysine-containing peptide is measured after 24 hours incubation with the test chemical at 25±2.5ºC. The peptide reactivity is reported as percent peptide depletion. The relevance to skin sensitisation is the presence of cysteine and lysine residuals in the skin proteins.

The profiler contains 24 structural alerts extracted from about 110 chemicals with experimentally measured lysine depletion values.

5.2.2.3.7 DPRA Cysteine peptide depletion.

As described above, this profiler contains 32 categories of alerts.

5.2.3. Databases

A number of intrinsic Toolbox databases were examined using the profilers as well as some additional databases from publicly available sources. This involved running the compounds with known data and experimental values against the profilers and determining the hits. The following databases of experimental toxicity data were examined:

5.2.3.1 Bacterial mutagenicity ISSSTY

This database was donated to the OECD QSAR Toolbox by the Istituto Superiore di Sanità (ISS), Rome, Italy. The database comprises 41,634 Ames test data points for 7,367 compounds.

The overall endpoint value (positive, negative, equivocal, inconclusive) outcome is determined as described in the help file:

- Positive: at least one strain is positive (with or without metabolic activation);
- Equivocal: no strain is positive, and at least one equivocal result is present in one of the following strains (with or without metabolic activation): TA1535, TA100, TA98, TA1538, TA1535, TA97;
- Negative: no positive or equivocal results are present in any strain, and negative outcomes exist for: a) at least one strain from among TA1535 or TA100 or TA97 (with and without metabolic activation); and b) at least one strain from among TA1538 or TA98 or TA1537 (with and without metabolic activation);
- Inconclusive: If none of the above criteria are fulfilled. When more than one experiment in one strain was available, the number of reported positive and negative studies was counted, and the strain overall outcome was determined as follows: if the percentage of Positive studies is lower than 40 %, then outcome = Negative; if the percentage of Positive studies

is between 40 - 60 %, then outcome = Equivocal; if the percentage of Positive studies is higher than 60 %, then outcome = Positive.

In order to record the data in a binary (positive/negative) form to allow for easy analysis of the profilers' predictivity, only positive and negative outcomes were included. The equivocal outcomes, which were for 168 compounds, were excluded from the analysis.

The number of compounds which showed an overall positive outcome in all strains was 2,847 and the total number with negative outcomes was 4,352.

5.2.3.2 Carcinogenicity and mutagenicity (ISSCAN)

This database was also donated by the Istituto Superiore di Sanità (ISS), and comprises 6,979 data points for 1,150 compounds. There are three endpoints for which data are presented; gene mutation, summary carcinogenicity, and TD50. The TD50 data were not used in this current study. Gene mutation data in Ames are reported in the same way as in the previous database, and a single datum point is available for each of the 832 compounds. Summary carcinogenicity data are represented as "positive", "negative" or "equivocal". Positives are carcinogenic in at least one experimental group; equivocal results are given to chemicals with equivocal results in at least one experimental group, together with negative results in the other experimental groups, and negatives are non-carcinogenic in all tests. Only positive and negative carcinogenic compounds were included in the analysis. Equivocal carcinogenic outcomes were excluded from the analysis.

5.2.3.3 Genotoxicity OASIS

Donated by The Laboratory of Mathematical Chemistry, Bourgas, Bulgaria.

The OASIS Genotox Database contains 7,500 compounds collected from seven sources. It contains data for mutagenic determined by the Ames test with and without metabolic activation. The database also includes chromosomal aberrations determined by *in vitro* tests using Chinese hamster lung cells (CHL, with and without S9). Micronucleus (MN) and mouse lymphoma gene mutation assay (MLA) were evaluated by Chinese hamster lung cells (CHL / IU) and by *in vitro* T-lymphoma cell lines, respectively. All endpoints were evaluated on a dichotomic scale, i.e. yes (active) or no (inactive). Data used in this study were the Ames test data, and chromosomal aberration as the Toolbox has profilers for these endpoints.

5.2.3.4 CRD-AGES

Data from the UK Chemicals Regulatory Directorate and the Austrian Agency for Health and Food Safety, comprising 179 pesticides with mutagenicity data and 100 with carcinogenicity data (Worth *et al.* 2010). Mutagenicity data are binary active/inactive from Ames tests and carcinogenicity were also binary (active/inactive) from a range of tests.

5.2.3.5 DSS Pesticide carcinogenicity

Summary carcinogenicity data for 1,282 pesticide compounds from the US EPA National Center for Computational Toxicology, (http://www.epa.gov/ncct/dsstox/index.html).

5.2.3.6 SAR carcinogenicity and genotoxicity databases:

These are high-quality data resources that can be used for building a predictive model, and for the 'read-across' of chemicals to find out their potential to be carcinogenic or mutagenic. Both databases contain summarised *in vitro* and *in vivo* cancer and mutagenicity endpoints along with chemical structures. These databases are used by Leadscope program to build predictive models. To ensure the high quality data for SAR, structure form, analysis of various salt forms of chemical compounds and their respective toxicity data have also been carried out to derive an overall endpoint for the active portion of the chemical. Several sources of experimental test results have been included in these databases, such as from FDA, NTP, CCRIS, CPDB and other primary sources. All chemical structures have been provided in SAR, neutral and tested form, and confirmed for accuracy.

The SAR carcinogenicity database includes 3,598 compounds with 11,538 test results and provides carcinogenicity study endpoint for male and female rats (1,774 and 1,725 compounds respectively) and male and female mice (1,640 and 1,675 compounds respectively). The SAR genotoxicity database provides compound-level calls for 46 genetic toxicity endpoints for 10,534 compounds. These include 32 bacterial mutagenicity endpoints, four *in vitro* mammalian, five *in vitro* chromosomal aberration and six *in vivo* micronucleus results.

5.2.3.7 EFSA Pesticides Mutagenicity

Ames test active/inactive classifications for 741 pesticides, compiled by, and downloaded from the European Food Safety Authority.

5.2.3.8. NISS mutagenicity database

A database of 1,863 compounds with binary active/inactive Ames test data. Downloaded from the US National Institute of Statistical Sciences, (https://www.niss.org/)

5.2.3.9. Inchemicotox Skin Sensitisation

A version of the Cronin and Basketter dataset (Cronin and Basketter 1994) from the Inchemicotox project (http://www.inchemicotox.org/), comprising 322 compounds, with results taken from the Guinea Pig Maximisation Test. The classification is derived from the percentage of animals sensitised in the test: non-sensitiser = 0-9%, weak sensitiser = 10-29%, moderate sensitiser = 30-79%, strong sensitiser = 80-100%.

In order to record the data in a binary (senstiser/non-senstiser) form to allow for easy analysis of the profilers' predictivity, only strong senstiser (80-100%) and non-senstiser (0-9%) outcomes were included. The weak and moderate senstiser outcomes were excluded from the analysis. The number of compounds which were classified as strong senstisers was 200 and as non-senstisers was 122.

5.2.3.10. CAESAR Skin Sensitisation

209 compounds from the EU CAESAR project (www.caesar-project.eu). For developing classification models, this data set was subdivided in two classes, sensitiser (S) and non-sensitisers (N), which gave a good distribution of the numbers of compounds in each class. The class S merges the first four ranges established by ECETOC: Extreme (EC3<0.1%), Strong (0.1%<EC3<1%), and Moderate (1%<EC3<10%) and Weak (EC3>10%) ranges; the class N regroups all compounds belonging to the non-sensitisers.

5.2.3.11. ECETOC Skin sensitisation

39 compounds with experimental results on skin and respiratory sensitisation. The compounds were selected as known sensitisers and non-sensitisers for the assessment of novel test techniques (http://www.ecetoc.org/technical-reports).

5.2.3.12 OECD Skin Sensitisation

1,036 compounds from two databases and includes chemicals tested by Local Lymph Node Assay (LLNA) or Guinea Pig Maximisation Test (GPMT). Based on the observed skin sensitisation effect the chemicals are classified in three classes: - strong sensitisers, weak sensitisers or non-sensitisers.

In order to record the data in a binary (senstiser/non-senstiser) form to allow for easy analysis of the profilers' predictivity, only strong senstiser and non-senstiser (0-9%) outcomes were included. The weak outcomes were excluded from the analysis. The number of compounds which were classified as strong senstisers was 430 and as non-senstisers was 488.

5.2.3.13 Lazar Opentox Rat Carcinogenicity

Lazy Structure-Activity Relationships (LAZAR) is an open-source tool for the prediction of complex toxicological endpoints such as carcinogenicity (female/male, hamster/ mouse/rat/rodent) and *Salmonella* mutagenicity. The compounds were selected from database of experimental toxicity data. Carcinogenicity models are based on the CPDB, while the *Salmonella* mutagenicity model uses a dataset of 3,895 compounds determined *in vitro* (https://lazar.in-silico.de/predict).

5.2.3.14 VEGA carcinogenicity

The VEGA platform serves to access a number of QSAR models for predicting mutagenicity and carcinogenicity. The compounds were selected from a set of 4,225 molecules tested in the Ames bacterial test and for carcinogenicity compounds were selected from a set of 805 chemicals from the Carcinogenic Potency Database (CPDB).

5.2.3.15 The Carcinogenic Potency Database (CPDB):

Data relating to cancer causing chemicals were compiled from the Carcinogenic Potency Database (CPDB), which is freely available from (http://toxnet.nlm.nih.gov/cpdb/cpdb.html). This database is a widely used and unique international resource comprising the results of 6,540 chronic, long-term animal carcinogenicity tests on 1,547 chemicals in rats, mice, dogs, hamsters and non-human primates.

5.2.4 Data analysis

The compounds in the databases (Section 5.2.3) were profiled using the appropriate profilers (Section 5.2.2).

For each compound, if any alert was triggered, the compound was allocated a score of 1, if no alerts were triggered, a score of 0 was allocated. The results were compared with the assigned binary activities from the original database (positive=1; negative=0).

Cooper statistics (Cooper *et al.,* 1979) were used to assess the results against the experimental values given in the databases, by calculating the sensitivity, specificity and accuracy of the alert triggers as follows:

Sensitivity (True positive rate) = TP/ TP+FN

Specificity (True negative rate) = TN/TN+FP

Accuracy = (TN+TP)/(TN+FP+FN+TP)

MCC = (TPxTN)-(FPxFN)/√(TP+FN)(TP+FP)(TN+FN)(TN+FP)

PPV (Positive predictive value) or (precision) = TP/ TP+FP

Where TP=True positive, TN=True negative, FP=False positive, FN=False negative

Sensitivity is defined as the percentage of correctly classified positive predictions among the total number of positive instances.

Specificity is the percentage of correct negative predictions compared to the total number of negatives.

Accuracy (concordance or "Q") is defined as the total number both positive and negatives correctly predicted among the total number of compounds.

PPV (positive predictive value) is defined as the total number of correctly classified positive predictions among the total number of both negative and positive instance.

MCC (Matthews correlation coefficient) is a weighted value that overcomes any imbalance in the data classes which might lead to over optimistic values of Q (Matthews, 1975). An *MCC* value of 1 indicates that the model can predict the data classes of unknown compounds perfectly, whilst a *MCC* value of 0 indicates that the predictions are no better than random guessing, and a *MCC* value of -1 indicates total disagreement between the predicted data and the actual data.

5.3: Results and discussion:

The results of the assessment of the profilers against the experimental data for mutagenicity, carcinogenicity and skin sensitisation are shown in Tables 5.1, 5.2 and 5.3 respectively. Further detailed analysis was undertaken to identify over-predictive structural alerts in the carcinogenicity profilers i.e. the Precision or PPV (positive predictive value) is lower than 0.5. This analysis was performed to determine structural alerts with little information or predictive capability to increase the sensitivity and overall accuracy of the profiler. Detailed analysis of 13 non-genotoxic carcinogenicity structural alert was conducted and is presented in Table 5.4.

An additional analysis for the Oncologic Primary Classification carcinogenicity profiler was performed for 30 structural alerts included in the profiler. The purpose of this analysis was to assess which structural alerts had a precision PPV (positive predictive value) lower than 0.5. This analysis is presented in Table 5.5.

The cut-off for value was set to be 0.5. This was to ensure that none of these profilers will have lower predictivity power as compared to Ames test. It is known that the Ames Test has been applied to predict rodent carcinogenicity. The high predictive power of a positive Ames ranges from 77% to 90% depending on the various factors. This makes it superior to any other *in vitro* genotoxicity assay, all of which have lower performance in terms of predicting genotoxicity (Kazius *et al*., 2006).

Table 5.1. Cooper statistics for the mutagenicity profilers in the Toolbox assessed against databases.

Profiler	DNA binding by OASIS v.1.1	DNA binding by OECD	genotox and nongenotox alerts by ISS	DNA alerts for AMES, MN and CA by OASIS	Ames test alerts by ISS	Micronucleus alerts by ISS
Bacterial mutagenicity (ISSSTY)						
Sensitivity	0.76	0.76	0.87	0.64	0.84	0.95
Specificity	0.65	0.59	0.55	0.77	0.63	0.28
Accuracy (Q)	0.69	0.65	0.67	0.72	0.71	0.53
MCC	0.56	0.45	0.56	0.62	0.66	0.32
Micronucleus (OASIS)						
Sensitivity	0.56	0.58	0.69	0.41	0.58	0.89
Specificity	0.58	0.53	0.42	0.71	0.57	0.18
Accuracy (Q)	0.57	0.55	0.55	0.57	0.57	0.51
MCC	0.16	0.12	0.12	0.13	0.16	0.10
Micronucleus (ISSSTY)						
Sensitivity	0.51	0.54	0.61	0.36	0.50	0.78
Specificity	0.62	0.55	0.50	0.79	0.68	0.21
Accuracy (Q)	0.56	0.54	0.56	0.54	0.58	0.54
MCC	0.13	0.08	0.11	0.16	0.18	-0.01
Genotox OASIS (Ames)						
Sensitivity	0.76	0.74	0.85	0.62	0.82	0.95
Specificity	0.63	0.56	0.52	0.78	0.62	0.22
Accuracy (Q)	0.70	0.65	0.69	0.70	0.72	0.58
MCC	0.51	0.38	0.50	0.53	0.59	0.27
Genotox (OASIS CA)						
Sensitivity	0.59	0.62	0.70	0.44	0.58	0.83
Specificity	0.66	0.61	0.50	0.80	0.66	0.28
Accuracy (Q)	0.62	0.61	0.61	0.61	0.62	0.57
MCC	0.28	0.25	0.22	0.28	0.26	0.13
Mutagenicity (ISSCAN)						
Sensitivity	0.80	0.78	0.86	0.62	0.82	0.95
Specificity	0.68	0.61	0.54	0.83	0.72	0.28
Accuracy (Q)	0.74	0.69	0.69	0.73	0.76	0.59
MCC	0.48	0.40	0.41	0.46	0.54	0.30
SAR Gentox Database						
Sensitivity	0.70	0.70	0.83	0.51	0.81	0.90
Specificity	0.69	0.55	0.57	0.85	0.66	0.33
Accuracy (Q)	0.72	0.63	0.70	0.86	0.74	0.62
MCC	0.39	0.26	0.41	0.38	0.48	0.27

Sensitivity = True positive rate; Specificity = True negative rate; MCC = Matthews Correlation Coefficient,

= Specificity below 0.5

Table 5.2. Cooper statistics for carcinogenicity profilers in the Toolbox assessed against databases.

Profiler	DNA binding by OASIS v.1.1	DNA binding by OECD	Carcinogenicity (genotox and nongenotox) alerts by ISS	Oncologic Primary Classification
DSS Pesticide Carcinogenicity				
Sensitivity	0.64	0.65	0.80	0.73
Specificity	0.60	0.54	0.54	0.44
Accuracy (Q)	0.62	0.60	0.68	0.60
MCC	0.24	0.19	0.36	0.18
CRD-AGES Carcinogenicity				
Sensitivity	0.45	0.42	0.68	0.66
Specificity	0.67	0.58	0.49	0.30
Accuracy (Q)	0.58	0.52	0.57	0.44
MCC	0.12	0.00	0.17	-0.05
VEGA Carcinogenicity				
Sensitivity	0.64	0.64	0.81	0.72
Specificity	0.57	0.54	0.52	0.42
Accuracy (Q)	0.61	0.59	0.68	0.59
MCC	0.22	0.18	0.34	0.15
ISSCAN Carcinogenicity				
Sensitivity	0.62	0.62	0.79	0.73
Specificity	0.58	0.52	0.51	0.38
Accuracy (Q)	0.60	0.57	0.64	0.54
MCC	0.20	0.14	0.31	0.11
CPDB Carcinogenicity				
Sensitivity	0.60	0.63	0.78	0.73
Specificity	0.61	0.55	0.53	0.43
Accuracy (Q)	0.61	0.58	0.64	0.57
MCC	0.21	0.18	0.31	0.16
LAZAR Carcinogenicity				
Sensitivity	0.63	0.65	0.76	0.70
Specificity	0.65	0.55	0.57	0.46
Accuracy (Q)	0.64	0.60	0.67	0.59
MCC	0.28	0.20	0.34	0.17
SAR carcinogenicity database				
Sensitivity	0.59	0.65	0.75	0.70
Specificity	0.56	0.51	0.51	0.41
Accuracy (Q)	0.58	0.60	0.67	0.60
MCC	0.14	0.15	0.26	0.10

Sensitivity = True positive rate; Specificity = True negative rate; MCC = Matthews Correlation Coefficient

= Specificity below 0.5

Table 5.3. Cooper statistics for skin sensitisation profilers in the Toolbox assessed against databases.

Profiler	Protein binding OASIS	DPRA Lysine peptide depletion	Keratinocyte gene expression	Protein binding potency	Protein binding by OECD	DPRA Cysteine peptide depletion	Protein binding alerts OASIS v1.1
Inchemitox skin sensitisation							
Sensitivity	0.53	0.12	0.19	0.12	0.47	0.22	0.50
Specificity	0.74	0.94	0.89	0.93	0.79	0.91	0.86
Accuracy (Q)	0.61	0.43	0.45	0.42	0.59	0.48	0.64
MCC	0.27	0.10	0.10	0.08	0.25	0.17	0.36
CAESAR							
Sensitivity	0.72	0.30	0.33	0.19	0.68	0.48	0.71
Specificity	0.36	0.77	0.73	0.90	0.46	0.55	0.40
Accuracy (Q)	0.54	0.54	0.54	0.55	0.56	0.52	0.55
MCC	0.09	0.08	0.07	0.12	0.14	0.03	0.11
ECETOC							
Sensitivity	0.60	0.30	0.43	0.20	0.63	0.37	0.60
Specificity	0.80	0.80	0.80	1.00	0.80	0.80	0.80
Accuracy (Q)	0.65	0.43	0.53	0.40	0.68	0.48	0.65
MCC	0.35	0.10	0.21	0.24	0.38	0.15	0.35
OECD Skin sensitisation							
Sensitivity	0.46	0.29	0.31	0.29	0.44	0.33	0.45
Specificity	0.83	0.86	0.85	0.86	0.83	0.85	0.84
Accuracy (Q)	0.67	0.58	0.59	0.58	0.66	0.61	0.68
MCC	0.31	0.18	0.19	0.17	0.30	0.21	0.32

Sensitivity = True positive rate; Specificity = True negative rate; MCC = Matthews Correlation Coefficient

= Specificity below 0.5

Table 5.4. PPV (positive predictive value) analysis for 13 structural alerts for non-genotoxic carcinogenicity included in carcinogenicity alert profiler by ISS in 7 carcinogenicity databases. = PPV lower than 0.5 and need to be improved or omitted

		TRUE POSITIVE							FALSE POSITIVE												
Non-genotoxic carcinogen structural alert	Alert	VEGA	LAZAR	ISSCAN	DDS	CPDB	CRDAG	SAR	VEGA	LAZAR	ISSCAN	DDS	CPDB	CRDAG	SAR	TOTAL FP		TOTAL TP	TOTAL	PPV	SUG
	PHCA	13	9	19	11	17	0	2	3	0	11	0	7	0	5	26		51	77.0	0.66	K
	BENZD	5	3	7	4	7	0	4	3	0	5	0	4	1	2	15		23	38.0	0.61	K
	TCE	5	4	16	5	10	0	4	1	0	1	0	1	0	1	4		35	39.0	0.90	K
	THCAR	10	1	15	8	11	0	8	8	0	20	0	27	0	7	62		44	106.0	0.42	I
	HALBN	10	10	2	10	4	5	13	15	0	0	0	0	6	8	29		34	63.0	0.54	K
	HALDB	1	2	6	1	4	0	3	1	0	1	0	0	0	1	3		14	17.0	0.82	K
	SACA	3	4	4	5	4	0	4	4	0	9	0	5	0	3	21		15	36.0	0.42	I
	QUERC	1	0	2	1	1	0	1	0	0	4	0	3	0	1	8		5	13.0	0.38	I
	PHTAL	1	2	2	1	3	0	1	1	0	5	0	3	0	2	11		7	18.0	0.39	I
	STERO	4	1	5	0	7	0	8	0	0	1	0	6	0	1	8		24	32.0	0.75	K
	IMIDA	5	3	5	4	6	0	11	6	0	12	0	8	0	2	28		27	55.0	0.49	K
	BENSU	2	2	4	2	4	0	5	0	0	3	0	5	0	1	9		15	24.0	0.63	K
	ALKHL	7	7	13	7	10	1	1	3	0	7	0	6	0	4	20		32	52.0	0.62	K
	TOTAL	67	48	100	59	88	6	65	45	0	79	0	75	7	38	244		326	570.0	0.57	

PHCA = (Poly)Halogenated Cycloalkanes, BENZD = 1,3-Benzodioxoles, TCE =Trichloro (or fluoro) ethylene and Tetrachloro (or fluoro) ethylene , THCAR= Thiocarbonyl , HALBN= Halogenated benzene , HALDB = Halogenated Dibenzodioxone , SACA = Substituted n-alkylcarboxylic acids , QUERC = Quercetin type flavonoids , PHTAL= Phthalate (or butyl) diesters and monoesters , STERO =Steroidal estrogens IMIDA = Imidazole, benzimidazole, BENSU = Benzenesulfonic ethers, ALKHL= Alkyl halides ,SUG = suggestion, I = need to Improve or omit , K= Keep .

Table 5.5. PPV analysis for 13 structural alerts for non-genotoxic carcinogenicity included in carcinogenicity alert profiler by ISS in 7 carcinogenicity databases. = PPV lower than 0.5 and need to be improved or omitted.

	ALERT	TOTAL	TOTAL FP	TOTAL TP	ppv	SUGGESTION
Non-genotoxic carcinogen structural alert	(Poly)Halogenated Cycloalkanes	77.0	26	51	0.66	Keep
	1,3-Benzodioxoles	38.0	15	23	0.61	Keep
	Trichloro (or fluoro) ethylene and Tetrachloro (or fluoro) ethylene	39.0	4	35	0.90	Keep
	Thiocarbonyl	106.0	62	44	0.42	Improve or omit
	Halogenated benzene	63.0	29	34	0.54	Keep
	Halogenated Dibenzodioxone	17.0	3	14	0.82	Keep
	Substituted n-alkylcarboxylic acids	36.0	21	15	0.42	Improve or omit
	Quercetin type flavonoids	13.0	8	5	0.38	Improve or omit
	Phthalate (or butyl) diesters and monoesters	18.0	11	7	0.39	Improve or omit
	Steroidal estrogens	32.0	8	24	0.75	Keep
	Imidazole, benzimidazole	55.0	28	27	0.49	Keep
	Benzenesulfonic ethers	24.0	9	15	0.63	Keep
	Alkyl halides	52.0	20	32	0.62	Keep
	TOTAL	570.0	244	326	0.57	
PPV after ignoring suggested structural alerts						0.64

Table 5.6. PPV analysis of each structural alert in oncologic carcinogenicity profiler through 7 carcinogenicity databases. = PPV lower than 0.5 and need to be improved or omitted

	ALERT	TOTAL	TOTAL TP	TOTAL FP	PPV	SUGGESTION
Oncologic Primary Classification	Acrylamide Reactive Functional Groups	25	18	9	0.72	Keep
	Acrylate Reactive Functional Groups	50	35	15	0.70	Keep
	Aldehyde Type Compounds	138	80	58	0.58	Keep
	Alkanesulfonoxy Ester Type Compounds	6	3	3	0.50	Keep
	Alkyl Sulfate and Alkyl Alkanesulfonate Type Compounds	28	16	12	0.57	Keep
	Alpha, beta-Haloether Reactive Functional Groups	131	65	66	0.50	Keep
	Aromatic Amine Type Compounds	976	565	413	0.58	Keep
	Arylazo Type Compound	0	0	0	0.00	Keep
	Carbamate Type Compounds	63	22	43	0.35	Improve or omit
	C-Nitroso and Oxime Type Compounds	17	12	5	0.71	Keep
	Coumarine and Furocoumarin Type Compounds|Lactone Type Reactive Functional Groups	3	3	0	1.00	Keep
	Epoxide Reactive Functional Groups	99	63	37	0.64	Keep
	Ethyleneimine Reactive Functional Groups	6	6	0	1.00	Keep
	Halogenated Linear Aliphatic Type Compounds	289	193	96	0.67	Keep
	Halogenated Aromatic Hydrocarbon Type Compounds	241	111	139	0.46	Keep
	Lactone Type Reactive Functional Groups	26	14	12	0.54	Keep
	Nitroalkane and Nitroalkene Type Compounds	52	26	26	0.50	Keep
	Nitrogen Mustards Reactive Functional Groups	18	13	5	0.72	Keep
	Nitrosamine Type Compounds	337	266	71	0.79	Keep
	Nitrosamide Type Compounds	3	3	0	1.00	Keep
	Organophosphorus Type Compounds	126	47	80	0.37	Improve or omit
	Peroxide Type Compounds	11	3	8	0.27	Improve or omit
	Phenol Type Compounds	276	117	159	0.42	Keep
	Polycyclic Aromatic Hydrocarbons - Homocyclic	10	9	1	0.90	Keep
	Reactive Ketone Reactive Functional Groups	21	2	19	0.10	Improve or omit
	Sulfur Mustard Reactive Functional Groups	2	2	0	1.00	Keep
	Sultone Reactive Functional Groups	4	3	1	0.75	Keep
	Thiocarbonyl Type Compounds	56	36	20	0.64	Keep
	Triazene Type Compounds	3	3	0	1.00	Keep
	Urea Type Compounds	10	3	7	0.30	Keep
	TOTAL	3027	1739	1305	0.57	
PPV after ignoring suggested structural alerts						0.593

5.3.1 Mutagenicity profilers

The accuracy (percentage of positives and negatives correctly predicted) of the mutagenicity profilers varies across the datasets from 51% to 76%. Clearly 51% is barely better than chance, whereas 76% is more acceptable because it is in line with the level of error generally seen in measured data in most databases. The micronucleus alerts appear to be general and, as such, significantly over-predicts mutagenicity. The most common alert triggered by this profiler is "Hacceptor-path3-Hacceptor". This alert indicates the non-covalent binding of the target chemical to DNA via two bonded atoms connecting two H bond acceptors (Snyder *et al*. 2006). However, it appears that such a functional group is common in both mutagens and non-mutagens. It is likely that the performance of this profiler would improve if this specific alert was omitted.

As expected, both DNA binding profilers work best with the data obtained from Ames type tests, and do not perform well for chromosome abnormality or micronucleus data.

The genotoxicity and non-genotoxicity alerts (ISS) have acceptable true positive results but fail to distinguish the negatives. Overall, these perform best with Ames type data.

The OASIS DNA alerts for Ames, micronucleus and chromosomal aberration predict the results in Ames datasets fairly well but for both micronucleus data and chromosomal aberration data, these profilers under-predict positive compounds with sensitivity rates ranging from 36-44%. The ISS Ames test alerts have accuracies over 70% for Ames datasets and MCC values greater than 0.5 indicate that the performance is independent of skewed sample categories. It, however, needs to be noted that the micronucleus/ CA alerts may not be suitable predictors of Ames. They need to be considered separately and used, along with Ames, to develop the overall weight of evidence.

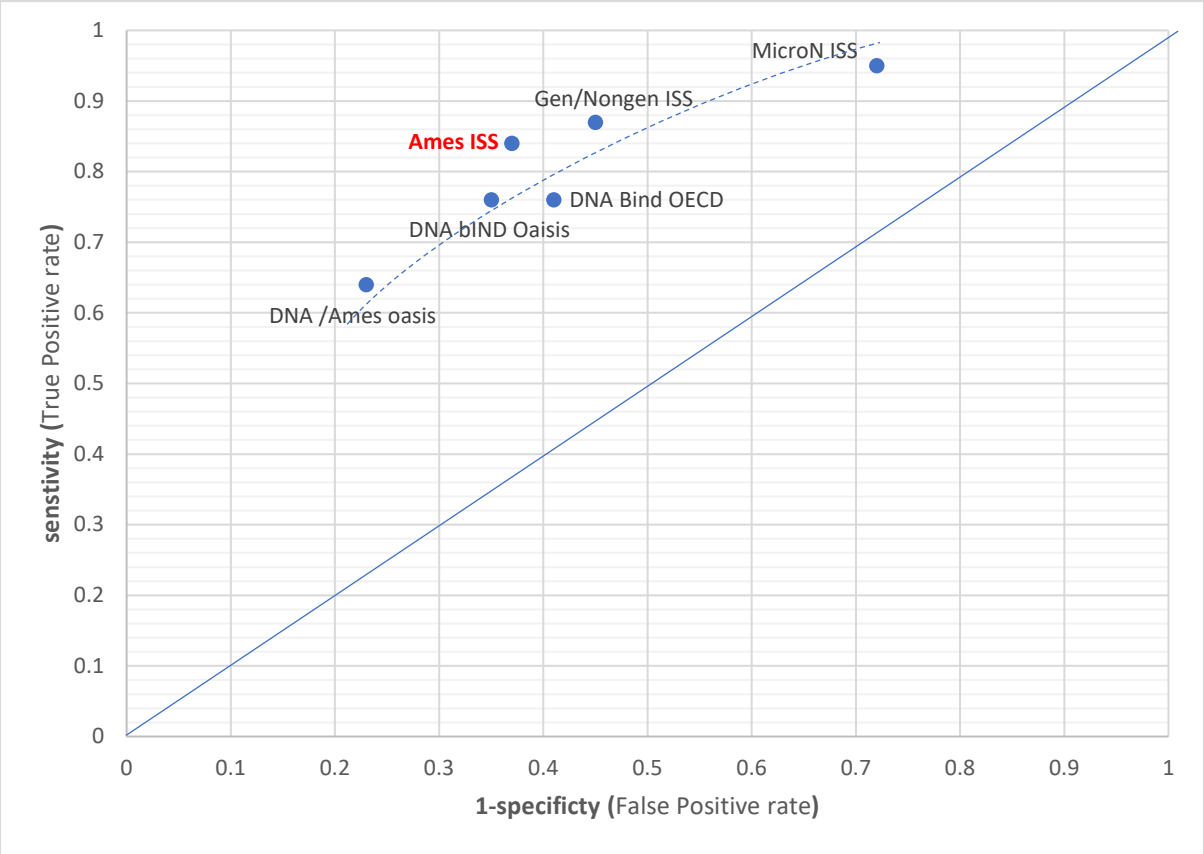


Figure 5.1. Receiver operating characteristic (ROC) curve analysis for mutagenicity profilers in the OECD QSAR Toolbox

The receiver operating characteristic (ROC) curve, which is defined as a plot of test sensitivity as the y coordinate versus its 1-specificity or false positive rate (FPR) as the x coordinate, is an effective method of evaluating the quality or performance of diagnostic tests. Figure 5.1 shows the ROC curve analysis for the in silico profilers. The ROC curve shows that Ames ISS profiler was the most efficient profiler that achieved the highest balanced accuracy with both high true positive rate and low false positive rate values.

5.3.2 Carcinogenicity profilers

Both DNA binding profilers perform equally poorly for carcinogens and non-carcinogens from all datasets, with accuracy values rarely above 60% and, MCC values indicating a performance barely better than chance.

The ISS carcinogenicity alerts fare a little better in predicting carcinogens, but a poor segregation of the non-carcinogens reduces the overall effectiveness of this profiler with accuracy levels between 57% and 68% for the sample datasets and modest to poor performance on skewed datasets as indicated by the MCC values of 0.17 to 0.36. The ROC analysis shown in Figure 5.2 indicates that the ISS carcinogenicity profiler preformed the as compared to the other three profilers in terms of both true positive rate and false positive rate.

As discussed in Chapter 2, genotoxic carcinogenicity structural alerts are more accuracy than non-genotoxic carcinogenic structural alerts. Based on that, 13 non-genotoxic structural alerts among ISS carcinogenicity profiler were analysed individually to test their performance in PPV (positive predictive value). As shown in Table 5.4, the overall PPV of ISS non-genotoxic carcinogen structural alerts was 57% where 326 substances are correctly predicted as non-genotoxic carcinogens out of total 570 substances that were detected to contain one of the 13 NGC structural alerts.

The precision value (PPV) for non-genotoxic carcinogenicity structural alerts ranged from 0.92 for (Trichloro (or fluoro) ethylene and Tetrachloro (or fluoro) ethylene) as the highest PPV to 0.39 for Quercetin type flavonoids. Four out of 13 NGC structural alerts seem to show over-prediction. These are thiocarbonyl, substituted n-alkylcarboxylic acids, quercetin type flavonoids, and phthalate (or butyl) diesters and monoesters. All four of these structural alerts predict non-carcinogenic substances as carcinogens in more than 50% of the total substances

that contain this structural alert, which as a result lowers the total accuracy of the ISS carcinogenicity profiler.

The thiocarbonyl NGC structural alert was flagged in 106 substances as the only structural alert. Any substance that contained more than one NGC structural alerts was not counted in this analysis to avoid any interference. Sixty-two non-carcinogenic substances were falsely predicted as carcinogenic substances by the thiocarbonyl structural alert with a sensitivity rate of 0.42. Due to this over-prediction of the thiocarbonyl alert, it can be suggested that ignoring this alert could increase sensitivity of the total NGC structural alerts and the ISS carcinogenicity profiler. This would, however, not be the ideal solution, as any thiocarbonyl NGCs would be completely out of the scope of the profiler. Instead, it is proposed that further research be carried out to see whether performance of this alert can be improved using a larger database of thiocarbonyl substances and attempting to revise and clarify its definition.

Likewise, the other three NGC structural alerts, i.e. substituted n-alkylcarboxylic acids, quercetin type flavonoids and phthalate (or butyl) diesters and monoesters also showed a precision value lower than 0.5 with a PPV of 0.42, 0.39 and 0.38 respectively. Again ignoring these four structural alerts increased the total precision value of NGC structural alerts and consequently of the performance of the ISS carcinogenicity profiler. The results (Table 5.4) showed that the precision value of ISS non-genotoxic structural alerts was improved from 0.57 to 0.64 by ignoring these four structural alerts. However, for the reasons mentioned above, it is proposed that further research should be carried out to improve the performance of these alerts within the profiler.

The Oncologic primary classification profiler over-predicts carcinogens with sensitivity rates of 66-73% at the expense of poor prediction of non-carcinogens (30-46%), resulting in an overall performance which is barely better than chance for most of the datasets. All 30

structural alerts of the Oncologic primary classification profiler were individually analysed for their precision as shown in Table 5.5. Four structural alerts showed over-prediction of non-carcinogenic substances as being carcinogenic in more than 50% of the total substances containing this structural alert. These four alerts were carbamate type compounds, organophosphorus type compounds, peroxide type compounds and reactive ketone reactive functional groups. Carbamate type compounds' structural alert for carcinogenicity was triggered in 63 substances with only 22 true positive carcinogenic substances. The other 43 substances that were flagged by this alert to be carcinogenic were non-carcinogenic in experimental tests. This give a low precision value for carbamate type compounds of 0.35. The second structural alert in oncologic primary classification profiler with low precision value (lower than 0.36) was the organophosphorus type compounds structural alert where 80 substances out of 126 flagged by this alert were wrongly predicted as carcinogenic substances. The peroxide type compounds structural alert showed only 0.27 precision (positive predictive value) rate with only three correctly predicted carcinogenic substances out of 11 substances flagged by the alert. The structural alert with the lowest precision (of 0.1) within the Oncologic primary classification profiler was for reactive ketone functional groups where there was an over-prediction for 19 out of 21 substances. This mean that only two substances that were flagged by this alert were correctly predicted as carcinogenic substances out of the total 21 substances.

It can therefore be suggested that all four of these structural alerts could be ignored from the Oncologic primary classification profiler to increase the total sensitivity and accuracy of the profiler. Indeed, as shown in Table 5.5, the overall precision of the profiler was improved by 0.023 which is nearly 3% improvement in the overall performance of the profiler.

Removal of alerts from profilers would, however, not be an ideal solution, as this will miss carcinogenic compounds and would be completely out of the scope of the profiler. Instead, it is proposed that further research be carried out to see whether performance of these four alerts can be improved using a larger database of relevant substances.

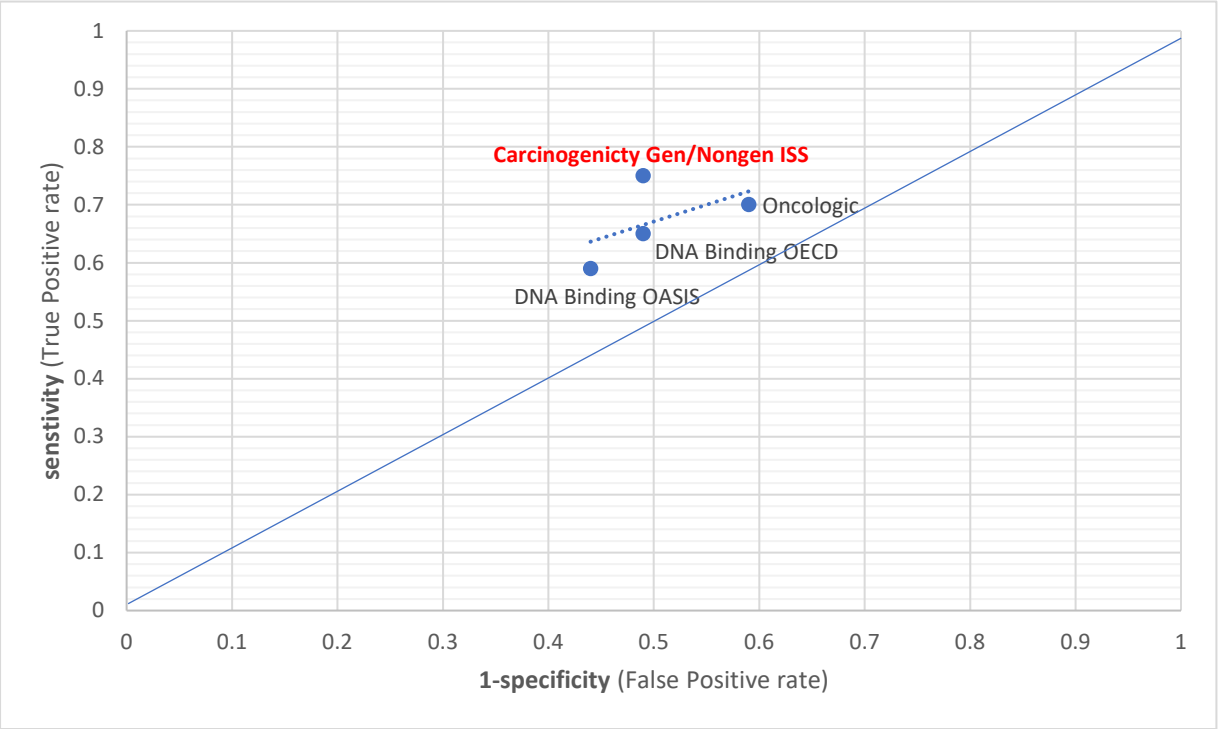


Figure 5.2. Receiver operating characteristic (ROC) curve analysis for carcinogenicity profilers in the OECD QSAR Toolbox.

5.3.3. Skin sensitisation profilers

The performance of the protein binding profilers is not consistent across the sample datasets. For the CAESAR dataset, these profilers tend to have a low predictivity for non-sensitisers, whilst for the other datasets it is the sensitisers which are not well predicted. Overall the performance of these profilers is moderate to poor for all the datasets.

The peptide depletion profilers showed a similar pattern, with performance being uniformly not better than chance for the CAESAR dataset but highly under-predicted for sensitisers in the other datasets. The protein binding potency profiler is also uniformly poor across all datasets, failing to detect the majority of sensitisers, with sensitivity rates between 12% and 29%. A similar pattern is seen with the keratinocyte gene expression profiler, where sensitivity rates were 19-43%.

The overall ROC analysis for all seven profilers shown in Figure 5.3 indicates that protein binding OASIS has relatively better performance compared to the other skin sensitisation profilers in the OECD QSAR Toolbox.

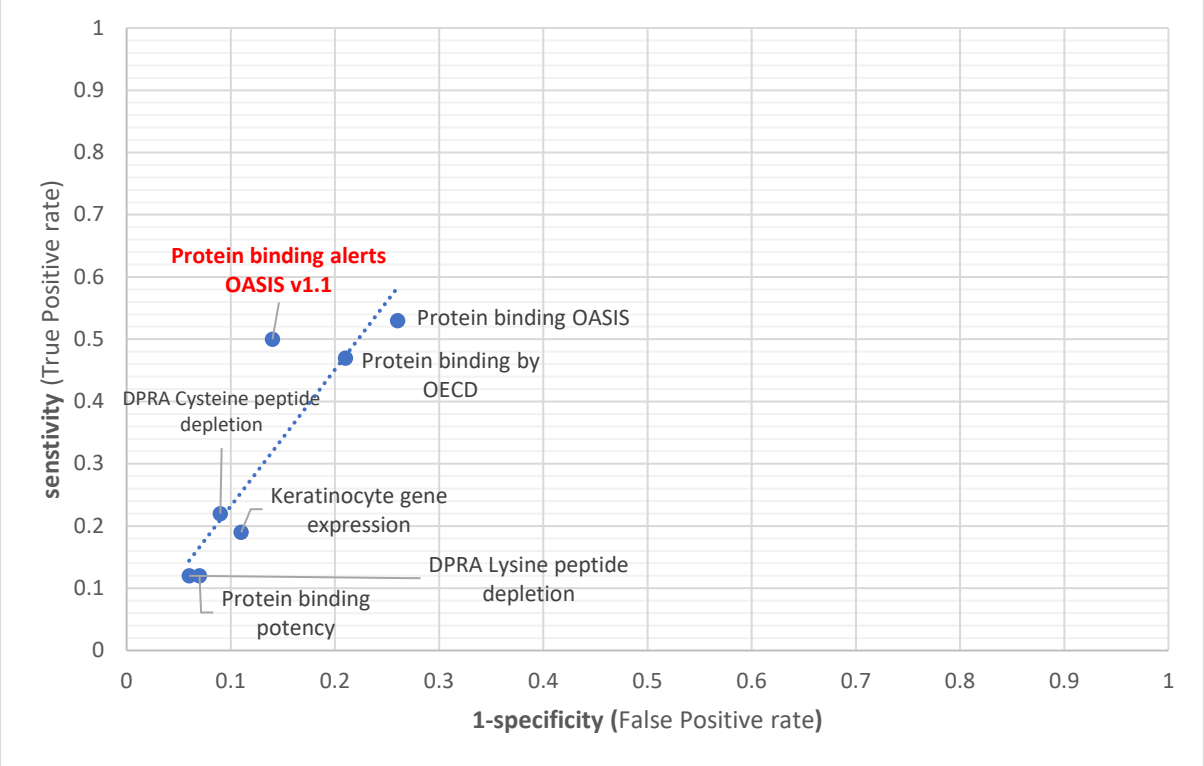


Figure 5.3. Receiver operating characteristic (ROC) curve analysis for the skin sensitisation profilers in the OECD QSAR Toolbox.

5.4 Discussion and Conclusions:

The profilers in the OECD QSAR Toolbox are provided for the purpose of constructing categories of mechanistic and identifying structural analogues of a target compound. As such, they provide a useful means for read-across from experimental data on analogous compounds to estimate a property or biological activity of an untested compound, although they are not necessarily intended to make toxicological predictions in themselves. The accuracy and reliability of a profiler in terms of predicting a target compound is, however, also important for defining the structural and functional features so that it is placed in the correct category/group of analogous substances. A number of the profilers are also used in other applications for indicating certain toxicity endpoints, such as Toxtree (http://toxtree.sourceforge.net/), and Oncologic (www.epa.gov/oppt/sf/pubs/oncologic.htm).

In this regard, those profilers that are equally likely to select endpoint-positive and endpoint-negative compounds into a grouping of analogues to be used in a read-across will, by definition, give rise to equivocal predictions for the target compound. It is therefore essential to know how accurately different profilers perform in terms of sensitivity, specificity and accuracy and to investigate possibilities for improvement. This requires an understanding of the role and merits of each individual alert within a profiler so that only the most relevant and reliable ones are kept as indicators for the particular endpoint. As this study has found, the alert "Hacceptor-path3-Hacceptor" in the micronucleus profiler is too ubiquitous to be a useful indicator of mutagenicity. Similarly in three NGC structural alerts within the ISS carcinogenicity profiler (thiocarbonyl, sub alkyl carboxylic acid, quercitin and phthalate), and the four structural alerts in oncologic primary classification profiler (carbamate type compounds, organophosphorus type compounds, peroxide type compounds and reactive ketone functional groups) proved to have extremely low precision values. As shown in the examples investigated in this chapter, the omission or substitution of these alerts, which unduly draw predictions towards equivocal

outcomes within a profiler, can improve the overall performance of the profilers. This, however, would bring the drawback of making some of the NGC alerts out of the scope of a profiler and therefore further research is needed to find out whether performance of these alert can instead be improved, e.g. by exploring larger datasets of relevant substances.

Another factor influencing the segregation of compounds by the alerts could be the way in which the categorical data found in the three endpoint datasets studied here are derived. Very often the binary categorisation of data is achieved by manipulation of the continuous data in some way to provide "cut-off" points for positive or negative assignment. The way in which this is done may affect the flagging of an alert in an essentially "negative" compound, or *vice versa*. By definition, most alerts have been derived from datasets of endpoint-positive compounds, because deriving "negative alerts", like proving a negative hypothesis, is generally not feasible. In this context, this study has given an insight into those alerts that may be found equally in endpoint-positive or negative compounds, and those which may be more effectively utilised to form groups of analogues for read-across predictions.

Further in-depth research in this area is necessary to study the suitability and merits of each of the alerts within the profilers in the OECD QSAR Toolbox and other *in silico* toxicity platforms to identify the root causes of the inadequacies and to investigate possibilities for improvement in the performance. This will, by implication, also improve the reliability of chemical read-across and grouping/categorisation for use in classification, labelling and risk assessment.

Chapter 6: Discussion

This chapter consists of two sections. The first provides a summary and discussion of the main research carried out, and the conclusions drawn, as detailed in Chapters 2 to 5. The second section provides insights for future work that could improve the assessment and evaluation of the currently available structural alerts for carcinogenicity, mutagenicity and skin sensitisation, and how such work could be translated into a practical tool to help the end-user. This will address the much-needed improvement in the reliability of *in silico* evaluations of carcinogenic, mutagenic and skin sensitising substances amongst the ingredients intended for use in cosmetic products during safety assessment.

6.1 Progress in the development and assessment of structural alerts for carcinogenic substances.

6.1.1 Summary of work

From the outset, the main focus of the work presented in this thesis was to evaluate the reliability of, and to identify the need for improvement in, the currently available structural alerts and *in silico* profilers of carcinogenicity and mutagenicity. For a broader comparative assessment of cosmetic ingredients, skin sensitisation profilers were also included later on in the study. The assessment of the profilers and structural alerts was essential to underpin confidence in their reliability in different software platforms, and to point out the need for refinements where necessary. Such refinements need to be in the form of a continuous process that aims to make the alerts and profilers more accurate and thus facilitate the safety assessment of the chemical ingredients used in cosmetic products.

6.1.2 The reliability of structural alerts in toxicity assessment

It is worth noting at the outset of this investigation that terms such as "carcinogenic/non-carcinogenic", "sensitiser/non-sensitiser" and "safe/unsafe" are used in this thesis for the sake of simplicity. It is fully recognised that assigning a chemical to be toxic or non-toxic depends on dose, exposure and threshold of toxicity for a particular endpoint that cannot be oversimplified with absolute terms. Structural alerts have gained wide regulatory acceptance for a number of reasons, foremost amongst them being that they are easily generated and interpreted. However, there has been growing concern about the accuracy of structural alerts to predict toxicity. The main concern about these alerts is that they represent only a part of the whole structure; i.e. functional groups that can be found in both toxic and non-toxic compounds; this may lead to over-prediction of toxicity. This over-prediction of toxicity with high sensitivity but low specificity was seen very clearly in the assessment of the predictivity of structural alerts undertaken in Chapters 3 and 5, as summarised in Tables 3.5, 5.1, 5.2 and 5.3. In Chapter 3, a total of 28 structural alerts were found to be inaccurate for use as part of the six mutagenic profilers studied. All of the structural alerts showed positive predictivity of less than 45% and more than ten substances were predicted to be positive for mutagenicity by these alerts. This phenomenon can be explained by the fact that the reactivity of these substructural alerts can be affected by other groups in the molecule, especially when the chemical properties of the substructures are dependent on the other groups in the same molecule (Alves *et al*., 2016). The scientific community has been debating the issue of the accuracy of predictions from structural alerts and hence their reliability. The OECD characterises read-across as a technique to predict a determined endpoint, but it requires that expert judgement is used and a justification of molecular similarity should be provided (OECD, 2007). The OECD sponsored the development of OECD QSAR Toolbox, a software application to predict (eco)toxicity based on chemical grouping and read-across, whilst leaving

the assessment of the prediction to the end user. The significance of structural alerts to predict toxicity was described in relation to the Toxtree software used in Chapter 2 to evaluate the reliability of structural alerts for nongenotoxic carcinogenicity. Toxtree also has a module for skin sensitisation that implements structural alerts. Contributing to the confusion over the significance of alerts as predictors of toxicity, the developers of Toxtree recently placed a statement on their website saying that they changed the name of the module from "Skin sensitisation alerts" to "Skin sensitisation reactivity domain" explaining that alerts provide only grouping into a reactivity mode of action and do not predict skin sensitisation potential. Although not explicitly reported, this conspicuous change in nomenclature is most likely due to pitfalls and deficiencies in the method. For instance, the use of simple categories led to the misclassification of 25% of compounds evaluated for respiratory sensitisation, including non-sensitisers containing alerts, and sensitisers that did not contain alerts (Alves *et al*., 2016; Enoch *et al.*, 2010). Given these shortcomings in the ability of structural alerts to predict toxicity, it can be argued that alerts may be useful for the initial flagging of potential toxic compounds but not necessarily for predicting toxicity. It is also important to point out that the original profiler alerts were also developed for grouping and read-across and hence were not intended to be predictive.

6.1.3 The feasibility of predicting human nongenotoxic carcinogenicity via structural alerts

The identification of nongenotoxic carcinogens remains one of the most challenging areas in toxicology. Even with a full dataset, setting protective levels for exposure is known to be problematic (Braakhuis *et al*., 2018). As with most toxicological endpoints, the majority of data relating to this endpoint are derived from animal experimentation with extrapolation to humans to allow for chemical risk assessment. This raises many potential problems, not least of which is the inter-species differences and potential for being over-protective with regard to

this endpoint, i.e. classification of compounds as carcinogens which are likely not to be harmful to humans. A further complication is that *in silico* models for toxicology can only be based on the data available, thus for nongenotoxic carcinogenicity, they will be based on information derived from standard rodent bioassays.

One of the possible shortcomings of structural alerts to predict nongenotoxic carcinogenicity is animal-to-human extrapolation. Nongenotoxic carcinogens act in species-specific, dose-dependent ways and include multiple mechanisms of action that involve many different chemical structures, which makes the extrapolation of predictivity from animal to human possibly inaccurate. For example, in long-term studies, chemicals that elicit the peroxisome proliferation phenomenon in rodents are associated with hepatocarcinogenesis. Upon treatment with these chemicals in high doses, the tumorigenic reaction appears to be related to both oxidative stress and increased cell proliferation. Non-rodent species have been shown to be highly resistant to the induction of peroxisome proliferation as compared to the rat (Corton *et al*., 2018). There are several potential explanations for the species-specific differences in response to peroxisome proliferation induction. One theory indicates that the differences in susceptibility to peroxisome proliferation between rats and humans is due to the variation in the comparative expression of PPARα between species (Lawrence *et al*., 2001). Expression of PPARα in human liver is relatively lower than in rat liver which limits the number of genes induced by the ligand upon exposure. The other explanation suggests that non-rodent species have a defect in the response element (PPRE) within the promoter that prevents the receptor from binding or regulating genes. This hypothesis was confirmed after analysing several human genomic samples that showed a defect in PPREs within the fatty acyl -CoA oxidase promoter (Lawrence *et al*., 2001). Advances in the human-relevant assessment of PPARa were reviewed recently by Felter *et al*. (2018) along with other modes of actions e.g. Constitutive Androstane Receptor (CAR).

The problem of *in silico* prediction of nongenotoxic carcinogenicity is well established. QSARs and structural alerts show the best performance for genotoxic endpoints as compared to non-genotoxic carcinogenicity (Carnesecchi *et al*., 2020). The reason for this is almost certainly that models for reactivity with DNA can be created relatively simply (e.g. structural alerts for covalent reactivity). For nongenotoxic carcinogenicity, there are a number of subtle mechanisms and initiating events, e.g. receptor binding, that are more difficult to model (Benigni and Bossa, 2019).

Whilst there are few, if any, reliable QSARs for human toxicity and chronic toxicity, specific endpoints such as human carcinogenicity are particularly poorly addressed - this is because of the emphasis on QSARs for regulatory endpoints and the lack of data (Gluck *et al*., 2018). However, toxicology and safety assessment are moving on and there is an opportunity in the future to improve the situation. Recent examples have demonstrated this with novel data sources and means of creating alerts (Golbamaki *et al*., 2016; Benigni *et al*., 2013). The opportunities for QSAR and structural alerts for nongenotoxic carcinogenicity include:

i) Ensuring that models are properly annotated and anchored to a mechanism of action. For instance, basing models around AOPs, and specifically initiating events, may be appropriate for this.

ii) Better definition of the domain of applicability of models, not only in terms of the chemistry but also the potential biology.

iii) Increasing knowledge of human non-genotoxic carcinogens with (where possible) new data sources e.g. using data from human cell lines.

Regarding the use of new data sources, there are several recent examples: Yamane *et al*. (2018) derived information for 20 compounds from human embryonic stem cells; Tung and Jheng

(2014) and Liu *et al*. (2011) used transcriptomics data to model non-genotoxic hepatocarcinogenicity. These and other researchers have also demonstrated that integrating mechanistic (biological) data and chemical structure information improves the prediction of nongenotoxic carcinogenicity. For instance *in vitro* and mechanistic data have been shown to improve the quality of QSARs (Guan *et al*., 2018; Chen *et al*., 2013; Lui *et al*., 2011).

Ultimately, there will always be uncertainty in using SARs / predictive methods and this is usually reflected in performance statistics. However, to predict non-genotoxic carcinogens the added uncertainty arising from extrapolation from one species to another must also be included. In order to achieve this, we need to define uncertainty and understand the consequences. Aspects of the models associated with high uncertainty could then be reduced - obvious areas of high uncertainty at the current time are relevance to humans and mechanistic underpinning of the models. Whilst we are currently not in an ideal position, it is likely that the models will be over-predictive. This is, in itself, consistent with the precautionary principle that implies we should accept the worst-case scenario and then provide evidence to reduce the risk e.g. PPAR only relevant to rodents. What is of greater concern is whether there are human-specific mechanisms of action that are not captured by the model. This will require knowledge of such mechanisms, again with reference to AOPs where possible (Rooney *et al*., 2018).

6.1.4 The significance of single alerts in a complex chemical structure.

Structural alerts highlight the importance of specific structural features as determinants of a compound's toxicity. However, biological effects are measured for the entire molecule, raising doubts about whether a fragment can always adequately define the property of the whole molecule. Several studies have been performed to examine the relationship and interplay between the fragment and the whole molecular structure (Alves *et al*., 2016). A small change

in molecular structure, i.e. replacement of one functional group with another in the same position, causes changes in many descriptor values which reflect the interconnectivity and mutual influence of all fragments in a molecule. Nitroaromatic compounds, which are used in hair dyes, are a good example to illustrate the mutual influence of substituents in the molecule on the overall toxic effect of these compounds. Kuz'min *et al*. (2008) developed a QSAR model for rat acute toxicity which demonstrated that the nitro group attached to the aromatic ring, which was known to increase toxicity, shows variations in its toxic effect depending on the number and nature of other substituents in the aromatic ring. Thus, the main finding of their study was that although an aromatic nitro group is considered to be a toxicophore, its contribution to toxicity could be significantly modified by other substituents. Chloro-substituted nitrobenzenes are a good example to illustrate this finding. Increasing the number of chlorine substituents in a nitrobenzene molecule was expected to increase toxicity but interestingly this was not completely true as it was determined that the influence of a chlorine substituent is not clear and depends strongly on the structural environment. For instance, a chlorine atom in the ortho-position to the nitro group is present in both the most toxic (2,6-dichloronitrobenzene) and the least toxic (2,3,5-trichloronitrobenzene) compounds. Overall, the insertion of a chlorine substituent in nitrobenzene increases its toxicity; the ortho-isomer is the most toxic. Introduction of the second chlorine results in large changes in toxicity, that are observed for dichloronitrobenzenes. Addition of chlorine substituents decreases the difference in toxicity between the isomers. Moreover, the accumulation of chlorine atoms in the benzene ring decreases their influence on toxicity, i.e., the increase in toxicity is not proportional to the number of chlorine atoms or, even more, the addition of chlorine decreases the toxicity (Kuz'min *et al*., 2008). The effect of sequential insertion of chlorine substituents into the benzene ring was also analysed. In Figure 6.1, the toxicity of each molecule is represented as six separate contributions of the corresponding carbon of the aromatic ring and its substituent.

Insertion of a chlorine atom in the ortho-position to the nitro group leads to an increase in toxicity in comparison with nitrobenzene. This effect is not limited to the chlorine atom alone. In fact, the contributions to toxicity of all other atoms are augmented (except C–H bond in ortho-position to the C–Cl bond, Figure 6.1). Insertion of an additional chlorine adjacent to the previous ortho-chlorine has only a small effect on toxicity. Although the new C–Cl bond (position 3) increases the toxicity of the molecule, the contributions of the nitro group and other C–Cl fragment (position 2) have been diminished. Thus, in spite of the redistribution of influence on toxicity between different fragments of 2,3-dichloronitrobenzene, the toxicity of the whole compound hardly changes compared to 2-chloronitrobenzene. A dramatic change in toxicity was predicted for 2,3,5-trichloronitrobenzene. However, substitution of hydrogen by chlorine in position 5 results in substantial lowering of toxicity. This resulted in the diminishing toxicity of all fragments analysed, especially the chlorine in the 2-position (Kuz'min *et al*., 2008; Alves *et al*., 2016). These examples emphasise an important conclusion that compound toxicity can be substantially affected by the mutual interactions and influences between its structural components. Moreover, individual substructures do not act directly and independently as is saliently presumed by the concept of structural alerts. Instead, various substructures, even including distant neighbours, mutually influence their contributions.

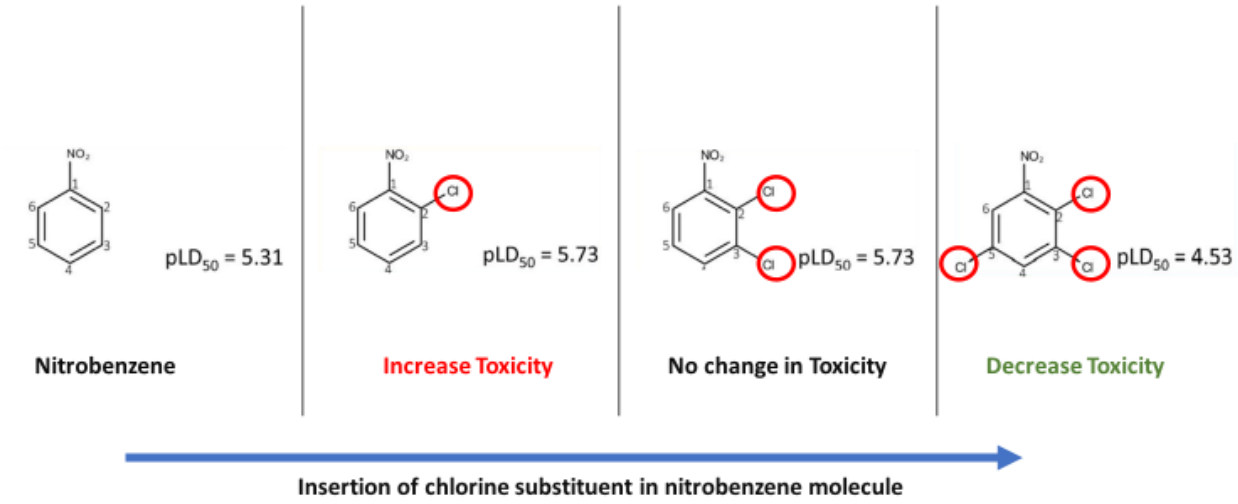


Figure 6.1. Relative influence of structural fragments on the rat acute toxicity of chlorosubstituted nitrobenzenes, where pLD_{50} is the negative logarithm of the molar dose that causes 50% lethality (Alves *et al*., 2016).

6.1.5 Assigning cut-off values for the identification and evaluation of structural alerts

A cut-off value of 0.5 PPV was applied in the evaluation of the reliability of carcinogenicity, mutagenicity and skin sensitisation structural alerts performed in Chapters 3 and 5. Thus, any structural alerts showing positive predictivity lower than 50% were classified as inaccurate. This was to ensure that none of these profilers have lower predictivity power compared to the Ames test. One of the key purposes of the Ames test is to predict rodent carcinogenicity. The high predictive power of a positive Ames ranges from 77% to 90% depending on the various factors. This makes it superior to any other *in vitro* genotoxicity assay, all of which have lower performance in terms of predicting genotoxicity (Kazius *et al*., 2006).

In Chapter 4, the main goal of adjusting the cut-off value was to select the minimum number of carcinogenicity and mutagenicity scaffolds that would cover the largest possible number of carcinogenic and mutagenic compounds, ultimately in order to identify a precise structural alert. The ratio of activity (carcinogenicity or mutagenicity) (C1/S), where C1 represents total active compounds (carcinogenic or mutagenic) and S represents the number of active compounds that contain this scaffold, was adjusted to 0.7, which achieved predictive power comparable to the Ames test and should cover at least 10 compounds based on the selection criteria discussed above.

On the other hand, an essential factor influencing the segregation of compounds by the alerts could be the way in which the categorical data found in the three endpoint datasets studied here are derived. Very often the binary categorisation of data is achieved by manipulation of the continuous data in some way to provide "cut-off" points for positive or negative assignment. The way in which this is performed may affect the flagging of an alert in an essentially "negative" compound, or *vice versa*. By definition, alerts have been derived from datasets of endpoint-positive compounds, since deriving "negative alerts", similar to proving a negative hypothesis, is generally not feasible. In this context, this study has provided an insight into those alerts that may be found equally in endpoint-positive or negative compounds, and those that may be more effectively utilised to form groups of analogues for read-across predictions. Further in-depth research in this area is needed to study the suitability and merits of each of the alerts in the profilers in the OECD QSAR Toolbox and other *in silico* toxicity platforms to identify possibilities for improvement in performance. This will, by implication, also improve the reliability of chemical read-across and grouping/categorisation for classification, labelling and risk assessment.

6.*1.6 Conclusions*

It is widely known that carcinogenicity is one of the main causes of death around the world (Frankish, 2003). Testing chemicals for carcinogenicity, especially non-genotoxic carcinogenicity, is not straightforward, and *in silico* methods provide a means for rapid initial screening in this regard. The work presented in this thesis has assessed and evaluated the available structural alerts and profilers of carcinogenicity and mutagenicity in a number of *in silico* predicting programmes such as the OECD QSAR Toolbox, Toxtree and Leadscope. Many structural alerts were found to have unacceptably low positive predictivity for mutagenicity and carcinogenicity, which has a bearing on the performance of various profilers. These structural alerts need to be investigated further to improve their predictivity and, as a result, the predictivity of the respective profilers. The study also identified a number of carcinogenicity scaffolds through the use of the SAR carcinogenicity database. Although these are linked to carcinogenicity, the chemistry-based alerts are not likely to be useful for the formation of chemical categories or read- across because they lack a mechanistic interpretation of how a MIE is induced within the AOP paradigm.

This thesis is positioned as a programmatic statement that could change the thinking of both regulators and researchers. Informing the scientific community about the limitations of structural alerts that were discussed in previous sections, especially those that could be very useful in understanding the underlying mechanisms of toxicity, was not, however, the main goal of the discussion. The main goals were to show how toxicity prediction should not be performed by blindly relying on structural alerts, as well as how to boost safety assessment by combining the strongest parts of the alerts and QSAR models. It was demonstrated that blind reliance on structural alerts could lead researchers astray. Conversely, this discussion is not suggesting that QSAR models should be used instead of structural alerts. Although it was demonstrated that “black box” QSAR predictions usually provide the user with statistically

more accurate predictions, we also showed how alerts could serve as actionable structural hypotheses that could be validated by QSAR predictions. The best solution is to propose an integrated approach to desing new green chemicals by the structural modification of existing functional, but toxic, compounds using a combination of structural alerts and QSAR models. Another important point is that the influence of any part of a compound on its biological effect(s) is not constant and strongly depends on its structural environment. Thus, any alert, even one derived by mechanistic interpretation of statistically significant QSAR models does not have automatic predictive power. Alerts should be viewed as structural hypothesds of chemical action only and their true predictive power should be confirmed by QSAR predictions and, if possible, by experimental validation. The major recommendations discussed in this thesis are as follows:

1. In most cases, it is unreliable to use structural alerts alone to predict toxicity and this should be avoided.
2. Structural alerts act within the whole chemical structure, so their toxic effect depends mainly on the structural environment. Large datasets can be used to evaluate the extent of the interdependency of structural alerts.
3. The optimum way to confirm the significance of structural alerts is by using a QSAR model or, preferably, by experiment.
4. The accuracy of toxicity prediction of structural alerts can be improved by combining them with a QSAR model or a chemical biological read-across (CBRA) model.
5. Although structural alerts often fail in predicting chemical toxicity, they can still be useful in developing local QSAR models by spiltting large datasets into smaller subsets based on their mechanism of action.
6. Combining structural alerts with QSAR models in an intelligent way can be used to design functional non-toxic compounds e.g. for green chemistry applications.

6.2 Prospects for future work

The work presented in this thesis has enabled verification and refinement of the current structural alerts and profilers for carcinogenicity (both genotoxic and non-genotoxic), mutagenicity and skin sensitisation as well as the identified 18 carcinogenicity scaffolds to be included as structural alerts for carcinogenicity (see Tables 4.2, 4.3, 4.5, 4.6 and 4.9). These alerts can be used for both toxicity prediction and grouping. The information about structural alerts can be captured either in CSRML or SMARTS strings to be incorporated in software like Toxprint that can search for these alerts among a large number of chemicals. Publishing these findings will not be the only way to distribute these alerts: they will be donated to be used in software such as Toxtree and the OECD QSAR Toolbox to allow them to be used by the greatest possible number of scientists and researchers. However, further work is needed to develop new alerts using other databases such as ToxCast which, contains a huge number of peroxisome proliferator activated receptor assays for nearly 2,000 compounds. This could be an excellent resource for identifying new non-genotoxic carcinogenic structural alerts, refining the poorly predictive structural alerts presented in the previous chapters.

6.2.1 Combining structural alerts with QSAR models

A newly suggested technique to increase the predictive power of structural alerts is to combine them with QSAR prediction models. Structural alerts can be used to classify the investigated compounds based on their putative mechanisms of action. A good example is the skin sensitisation dataset. In the skin sensitisation process, the molecular initiating event (MIE) is protein binding that is represented by a number of protein binding structural alerts based on well-known organic chemistry principles, but they are not highly efficient to predict skin sensitisation. Structural alerts can be very helpful with large datasets in assigning a mechanism of action to the compounds investigated and in developing local QSAR models. The local QSAR models could be united with mechanism-uninformed global models in a consensus

ensemble that may have comparable or higher predictive power and coverage than separate models.

The shortcomings of individual structural alerts can also be avoided using a new approach called chemical biological read-across (CBRA). In contrast to classical read-across that predicts the toxicity of unknown compounds from their chemical analogues, CBRA uses both chemical and biological analogues, which achieves more accurate and reliable predictions (Low *et al*., 2014). The similarities between chemicals are assessed in CBRA based on two factors: chemical and biological. The chemical descriptors are usually obtained as computed structural and molecular properties while the biological descriptors can be obtained by experiment or the predicted result of biological measurement of chemical compounds. CBRA can achieve more understanding towards the prediction of complex toxicity, which is why it is described as "next generation read-across" (Low *et al*., 2014). As shown in Figure 6.2, CBRA represents the data for the user as a radial plot where the compound of interest is represented by a large central node. The chemical and biological nearest neighbours to the compound of interest are represented by smaller nodes surrounding the central node. The level of similarity of the biological and chemical neighbours to the compound of interest is visualised by two indicators. First, by the colour of the neighbouring node, indicating its observed activity (red=toxic, green=non-toxic) and second, by the relative position of the neighbouring node relative to the central node. The more similar two compounds are, the closer the neighbouring node is to the central node. In the radial plot, the nearest neighbour to the compound of interest in both chemical and biological descriptor space will be at the 12 o'clock position. CBRA provides a better understanding of the structure-activity relationship of the compound of interest, or for a group of similar compounds, thanks to its visual radial plots. The visual aspect makes it very useful for understanding the common chemical and biological neighbours and the activity landscape of the compound of interest, and this will help in the design of greener

chemicals. The chemical and biological models may show conflicting predictions when they are used as separate models; this aspect is solved by CBRA as it maximises the integration of both data streams.

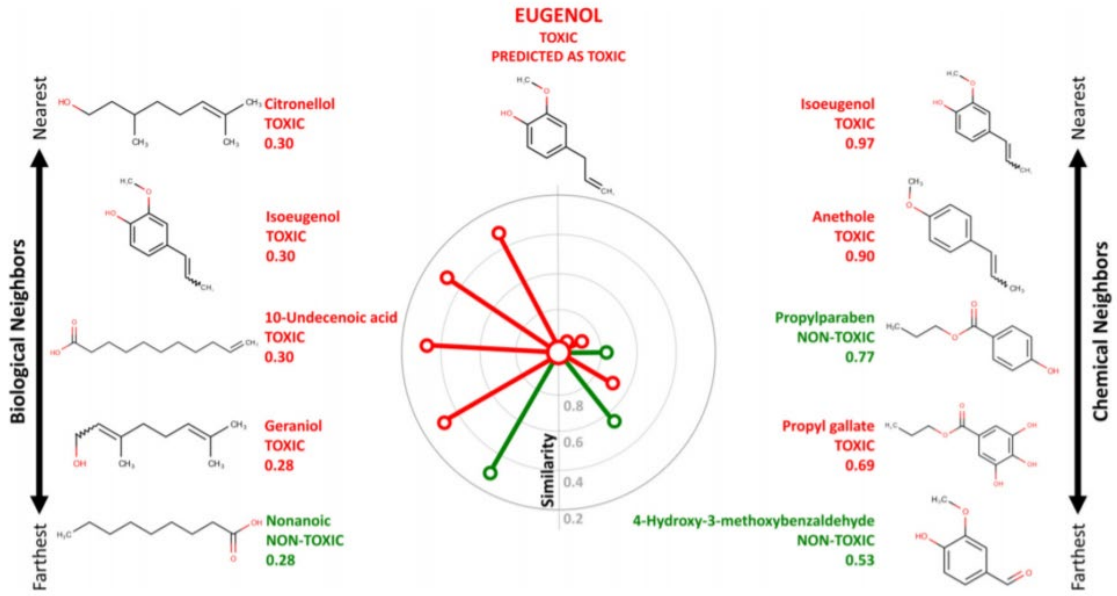


Figure 6.2. Eugenol radial CBRA plot showing its biological neighbours (left side) and chemical neighbours (right side) coloured based on their activity (red=toxic , green=non-toxic) (as per Alves *et al*., 2016).

6.2.2 Refinement of poorly predictive structural alerts for carcinogenicity and mutagenicity:

The analysis presented in Chapters 3 and 5 of the performance of the available profilers for carcinogenicity and mutagenicity yielded 28 poorly predictive mutagenic structural alerts and eight poorly predictive carcinogenic structural alerts (see Tables 3.12, 5.4 and 5.5). The work demonstrated the importance of continued analysis and assessment of the accuracy of current profilers within various *in silico* programmes such as Toxtree and the OECD QSAR Toolbox to improve their reliability. Therefore, it is envisaged that future work will involve further investigations to identify other shortcomings associated with the alerts.. All this information

will identify the core issues and pave the way for their improvement. The identified alerts with poor predictivity could also be used to determine whether there are certain structural features linked to over-predictions in terms of false positive (or false negative) results.

6.2.3 Use of in vitro/in chemico data to discern mechanistic information for chemistry-based alerts

The work performed in Chapter 4 enabled the development of 17 carcinogenic scaffolds and 23 mutagenic scaffolds that are chemistry-based structural alerts and as such do not have a mechanistic basis associated with them. As the chemistry-based structural alerts can trigger predictions for various types of chemicals, the probability that they will be useful in identifying carcinogenicity or mutagenicity via more than one mechanism is higher than that of the mechanism-based structural alerts. In view of this, the chemistry-based structural alerts can be refined in conjunction with mechanism-based alerts to increase their reliability and accuracy. This could also be done by further combining *in vitro* and *in chemico* data to separate the mechanistic information for each chemical structure, which may have the same chemical alert but initiate a different MIE (see Table 6.1 for some *in chemico* approaches). The association of a mechanistic hypothesis with each chemical structure will eventually solve the issues associated with structural alerts that may exert their action via multiple mechanisms and thus help in refining the usefulness of chemical-based alerts for toxicity assessment of untested chemicals.

Table 6.1. Examples of *in chemico* reactivity for mutagenicity and skin sensitisation measured during non-pharmaceutical research and development (Cronin *et al*., 2009).

Toxicity endpoint	*In chemico* approach
DNA binding	Reactivity toward 2´-deoxyguanosine
Skin sensitisation	Depletion of glutathione assessed by using UV
	Rate constant for reaction with n-butylamine (as part of the Relative Alkylation Index, RAI)
	High-throughput kinetic profiling approach for covalent binding to peptides, providing second-order rate constants

6.2.4 Development of additional carcinogenic and mutagenic alerts:

Although the work carried out in this thesis has developed a number of chemistry based structural alerts for carcinogenicity and mutagenicity, the need to develop additional alerts, both mechanistically and chemically based, to cover the relatively small chemical space is still high. Databases such as ToxCast, ChEMBL, TGGates can be used to develop additional mechanism- and chemistry- based alerts. In the Toxcast databases nearly 2,000 chemicals from a wide range of different sources were evaluated, including: industrial and consumer products, food additives and potentially "green" chemicals that could be safer alternatives to existing chemicals. Chemicals were evaluated in over 700 high-throughput assays covering a range of high-level cellular responses and approximately 300 signalling pathways (Epa.gov, 2015). This database contains a large number of assays for binding to the peroxisome proliferator activated receptor (PPAR) which is one of the main non-genotoxic carcinogenic mechanisms. The data are freely available at http://www.epa.gov/ncct/toxcast/.

It is envisaged that the scaffold analysis process used in Chapter 4 could be used to develop one or more new carcinogenic and mutagenic structural alerts.. Thus, it is expected that adding further structural alerts to current carcinogenicity and mutagenicity profilers would expand the

chemical domain covered by these profilers and reduce the false negative results in the *in silico* profilers. It is envisaged that carcinogenicity and mutagenicity structural alerts (both those derived in this thesis and those that could be developed in future) can be used in *in silico* predictive tools such as the OECD QSAR Toolbox or predictive software such as KNIME. Several benefits might be achieved using these newly developed structural alerts. First, they can be used to screen chemical inventories in order to detect and identify chemicals having the potential to induce carcinogenicity and mutagenicity. Second, these alerts, in combination with other alternative techniques and information obtained from the scientific literature, could help in the development of AOPs for other mechanisms of carcinogenicity, especially for non-genotoxic carcinogens.

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8: Appendices

Appendix I. List of the 43 inorganic mutagenic chemicals in CCRIS DB that failed to be identified by any of six mutagenic profilers in the OECD QSAR Toolbox (Chapter 3).

ID	Chemical Name	SMILES
1	Sodium nitrite	[Na+].[O-]N=O
2	Ozone	[O-][O+]=O
3	Sodium selenite	[Na+].[Na+].[O-][Se]([O-])=O
4	Cadmium chloride	Cl[Cd]Cl
5	Selenic acid	[Na+].[Na+].[O-][Se]([O-])(=O)=O
6	Diborane	BB
7	Lead	[Pb]
8	Cadmium; Cadmium compounds	[Cd]
9	Selenium Sulfide	S=[Se]
10	Aluminum chloride	Cl[Al](Cl)Cl
11	Potassium bromate	[K+].[O-]Br(=O)=O
12	Zirconium, dichloro-di-pi-cyclopentadienyl	Cl[Zr](Cl)(C1C=CC=C1)C1C=CC=C1
13	Titanium, dichlorobis(eta5-2,4-cyclopentadien-1-yl)	Cl[Ti](Cl)(C1CC=CC=1)C1CC=CC=1
14	Magnesium oxide (MgO)	O=[Mg]
15	Hypochlorous acid	[Na+].[O-]Cl
16	Hydrogen peroxide,	OO
17	Potassium nitrite	[K+].[O-]N=O
18	Cyanoguanidine	NC(=N)NC#N
19	Manganese	[Mn]
20	Manganese chloride	Cl[Mn]Cl
21	Manganese(II) sulfate	[Mn+2].[O-]S([O-])(=O)=O
22	Nickel chloride (NiCl2)	Cl[Ni]Cl
23	Permanganic acid (HMnO4)	[K+].[O-][Mn](=O)(=O)=O
24	Platinate(2-), hexachloro-, dihydrogen, hexahydrate	[H+].[H+].O.O.O.O.O.O.Cl[Pt-2](Cl)(Cl)(Cl)(Cl)Cl
25	Dipotassium hexachloroplatinate	[K+].[K+].Cl[Pt-2](Cl)(Cl)(Cl)(Cl)Cl
26	Potassium tetrachloroplatinate(II)	[K+].[K+].Cl[Pt-2](Cl)(Cl)Cl
27	cis-Dichlorobis(2-methyl-2-propanamine)platinum	Cl[Pt]Cl.CC(C)(C)N.CC(C)(C)N
28	Platinum, diamminedibromo-, (SP-4-2)- (9CI)	N.N.[Br-].[Br-].[Pt+2]
29	Dichloro-(S,S)-(N,N'-diethyl-2,4-pentanediamine)platinum(II)	[Cl-].[Cl-].[Pt+2].CCNC(C)CC(C)NCC
30	Potassium superoxide;	[K+].O=O
31	Triammonium hexachlororhodate; Rhodate(3-),	[N+H4].[N+H4].[N+H4].[Cl-].[Cl-].[Cl-].[Cl-].[Cl-].[Cl-].[Rh+3]
32	Dipotassium pentachlororhodate	[K+].[K+].Cl[Rh-2](Cl)(Cl)(Cl)Cl
33	Silane	[Si]
34	Silane, dichloromethylvinyl	C[Si](Cl)(Cl)C=C
35	Silver iodide (AgI)	[Ag]I
36	Sodium sulfide, nonahydrate	O.O.O.O.O.O.O.O.O.[Na+].[Na+].[S-2]
37	Titanium chloride (TiCl3)	Cl[Ti](Cl)Cl
38	Ammonium Hexachloroplatinate (iv)	[N+H4].Cl[Pt-2](Cl)(Cl)(Cl)(Cl)Cl
39	Boric acid, sodium salt	[Na+].[Na+].[Na+].[O-]B([O-])[O-]

40	Bis(chlorotriammineplatinum) tetrachloroplatinate(II)	N.N.N.N.N.N.Cl[Pt+3].Cl[Pt+3].Cl[Pt-2](Cl)(Cl)Cl.Cl[Pt-2](Cl)(Cl)Cl.Cl[Pt-2](Cl)(Cl)Cl
41	Bis(p-methoxyphenyl)selenide	COc1ccc([Se]c2ccc(OC)cc2)cc1
42	cis-Dichlorobis(3,5-dimethylpyridine)platinum	Cl[Pt]Cl.Cc1cncc(C)c1.Cc1cncc(C)c1
43	Dichloro-(s)-3-aminohexahydropyridine Platinum (ii)	Cl[Pt]Cl.NC1CCCCN1

Appendix II. List of the 112 various organic mutagenic chemicals in CCRIS DB that failed to be identified by any of six mutagenic profilers in the OECD QSAR Toolbox and do not have any chemical unifying characters to be grouped or used to initiate a new rule for a new chemical structural alert (Chapter 3).

ID	Chemical Name	SMILES
1	2-Mercaptobenzothiazole	S=C1Nc2ccccc2S1
2	Thiodiphosphoric acid (((HO)2P(S))2O), tetraethyl ester (9CI)	CCOP(=S)(OCC)OP(=S)(OCC)OCC
3	1,3-Dioxane	C1COCOC1
4	1,2,3,5-Tetramethylbenzene	Cc1cc(C)c(C)c(C)c1
5	1-Naphthalenol, methylcarbamate;	CNC(=O)Oc1cccc2ccccc12
6	Cyclohexanone, oxime	ON=C1CCCCC1
7	Dicyclohexylamine	C1CCC(CC1)NC1CCCCC1
8	1,3-Diphenylguanidine	N=C(Nc1ccccc1)Nc1ccccc1
9	Ferrocene	[Fe+2].c1cc[c-H]c1.c1cc[c-H]c1
10	1,3-Butadiene	C=CC=C
11	Acrylonitrile	C=CC#N
12	Propargyl alcohol	OCC#C
13	Acetaldehyde oxime	CC=NO
14	o-Chloropyridine	Clc1ccccn1
15	Butyraldehyde oxime	CCCC=NO
16	Propene	CC=C
17	Benzothiazyl disulfide	S(Sc1nc2ccccc2s1)c1nc2ccccc2s1
18	Methyl styryl ketone	CC(=O)C=Cc1ccccc1
19	Decane	CCCCCCCCCC
20	2',3',4'-Trichloroacetophenone	CC(=O)c1ccc(Cl)c(Cl)c1Cl
21	3-((Methoxycarbonyl)amino)phenyl N-(3-methylphenyl)carbamate (phenmedipham)	COC(=O)Nc1cccc(OC(=O)Nc2cccc(C)c2)c1
22	2-Methyl-3-butenenitrile	CC(C=C)C#N
23	Phthalide, 3-propylidene (6CI,8CI)	CCC=C1OC(=O)c2ccccc12
24	Benzenamine, 4-butyl-N-((4-methoxyphenyl)methylene)	CCCCc1ccc(cc1)N=Cc1ccc(OC)cc1
25	Di(N-octyl)tin-S,S'-bis(isooctylmercaptoacetate)	CCCCCCCC[Sn](CCCCCCCC)(SCC(=O)OC(C)CCCCCC)SCC(=O)OC(C)CCCCCC
26	4-Butyloxybenzal-4'-ethylaniline	CCCCOc1ccc(cc1)C=Nc1ccc(CC)cc1
27	Trans-2,3-dibromo-2-butene-1,4-diol	OCC(Br)=C(Br)CO

28	Pentanedinitrile, 2-methyl-	CC(CCC#N)C#N
29	Linolenic acid (8CI)	CCC=CCC=CCC=CCCCCCCCC(O)=O
30	3-Pentenenitrile; pent-3-enenitrile	CC=CCC#N
31	Bromopicrin	[O-][N+](=O)C(Br)(Br)Br
32	1,3,5-Tris(hydroxy-ethyl)s-hexahydrotriazine	OCCC1NC(CCO)NC(CCO)N1
33	Trimethylene oxide (8CI)	C1COC1
34	Trimethyloxonium hexachloroantimonate(1-)	C[O+](C)C.Cl[Sb-](Cl)(Cl)(Cl)(Cl)Cl
35	Allyl urea	NC(=O)NCC=C
36	Urea	NC(N)=O
37	2-Chloro-2-nitropropane	CC(C)(Cl)[N+]([O-])=O
38	1,1-Dichloro-1-nitroethane	CC(Cl)(Cl)[N+]([O-])=O
39	Methyl sulfoxide	C[S+](C)[O-]
40	1,4-Benzenediamine, N,N'-diphenyl-	N(c1ccccc1)c1ccc(Nc2ccccc2)cc1
41	2,6-Octadiene, 1,1-diethoxy-3,7-dimethyl-	CCOC(OCC)C=C(C)CCC=C(C)C
42	Chloropicrin	[O-][N+](=O)C(Cl)(Cl)Cl
43	o-Tolyl phosphate	Cc1ccccc1OP(=O)(Oc1ccccc1C)Oc1ccccc1C
44	Methacrylic acid	CC(=C)C(O)=O
45	Benzoic acid, p-(dichlorosulfamoyl)	OC(=O)c1ccc(cc1)S(=O)(=O)N(Cl)Cl
46	1,3-Dimethyl-2-nitrobenzene	Cc1cccc(C)c1[N+]([O-])=O
47	1-Chloronaphthalene	Clc1cccc2ccccc12
48	N,N'-Di-2-naphthyl-p-phenylenediamine	N(c1ccc(Nc2ccc3ccccc3c2)cc1)c1ccc2ccccc2c1
49	2,3,6-Trichlorophenol	Oc1c(Cl)ccc(Cl)c1Cl
50	2,4,5-Trichlorophenol	Oc1cc(Cl)c(Cl)cc1Cl
51	Butanone oxime	CCC(C)=NO
52	2,2'-Methylene-bis (4-chlorophenol)	Oc1ccc(Cl)cc1Cc1cc(Cl)ccc1O
53	2-Vinylpyridine	C=Cc1ccccn1
54	Bis(cyclopentadienyl)vanadium chloride	Cl[V](Cl)(C1C=CC=C1)C1C=CC=C1
55	Bis(1,5-cyclooctadiene)nickel	[Ni].C1CC=CCCC=C1.C1CC=CCCC=C1
56	(1S,4S,4aS,6S,8aS)-4-isopropyl-1,6-dimethyldecahydronaphthalene	CC1CCC2C(C)CCC(C(C)C)C2C1
57	2-Methylpropanenitrile	CC(C)C#N
58	C.I. Natural Orange 4	CC(C=CC=C(C)C=CC(O)=O)=CC=CC=C(C)C=CC=C(C)C=CC(O)=O
59	Retinol palmitate	CCCCCCCCCCCCCCCC(=O)OCC=C(C)C=CC=C(C)C=CC1=C(C)CCCC1(C)C
60	MENTHONE	CC(C)C1CCC(C)CC1=O
61	sodium dimethyldithiocarbamate	[Na+].CC(C)C([S-])=S
62	Anisole, p-propenyl-, trans-;	COc1ccc(C=CC)cc1
63	Biphenyl-4-ol	Oc1ccc(cc1)-c1ccccc1
64	5-beta-Cholan-24-oic acid, 3-alpha,7-alpha-dihydroxy-	CC(CCC(O)=O)C1CCC2C3C(O)CC4CC(O)CCC4(C)C3CCC12C
65	Deterrol	CC(=C)c1ccc(C)c2ccc(CO)c2c1
66	7H-Benz(de)anthracen-7-one	O=C1c2ccccc2-c2cccc3cccc1c32
67	1,2,3-Trimethylbenzene	Cc1cccc(C)c1C
68	2,3'-bipyridine	c1ccc(nc1)-c1cccnc1
69	4,4'-bipyridine	c1cc(ccn1)-c1ccncc1
70	tributylborane	CCCCB(CCCC)CCCC
71	2-Nitrobutane	CCC(C)[N+]([O-])=O
72	2(1H)-Quinolinone	O=C1Nc2ccccc2C=C1
73	Ursodeoxycholic acid	CC(CCC(O)=O)C1CCC2C3C(O)CC4CC(O)CCC4(C)C3CCC12C

74	Deoxycholic acid	CC(CCC(O)=O)C1CCC2C3CCC4CC(O)CCC4(C)C3CC(O)C12C
75	Cholic acid	CC(CCC(O)=O)C1CCC2C3C(O)CC4CC(O)CCC4(C)C3CC(O)C12C
76	1,3,5-Cycloheptatriene, 1-methoxy-	COC1CC=CC=CC=1
77	nitrocyclopentane	[O-][N+](=O)C1CCCC1
78	Nitrocyclopentane nitronate	[O-][N+]([O-])=C1CCCC1
79	1,3-dithiane	C1CSCSC1
80	p-Dithiane	C1CSCCS1
81	Isonicotinaldehyde oxime	ON=Cc1ccncc1
82	Naphthoresorcinol	Oc1cc(O)c2ccccc2c1
83	1,6-Naphthalenediol	Oc1ccc2c(O)cccc2c1
84	Sesquiterpene, stearic acid ester	CCCCCCCCCCCCCCCCCC(=O)OCC1=CC=C2C(C)=CCC(C=C12)C(C)=C
85	1H-Phenalen-2-amine, 2,3-dihydro-N-methyl-, hydrochloride	Cl.CNC1Cc2cccc3cccc(C1)c23
86	Phenalen-1-one	O=C1C=Cc2cccc3cccc1c23
87	Bithionol sulfoxide	Oc1c(Cl)cc(Cl)cc1[S+]([O-])c1cc(Cl)cc(Cl)c1O
88	Phosphoramidothioic acid, O,S-dimethyl ester	COP(N)(=O)SC
89	2-Nitropropane nitronate	CC(C)=[N+]([O-])[O-]
90	1,6-Pyrenequinone	O=C1C=Cc2ccc3C(=O)C=Cc4ccc1c2c43
91	1,8-Pyrenequinone	O=C1C=Cc2ccc3C=CC(=O)c4ccc1c2c34
92	2,3-dichloropyridin	Clc1cccnc1Cl
93	Pyridine, 2-fluoro-	Fc1ccccn1
94	1-Methyl-4-phenyl-2,3-dihydropyridinium perchlorate	[O-]Cl(=O)(=O)=O.C[N+]1CCC(=CC=1)c1ccccc1
95	Thiazolidine	C1CSCN1
96	Vanadic acid, ammonium salt	N.O[V](=O)=O
97	2-Mercaptoethanol thionitrite	OCCSN=O
98	Chlorinated trisodium phosphate	[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[Na+].[O-]Cl.[O-]P([O-])([O-])=O.[O-]P([O-])([O-])=O.[O-]P([O-])([O-])=O.[O-]P([O-])([O-])=O
99	3,3'-Dipyridyl	c1cncc(c1)-c1cccnc1
100	2,4'-Dipyridyl	c1ccc(nc1)-c1ccncc1
101	1,4-pentadiene-3-ol; Penta-1,4-dien-3-ol	OC(C=C)C=C
102	4-Biphenylyl methylcarbamate	CNC(=O)Oc1ccc(cc1)-c1ccccc1
103	1,2,3,4,4a,10a-hexahydrophenanthrene	C1CCC2C(C1)C=Cc1ccccc21
104	1,2,3,9,10,10a-hexahydrophenanthrene	C1CC=C2C(C1)CCc1ccccc12
105	1,1'-Dimethyl-3,3'-bipyridinium diiodide	[I-].[I-].C[n+]1cccc(c1)-c1ccc[n+](C)c1
106	4-(2'-Pyridyl)-1-methylpyridinium iodide	[I-].C[n+]1ccc(cc1)-c1ccccn1
107	(4aR,10aS)-6-methoxy-1,2,3,4,4a,10a-hexahydrophenanthrene	COc1ccc2C=CC3CCCCC3c2c1
108	9-Methyl-trans-1,2,3,4,4a,10a-hexahydrophenanthrene	CC1=CC2CCCCC2c2ccccc12
109	4-(1-phenylureido)benzoic acid	NC(=O)N(c1ccccc1)c1ccc(cc1)C(O)=O
110	10-Methoxyacenaphtho(1,2-b)quinoline	COc1ccc2nc3c(cc2c1)-c1cccc2cccc-3c21
111	4-(5-cyano-3-oxopentyl)benzoic acid	OC(=O)c1ccc(CCC(=O)CCC#N)cc1
112	2,5,9,11-Tetramethyl-5H-quinindoline	CC1C2C(=Nc3ccc(C)cc23)N(C)c2ccc(C)cc12