Proposal of an *in silico* profiler for categorisation of repeat dose toxicity data of hair dyes

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Abstract

This study outlines the analysis of repeat dose toxicity data taken from Scientific Committee on Consumer Safety (SCCS) opinions for commonly used hair dyes in the European Union. Structural similarity was applied to group these chemicals into categories. Subsequent mechanistic analysis suggested that toxicity to mitochondria is potentially a key driver of repeat dose toxicity for chemicals within each of the categories. The mechanistic hypothesis allowed for an *in silico* profiler consisting of mechanism-based structural alerts to be proposed. This *in silico* profiler is intended for grouping chemicals into mechanism-based categories within the Adverse Outcome Pathway paradigm.

Introduction

Significant changes in the European cosmetic and chemical legislations during the last decade have concentrated efforts in the development of alternative methods to animal experimentation for safety testing purposes (Commision, 2003; Commision, 2007). The Adverse Outcome Pathway (AOP) paradigm has emerged as a promising approach in that it enables key events in the pathway that leads to a toxicological outcome to be identified (Ankley *et al.*, 2010; Vinken, 2013; Vinken *et al.*, 2013). Key amongst these events is the Molecular Initiating Event (MIE) which has been the focus for the development of *in silico* profilers (Przybylak and Schultz, 2013). These profilers define the chemical features associated with a given MIE in terms of collections of structural alerts and are intended to be used to categorise chemicals based on a common MIE (Enoch *et al.*, 2011a; Enoch *et al.*, 2013a; Enoch and Roberts, 2013; Przybylak and Schultz, 2013; Sakuratani *et al.*, 2013a; Sakuratani *et al.*, 2013b; Vinken, 2013; Vinken, Whelan and Rogiers, 2013). The mechanism-based categories of chemicals that result from such AOP-derived profilers are applicable to predict hazard via read-across and hence assist in the filling of data gaps. In addition, these groupings also form the basis for the more in-depth analysis that is required for an overall risk assessment. In such a situation, additional testing using *in vitro* and/or *in chemico* methods to assess other key steps in the AOP is likely to be required. The ability to group chemicals into mechanism-based categories using *in silico* profilers enables advanced testing strategies to be developed based on the prioritisation of chemicals and their testing in the more elaborated and costly assays (Gutsell and Russell, 2013).

Repeat dose toxicity results are, however, available for cosmetic ingredients present on the Annexes of Cosmetic Regulation 1223/2009. Indeed, for cosmetic substances for which some concern exists with respect to human health, e.g. colorants, preservatives, UV-filters and hair dyes. These data, consisting of No Observable Adverse Effect level (NOAEL)-values are available through the so-called opinions of the Scientific Committee on Consumer Safety (SCCS) and its predecessors, the Scientific Committee on Cosmetic products and Non-Food Products intended for consumers (SCCNFP) and the Scientific Committee on Consumer Products (SCCP). Clearly, such data could provide a useful starting point for developing MIEs and identifying the chemistry required for the grouping of chemicals for read-across.

In particular for hair dyes, high quality toxicological data became available as a consequence of the step-wise strategy of the European Commission to regulate all hair dyes listed as substances in cosmetic products. As such, industry was required to submit safety dossiers for hair dye components and possible mixtures for evaluation by the Scientific Committees. The trigger for this action was the major concern of the scientific community for a putative link between the use of hair dyes and the development of cancer, with a focus on leukaemia and bladder cancer (Gago-Dominguez *et al.*, 2001). Despite the requirement to assess the toxicity of hair dyes, few models or structural alerts for their toxic effects, or rationale for their grouping, is currently available.

Therefore, the aim of this study is to propose an *in silico* profiler from the retrospective analysis of the oral repeat dose toxicity data available for hair dyes and retrieved from the Scientific Committees opinions published between 2000 and 2013. Mechanistic information relating these structural alerts to potential MIEs was sought from the peer reviewed literature.

Methods

Experimental data

NOAEL values from oral 90-day rat studies for 94 hair dyes were extracted from the opinions of the SCCS and its predecessors between 2000 and 2013. Chemical names, CAS numbers and chemical structures were also taken from these reports. These data were used in the chemoinformatics analysis leading to the development of mechanism-based structural alerts. All data are available as supplementary information in the form of an Excel workbook.

Development of mechanism-based structural alerts

The development of mechanism-based *in silico* profilers suitable for category formation is a time-consuming, literature-intensive process. Previous research leading to the establishment of *in silico* profilers for toxicological endpoints such as skin and respiratory sensitisation utilised a mechanistic hypothesis as a starting point for structural alert development (Enoch *et al.*, 2008; Enoch *et al.*, 2012a). However, for complex endpoints such as organ-specific toxicity for which knowledge relating to possible MIEs is lacking, a chemoinformatics approach, coupled with *a posteri* mechanistic rationalisation, has been shown to be successful (Hewitt *et al.*, 2013). Given the complexity of potential mechanisms driving oral repeat dose toxicity, the current study employed the latter approach using the protocol described hereafter.

Structural similarity-based category formation

All chemical structures were encoded as SMILES strings, neutralised and salts removed *prior* to chemical similarity analysis. Structural similarity of each chemical to all others in the dataset was calculated using the atom environments/Tanimoto coefficient approach as implemented in the freely available Toxmatch software (V1.07). Categories were developed for each chemical in the dataset using an in-house code implemented in Excel software that identified analogues with a similarity index of 0.7 or greater. Categories containing three or more chemicals were selected for further analysis.

Structural alert-based category formation

Each similarity-based category containing three of more chemicals was visually inspected in order to identify key structural fragments present in all category members. This structural fragment was then encoded as a SMARTS pattern-based structural alert. Each chemical in the dataset was subsequently profiled against these structural alerts in order to expand the groupings to include chemicals that were not found by the structural similarity analysis. This is an important step in the protocol as pure structural similarity-based categories are frequently unable to detect chemicals containing the key structural fragments. Structural alerts were then subjected to a mechanistic analysis involving detailed literature work in order to outline an MIE for the corresponding category members. This mechanistic analysis involved establishing potential MIEs related to chronic toxicity and linking them to the

chemistry of the structural alerts. Structural alerts were only considered as robust if a clear correlation between their chemistry and an MIE identified from relevant scientific literature could be established.

Development of a refined set of structural alerts and in silico profiler

The final stage in the analysis was to use the mechanistic knowledge to extend the applicability domain of the structural alerts enabling an *in silico* profiler to be developed. This analysis involved identifying additional structural alerts capable of triggering the same MIEs based on chemical information. The mechanistic rationale for these additional structural alerts was supported by evidence drawn from the scientific literature. All structural alerts identified in this study were then collated into an *in silico* profiler that allowed chemicals capable of causing the same MIE to be assigned to a single category. In keeping with the development of previous *in silico* profilers, the structural alerts were described within the resulting *in silico* profiler based on commonality of the underlying chemistry.

Results and discussion

The aim of this study was to develop an *in silico* profiler suitable for chemical categorisation of oral repeat dose toxicity data of hair dyes. The analysis involved utilising chemical similarity to identify groups of chemicals from a dataset of 94 hair dyes. Data related to repeat dose toxicity, as obtained from 90-day oral rat studies, were extracted from published SCC(NF)P and SCCS opinions with NOAEL values ranging from 0.3 mg/kg/day up to 1000 mg/kg/day. The similarity analysis identified four categories of hair dyes containing either a 2-nitroaminobenzene, 4-nitroaminobenzene, aromatic azo or anthraquinone moieties. These key structural fragments were used to develop a mechanistic hypothesis for the MIE for each category. This analysis resulted in the definition of four structural alerts related to the ability of aromatic chemicals to disrupt mitochondrial function due to their free radical chemistry. This mechanistic chemistry allowed an *in silico* profiler containing a refined set of structural alerts to be defined. The resulting *in silico* profiler assigned 56 of the 94 chemicals in the dataset to a mechanism-based chemical category. However, further experimental analysis is required to identify additional key steps to allow an AOP (or AOPs) to be defined.

Development of mechanism-based structural alerts for category formation

The chemo-informatics analysis identified four similarity-based categories in the dataset, defined as a cluster containing three of more analogues. These included 2-nitroaminobenzenes, 4-nitroaminobenzenes, aromatic azos and anthraquinones. In all datasets, a structural alert was defined based on the key fragment in each of the clusters. These structural alerts were used to identify additional related chemicals not identified by the structural similarity analysis. This is a crucial step in the development of mechanism-based structural alerts when using chemical similarity to cluster the initial dataset as related chemicals are frequently omitted. The resulting structural alerts and the number of analogues identified using them to re-analyse the data are summarised in Table 1.

[Table 1 here]

2-nitroaminobenzene and 4-nitroaminobenzene structural alerts

A total of 26 chemicals were identified using the 2-nitroaminobenzene and 4-nitroaminobenzene structural alerts. The nitro group in these chemicals can be readily reduced to an amino moiety by nitroreductase via a hydroxylamine intermediate in the gut and the liver resulting in the production of 1,2- and/or 1,4-diaminobenzenes (Gorontzy *et al.*, 1993; Roldan *et al.*, 2008). These chemicals are then prone to oxidation to the corresponding 1,2- and/or 1,4-phenylenediamines (Figure 1).

[Figure 1 here]

Importantly, the conversion of 1,2-diaminobenzenes into 1,2-phenylenediamines is reversible implying that these chemicals are capable of cycling electrons. This also holds true for the corresponding 1,4-diaminobenzenes. It is known that this electron cycling mechanism allows these types of chemicals to interfere with the electron transport chain within the mitochondria (Wallace and Starkov, 2000). The mechanism leading to disruption could therefore involve the 1,2-diaminobenzene moiety within a chemical accepting an electron from respiratory complex I. This could oxidise the 1,2-diaminobenzene moiety to a 1,2-phenylenediamine which thereafter could transport the electron several steps down the respiratory chain directly into complex VI. The release of the electron would then reduce 1,2-phenylenediamine back to a 1,2-diaminobenzene allowing the process to be repeated in a cyclic fashion (Figure 2). This disruption ultimately could lead to a reduction in mitochondrial membrane potential and a subsequent reduction in ATP production (Bironaite *et al.*, 1991; Chan *et al.*, 2005; Munday, 1992; Wallace and Starkov, 2000).

[Figure 2 here]

The aromatic amine moiety of the reduction products is also known to induce uncoupling of oxidative phosphorylation via a protonophoric mechanism (Terada, 1990) (Figure 3). The deprotonated form of these compounds scavenges a free proton from the inner membrane space. Upon protonation the compound is able to migrate across the inner mitochondrial membrane into the mitochondrial matrix. Due to the increased alkaline environment within the matrix the proton dissociates and the deprotonated compound returns to the inner membrane space enabling the cycle to continue. The continuation of this cycle increases oxygen consumption and heat production, alongside a reduction in the electrochemical gradient and ATP production (Chan, Truong, Shangari and O'Brien, 2005; Pessayre *et al.*, 2012; Terada, 1990; Wallace and Starkov, 2000). Therefore, it is suggested that both mechanisms might contribute to the observed mitochondrial dysfunction.

[Figure 3 here]

Anthraquinone structural alert

The structural alert based on the anthraquinone moiety identified a total of 5 chemicals in the dataset. These chemicals have also been shown to be capable of disrupting the electron transport chain in mitochondria by transporting electrons from respiratory complex I directly to complex IV (Henry and Wallace, 1995; Kitani *et al.*, 1981). This process is similar to that outlined for 1,2- and 1,4-diaminobenzenes in that the anthraquinone moiety accepts an electron from complex I to become a semi-quinone radical. This radical species could transport an electron directly to complex IV, being oxidised back to the anthraquinone in the process (Figure 4). Again, this reaction is reversible allowing the anthraquinone moiety to cycle electrons repeatedly from respiratory complex I to complex IV. In addition to acting as direct electron transport agents, the production of the semi-quinone radical has also been suggested to cause indirect mitochondrial toxicity due to their ability to react with molecular oxygen to produce reactive oxygen species. The chemical species include hydroxyl and superoxide radicals that are capable of evoking widespread damage to mitochondrial DNA, proteins and lipids (Kappus, 1986; Ohkuma *et al.*, 2001).

[Figure 4 here]

Aromatic azos structural alert

The final structural alert identified from the similarity analysis related to chemicals containing an aromatic azo moiety and identified 6 chemicals from the dataset. Chemicals containing an aromatic azo linkage are readily reduced to the free amine by the enzyme azoreductase (Nam and Renganathan, 2000). The presence of an additional nitro, amine or hydroxyl group in the 2- or 4-position on at least one of the aromatic rings could result in the possibility of the production of a 1,2- or 1,4-diaminobenzene moiety (Figure 5). This moiety might then act as an electron cycling agent resulting in the disruption of the respiratory chain in the mitochondria, as outlined previously for the 2-nitroaminobenzene and 4-nitroaminobenzene clusters.

[Figure 5 here]

Additional chemicals capable of electron cycling

The mechanistic chemistry outlined for the four structural alerts identified from the similarity analysis suggests that the ability to cycle electrons might represent a key MIE for mitochondrial toxicity for aromatic chemicals of this type. The mechanistic analysis further suggests that chemicals capable of forming free radicals could trigger this type of MIE resulting in toxicity. Therefore, it should be possible to develop additional structural alerts based around this mechanistic chemistry to increase the applicable chemical space relating to the MIE with respect to electron cycling. For example, based on the analysis of the chemistry outlined above for the 2-nitroaminobenzene category, it is conceivable to assume that chemicals containing a 1,2-diaminobenzene moiety would also be capable of cycling electrons, as this structure is one of the key intermediates in the mechanism proposed in Figure 1. Table 2 defines a refined set of structural alerts of mechanistically related chemicals. It should be noted, however, that the quinone structural alert includes chemicals containing an anthraquinone moiety. The aromatic azo structural alert is included in Table 2 for completeness.

[Table 2 here]

The majority of the mechanistic chemistry for these structural alerts is as discussed previously (Table 2). The scope of the pro-quinone structural alert is significantly extended as evidenced by the number of chemicals assigned to the resulting category. This is due to the extensive additional mechanistic chemistry knowledge in the wider literature relating to the types of chemicals readily converted to the corresponding quinones (Enoch *et al.*, 2011b;

Kalgutkar *et al.*, 2005). In addition, the anthraquinone structural alert has been extended to cover quinones, based on the related chemistry and the proven ability of these chemicals to disrupt the respiratory chain in mitochondria via the same mechanism (Henry and Wallace, 1995; Kitani, So and Miller, 1981; Scatena *et al.*, 2007). In contrast, the mechanistic chemistry for the three 1,3-substituted structural alerts is somewhat different to the remaining structural alerts in that these chemicals are not able to form quinone-type species due to the 1,3-motif. However, it has been reported that they are capable of causing toxicity via a free radical mechanism (Aptula *et al.*, 2009) (Figure 6). Thus, the inclusion of these three structural alerts can be justified based on the hypothetical mechanistic rationale that a key MIE for mitochondrial toxicity could be electron cycling due to free radical formation.

[Figure 6 here]

Mitochondria and repeat dose toxicity

The hypothetical mechanistic analysis presented above suggests that chemicals capable of free radical chemistry might disrupt the respiratory chain in the mitochondria leading to chronic toxicity. This is in keeping with previous research into the cardiotoxicity of anthracyclines upon extended low dose exposure (Montaigne *et al.*, 2012). This adverse reaction has been shown to be related to mitochondrial dysfunction which results in the activation of a number of protein kinases. The MIE for this toxicity has been suggested to involve the ability of the quinone moiety within these drugs to form a semi-quinone radical and thus cycle electrons (Figure 3). In addition, these chemicals have been shown to form a variety of reactive oxygen species also capable of disrupting the normal function of mitochondria. It has also been suggested that mitochondrial dysfunction is a key driver in chronic toxicity (Kovacic and Jacintho, 2001a; Kovacic and Jacintho, 2001b; Porceddu *et al.*, 2012; Vinken, Whelan and Rogiers, 2013). A recent study also outlined how for the same chemical the mechanism driving toxicity can change on-going from acute to chronic exposure (Nikam *et al.*, 2013). The importance of mitochondrial dysfunction as a driver of chronic toxicity has recently also led to the definition of a number of structural alerts, one of which (2-aminonitrophenol) was included in the current study (Naven *et al.*, 2013).

[Table 3 here]

The data in Table 3 highlights that a variety of adverse effects within multiple organs are associated with the NOAEL values for chemicals assigned to each category. This variability

in the toxicity profile adds weight to the hypothesis that the observed toxicity might have been initiated by mitochondria dysfunction. This is due to the fact that mitochondria are present within most organ systems, performing a number of roles vital to normal cellular functioning. There is an extensive body of literature outlining a range of chemicals that inhibit the mitochondrial physiology resulting in toxicity at the organ level (Dykens and Will, 2008). Typically, the most susceptible organs are those containing a higher concentration of mitochondria, those exposed to a higher concentration of the compound and/or those with a higher aerobic energy demand, such as the liver, kidney and cardiac muscle (Amacher, 2005; Dykens and Will, 2008; Dykens and Will, 2007). In addition, it has been recognised by the pharmaceutical industry that mitochondrial dysfunction may be a cause of numerous toxicities within a variety of organs, and has led to the withdrawal of a number of therapeutic drugs (Amacher, 2005; Dykens and Will, 2008; Dykens and Will, 2007; Pessayre, Fromenty, Berson, Robin, Letteron, Moreau and Mansouri, 2012).

Adverse Outcome Pathway concept, perspectives and proposed future work

The analysis presented above outlines how structural alerts related to potential MIEs could be derived. The main focus of this type of analysis is the development of the mechanistic chemistry relating the structural alerts to a possible MIE. This focus is a process that involves an in-depth survey of relevant scientific literature in support of the mechanistic hypothesis made, enabling *in silico* profilers to be developed for a given MIE. The current study has resulted in the development of a profiler capable of identifying chemicals that could cycle electrons and thus lead potentially to the disruption of the respiratory chain in the mitochondria. An important aspect of the on-going development of *in silico* profilers is the experimental verification of the mechanistic hypothesis, which increases confidence in the prediction of an MIE for an untested chemical. Such analysis has been recently undertaken for the *in silico* profilers relating to covalent protein binding in the OECD QSAR Toolbox (Enoch *et al.*, 2012b; Enoch *et al.*, 2013b; Nelms *et al.*, 2013; Rodriguez-Sanchez *et al.*, 2013). In terms of the current study, future work would consist of testing of a representative number of hair dyes/chemicals from each of the categories outlined to cause mitochondrial toxicity in an *in vitro* experimental set-up. In the longer term, the applicability domain of the *in silico* profiler could then be much better defined through the use of directed and intelligent testing of compounds using assays appropriately defined by the key events of the AOP.

To be able to predict oral repeat dose toxicity reliably, it is necessary, in addition to defining the applicability domain of the *in silico* profiler and by extension the MIE associated with the profiler, to generate extensive knowledge of subsequent key events in the AOP leading to toxicity. This requirement is highlighted by the broad range of oral NOAEL values for the categories derived in the current study which vary between one and two orders of magnitude (Table 2 for the ranges, Table 3 for each chemical within each category). Importantly, these values show the limitations of the *in silico* profilers ability to predict oral repeat dose toxicity. Assuming no additional information is available, the most realistic prediction for an untested chemical, assigned to one of the categories, would be to state that the oral NOAEL value would be likely to fall within the range of the values for the other category members. However, even this type of prediction may not be appropriate, in that the new untested chemical could be capable of altering a downstream key event in the AOP in a different manner to the remaining category members. It is also possible that the chemical may have a different toxicokinetic and/or dynamic profile to the other category members. It is therefore essential that the mechanistic information relating to the MIE contained within an *in silico* profiler is complimented with information derived from other existing *in vivo* data, *in vitro*, *in silico* or *in chemico* tests designed to target other key events in the AOP (and relating to toxicokinetics and dynamics). Only when a significant proportion of this information is available will the estimation of values such as NOAELs become possible without using animal models.

Conclusions

This study proposes an *in silico* profiler for chemicals used as hair dyes capable of causing mitochondrial dysfunction. It is based on a retrospective analysis of oral repeat dose toxicity data for 94 hair dye chemicals and is intended for use in grouping and category formation. It is important to note that the proposed profiler does not predict oral repeat dose toxicity; instead it provides arguments for a key MIE that might be responsible for initiating an AOP leading to chronic toxicity. This work generally shows that detailed mechanistic analysis is required for the development of *in silico* profilers and explains how such analysis can be used to identify potential MIEs. Clearly future *in vitro* work must be undertaken to outline additional key events in biological pathway before a relevant and complete AOP could be established.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Figure 1: Reduction of 2-nitroaminobenzene to the corresponding 1,2-diaminobenzene and then subsequent oxidation to a 1,2-phenylenediamine

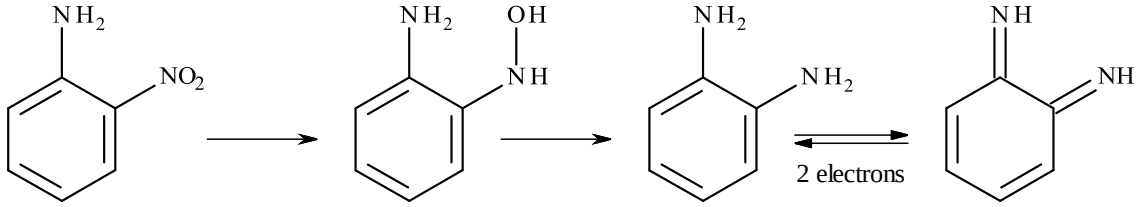
Figure 2: Electron cycling process leading to disruption of the respiratory chain in the mitochondria due to the presence of an alternate electron acceptor

Figure 3: Cycling of the compound within the inner membrane space (IMM), scavenging hydrogen ions from within the inner membrane space (IMS) and transporting them to the mitochondrial matrix (MM)

Figure 4: Activation of the anthraquinone moiety into a semi-quinone radical

Figure 5: Reduction of aromatic azo compounds producing a 1,4-diaminobenzene and 1,4-phenylenediamine capable of cycling electrons

Figure 6: Free radical mechanism for 1,3-diaminobenzene (an analogous mechanism is possible for the 1,3-dihydroxybenzene and 3-hydroxyaminobenzene containing chemicals)



hydroxylamine 1,2-diaminobenzene 1,2-phenylenediamine

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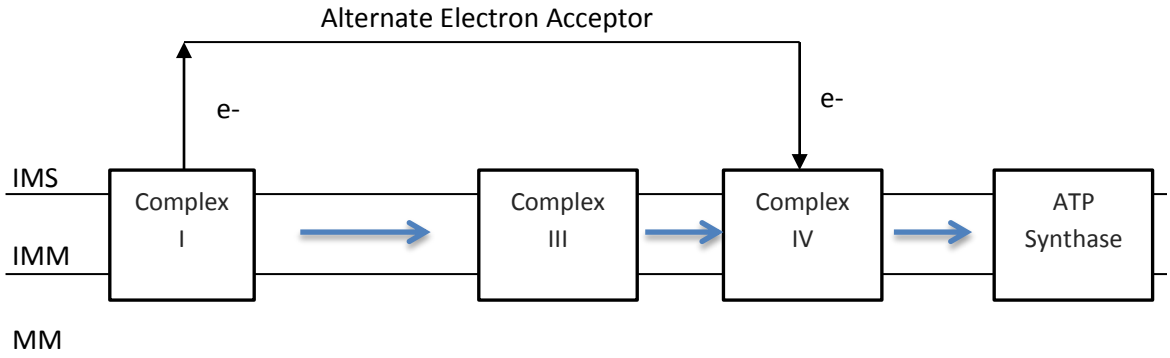
Alternate Electron Acceptor
e-
e-
IMS
IMM
MM
Complex I
Complex III
Complex IV
ATP Synthase

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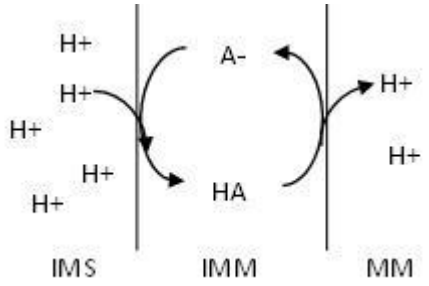

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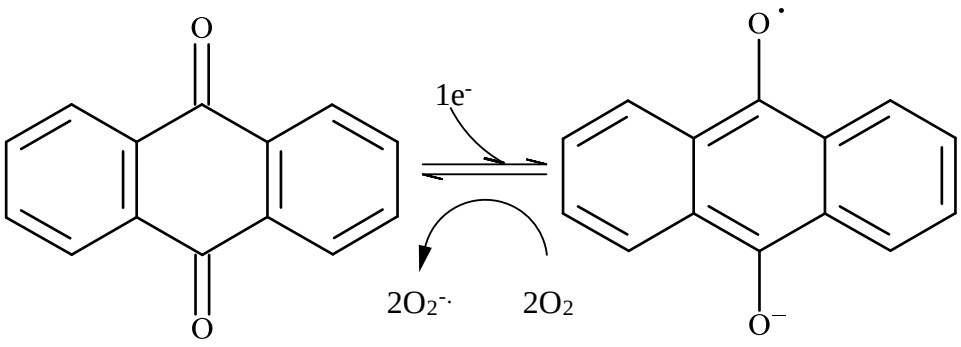

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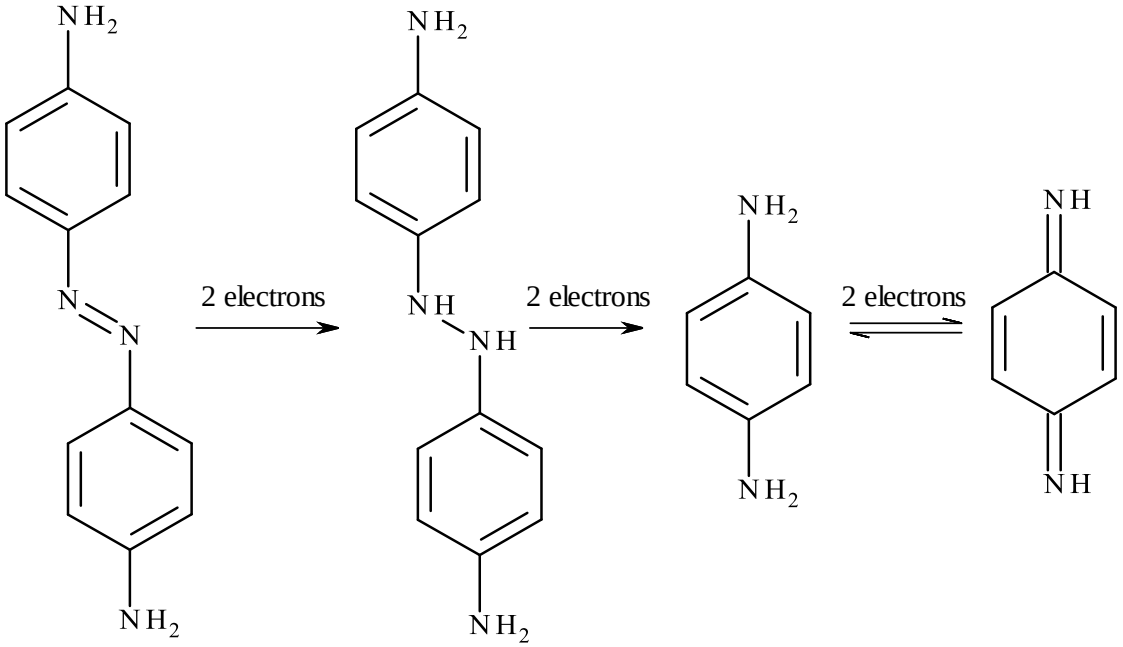
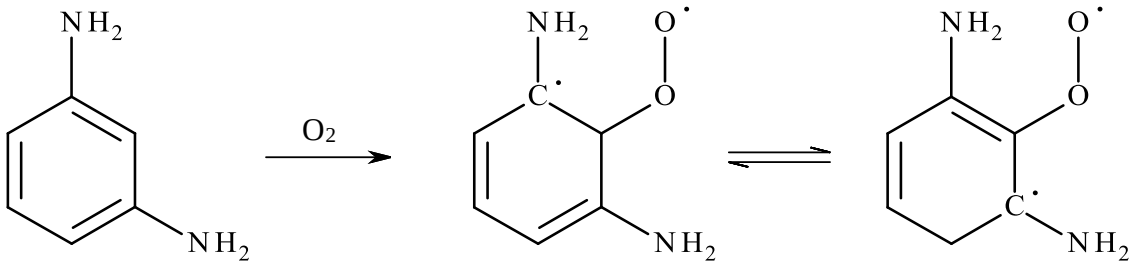

NH2
N
N
NH2
2 electrons
NH2
NH
NH
NH2
2 electrons
NH2
NH2
2 electrons
NH
NH

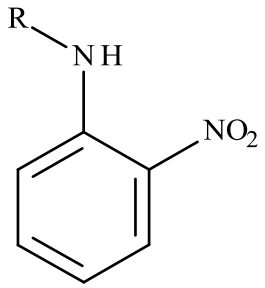
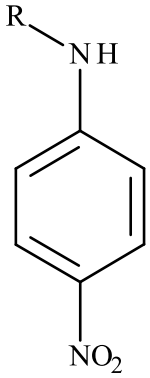
473

474



475

476 Table 1: Structural alerts identified from the similarity analysis carried out on the 93 hair dye
477 chemicals

Structural alert	Key structural fragment	Number of analogues
2-nitroaminobenzenes	 R = hydrogen, carbon	20
4-nitroaminobenzenes	 R = hydrogen, carbon	6

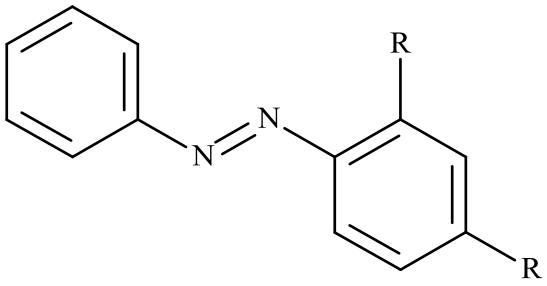
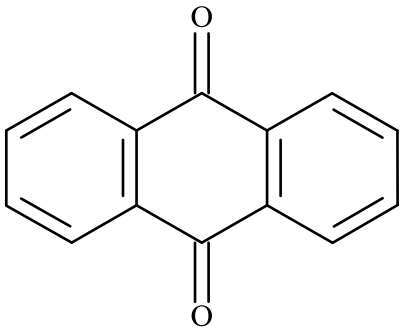
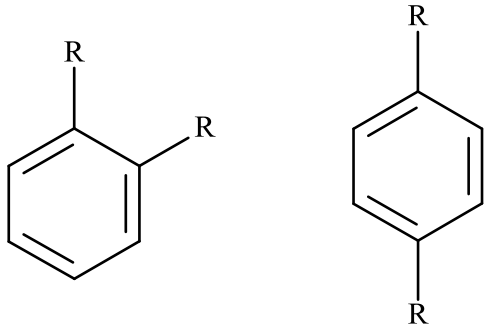
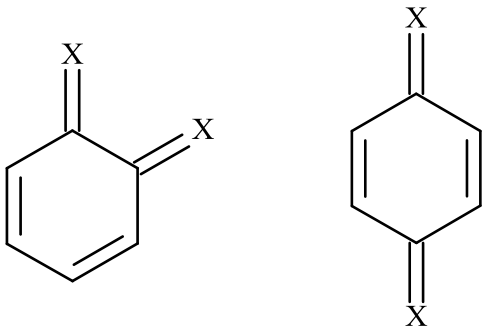
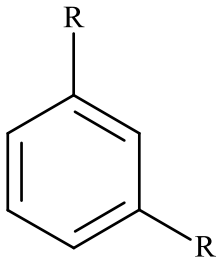
Aromatic azos	 R = at least must be NH_2, NH	6
Anthraquinones		5

Table 2: Refined set of structural alerts capable of free radical cycling chemistry (NOAEL values relate to 90-day oral rat studies)

Name	Key structural features	Number of chemicals	oral NOAEL ranges (mg/kg/day)	Figure
Pro-quinones (R = OH, NH_2, NH, NO_2)		39	1.4 – 250.0	1

Quinones (X = NH,O)		7	2.0 – 200.0	3
Meta-substituted benzenes (R = NH_2, OH)		4	50.0 – 100.0	5

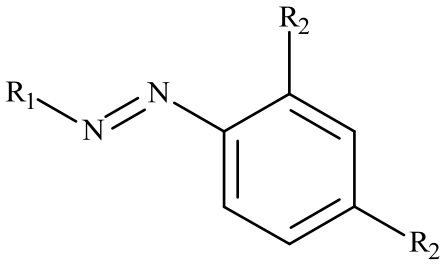
Aromatic azos (R_1 = aromatic carbon) (R_2 = NH_2, NH, OH)		6	0.3 – 52.6	4

Table 3: Repeat dose data the chemicals grouped into categories by the structural alerts defined in the current study (AAT – alanine aminotransferase, APTT – activated partial thromboplastin time, AST – aspartate aminotransferase, BWG – body weight gain, GI – gastrointestinal, MCH – Mean Corpuscular/cell Haemoglobin, MCV – Mean Corpuscular/cell Volume, PT – Prothrombin Time, RBC – Red Blood Cell)

ID	Category	Chemical	NO(A)EL (mg/kg bw/day)	LO(A)EL (mg/kg bw/day)	Reported adverse effects
1	Quinone	Disperse Violet 1	2	20	↑ Centrilobular/Midzonal hepatocyte hypertrophy ↑ Triglycerides (♀) ↑ Cholesterol ↓ Motor activity
2	Quinone	Lawsone	2	7	↓ Erythrocyte count (♀) ↓ Blood urea (♀) ↓ Albumin:Globulin ratio (♀) ↑ Bilirubin (♀) ↑ Kidney weight (♀) ↓ Blood glucose (♂) ↑ Triglycerides (♂) ↑ Haematopoiesis, spleen (♂) ↑ (Multi)focal ulceration of mucosa, forestomach ↑ Interstitial oedema, forestomach
3	Quinone	Acid Green 25	100	300	↑ Kidney weight
4	Quinone	HC Green No. 1	100	300	↓ Food consumption (♀) ↓ Body weight (♀) ↑ Hypokalemia ↑ Oliguria (♂)
5	Quinone	Acid Blue 62	300	1000	↑ Kidney weight ↑ Liver weight

					↑ Ptyalism ↑ Tubular nephrosis ↑ Centrilobular hepatocyte hypertrophy ↑ Blood Urea ↑ Albumin ↑ Cholesterol ↑ AAT ↓ Body weight ↓ Glucose
6	Quinone	Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate	200	800	↓ Absolute thymus weight (♀) ↓ Body weight (♂) ↓ Relative thymus weight
7	Quinone	Acid Violet 43	300	1000	↑ PT ↑ APTT
8	Pro-quinone	Toluene-2,5-diamine	10	20	↑ AST ↑ Mononuclear cell infiltrates, diaphragm ↑ Mononuclear cell infiltrates, eye ↑ Mononuclear cell infiltrates, thigh ↑ Mononuclear cell infiltrates, tongue ↑ Muscular degeneration, diaphragm ↑ Muscular degeneration, thigh ↑ Muscular degeneration, tongue ↑ Muscular regeneration, diaphragm
9	Pro-quinone	Picramic acid	5	15	↑ Ulceration of GI tract ↑ Inflammation of GI tract ↑ Fibrosis of GI tract ↑ Tubular cell swelling ↑ MCV ↑ MCH ↑ Reticulocyte count
10	Pro-quinone	HC Red No. 13	No NO(A)EL	5	↑ Creatinine (♀)

					↑ Kidney weight ↑ PT (♂) ↓ Albumin:Globulin ratio (♀) ↓ Glucose (♀) ↓ MCH (♂) ↓ MCV
11	Pro-quinone	2,2'-Methylenebis-4-aminophenol	5	15	↑ Cast formation, kidney ↑ Thickened basement membrane, kidney ↑ Tubular basophilia, kidney ↑ Tubular degeneration, kidney
12	Pro-quinone	4-Nitrophenyl aminoethylurea	5	25	↓ RBC count ↓ Haemoglobin concentration ↑ MCV ↑ Reticulocyte count ↑ Extramedullary haematopoiesis, spleen ↑ Haemosiderosis (♀) ↓ Packed cell volume (♂)
13	Pro-quinone	HC Red No. 1	5	20	↓ Erythrocytes (♀) ↑ Leukocytes (♀) ↑ Lymphocytes (♀) ↓ Thymus weight (♂) ↑ MCH (♂)
14	Pro-quinone	Tetrahydro-6-nitroquinoxaline	5	25	↑ Ptyalism ↑ Liver weight ↑ Spleen weight
15	Pro-quinone	p-Phenylenediamine	8	16	↑ Myodegeneration, skeletal muscle
16	Pro-quinone	2-Chloro-6-ethylamino-4-nitrophenol	10	30	↑ Liver weight
17	Pro-quinone	Dihydroxyindoline	10	20	↑ Pigmentation, kidney
18	Pro-quinone	PEG-3-2',2'-di-p-phenylenediamine	10	25	↑ Intracellular pigmentation, kidney tubules ↑ Pigmentation, thyroid epithelium ↑ Pigmentation, duodenum

19	Pro-quinone	p-Methylaminophenol sulphate	10	30	↑ Tubular epithelial degeneration, kidney ↑ Single cell necrosis, kidney ↓ Specific gravity (♂) ↑ Urinary volume (♂)
20	Pro-quinone	2-Hydroxyethyl picramic acid	15	60	↑ Protein cylinders, kidneys ↑ Activation of thyroid epithelial cells
21	Pro-quinone	HC Yellow No. 13	21	90	↑ Degeneration, Islet cells ↑ Inflammation, endocrine pancreas ↑ Fibrosis, endocrine pancreas ↑ Serum cholesterol (♂)
22	Pro-quinone	3-Methylamino-4-nitrophenoxyethanol	25	100	↑ Ptyalism ↓ Lymphoid in thymus
23	Pro-quinone	HC Orange No.1	25	No LO(A)EL	Nothing reported
24	Pro-quinone	2-Amino-6-chloro-4-nitrophenol	30	90	↓ Body weight ↑ Kidney weight
25	Pro-quinone	4-Hydroxypropylamino-3-nitrophenol	30	90	↑ Thyroid weight ↓ AST
26	Pro-quinone	Acid Yellow 1	30	100	↑ Mean absolute reticulocyte ↑ Haematopoiesis ↑ Lesions, caecum ↑ Lesions, intestine ↑ Lesions, liver ↑ Lesions, spleen ↑ Haemosiderosis (♀) ↑ MCV (♀) ↑ Spleen weight (♂)
27	Pro-quinone	1,2,4-Trihydroxybenzene	50	100	↑ Piloerection ↑ Ptyalism ↑ Mean RBC volume ↑ MCH ↑ Platelets ↓ Haematocrit

					↓ RBC count ↓ Haemoglobin ↑ Kidney weight ↑ Liver weight ↑ Spleen weight ↓ Body weight (♂)
28	Pro-quinone	4-Amino-3-nitrophenol	50	250	↑ Liver weight (♂)
29	Pro-quinone	HC Violet No. 2	50	200	↑ Liver weight ↓ RBC ↓ PT
30	Pro-quinone	HC Yellow No. 11	50	200	↑ Acidophilic globules in cortical tubular epithelium ↑ Liver weight (♀) ↑ Kidney weight ↓ Thymus weight ↓ Creatinine
31	Pro-quinone	HC Yellow No. 2	50	No LO(A)EL	Nothing reported
32	Pro-quinone	2-Nitro-4-amino-diphenylamine-2'-carboxylic acid	60	180	↑ Thrombocytes ↑ Water consumption (♀)
33	Pro-quinone	4-Amino-m-cresol	60	120	↑ Spleen weight
34	Pro-quinone	HC Blue No. 12	60	No LO(A)EL	Nothing reported
35	Pro-quinone	HC Blue No. 11	80	160	↑ Kidney weight ↑ Vacuolated tubular cell
36	Pro-quinone	HC Red No. 3	90	250	↓ Body weight
37	Pro-quinone	2-Hydroxyethylamino-5-nitroanisole	100	500	↑ Liver weight ↑ Spleen weight ↑ PT ↑ Fibrinogen level ↑ Blood urea nitrogen ↑ AAT (♂) ↑ Urinary volume

38	Pro-quinone	HC Orange No. 3	100	300	↑ Kidney weight ↑ Liver weight ↑ Spleen weight ↑ AAT ↑ AST
39	Pro-quinone	HC Yellow No. 10	100	500	↑ Staining, body ↑ Staining, fur ↑ Body weight ↑ Ptyalism ↑ Food consumption ↑ Liver weight ↑ Spleen weight (♂)
40	Pro-quinone	HC Orange No. 2	150	500	↑ Ptyalism ↓ BWG ↓ Food consumption ↓ Blood glucose
41	Pro-quinone	Acid Blue 62	300	1000	↑ Kidney weight ↑ Liver weight ↑ Ptyalism ↑ Tubular nephrosis ↑ Centrilobular hepatocyte hypertrophy ↑ Blood urea ↑ Albumin ↑ Cholesterol ↑ AAT ↓ BWG ↓ Glucose
42	Pro-quinone	2-Nitro-5-glyceryl methylaniline	200	800	↑ Ptyalism ↑ Vacuolated pancreatic cells ↑ Vacuolated renal tubular cells ↑ Tubular nephrosis ↑ Piloerection

					↑ Hunched back ↑ Hypokinesia ↑ Bilateral opacity ↑ Adrenal weight ↑ Kidney weight ↑ Liver weight ↓ BWG
43	Pro-quinone	3-Nitro-p-hydroxyethylaminophenol	200	No LO(A)EL	Nothing reported
44	Pro-quinone	N,N'-bis(hydroxyethyl)-2-nitro-p-phenylenediamine	240	720	↑ Kidney weight ↑ Liver weight ↓ Activity (♀) ↓ Ataxia (♀) ↑ Ptyalism (♀) ↑ Ocular discharge (♀) ↑ Lethargy (♀) ↑ Hunched posture (♀) ↑ Triglycerides (♂) ↑ Urea (♂) ↑ Urinary specific gravity
45	Pro-quinone	HC Yellow No. 4	250	500	↓ Body weight ↑ Thyroid lesions ↑ Uterine lesions (♀) ↑ Kidney lesions (♂) 1 Mortality
46	Pro-quinone	HC Yellow No. 9	250	No LO(A)EL	Nothing reported
47	Meta-hydroquinone	5-amino-6-chloro-o-cresol	No NO(A)EL	100	↑ Centrilobular hepatotrophy, liver ↑ MCV ↑ Mean corpuscular Hb (♀) ↑ MCH concentration (♀)
48	Meta-hydroquinone	3-Amino-2,4-dichlorophenol	80	160	↑ Liver degeneration ↑ Liver necrosis

					↑ Foci mononuclear cell infiltration ↑ Kidney degeneration ↑ Kidney necrosis ↑ Tubular epithelial cell hypertrophy ↑ Phosphorus (♂) ↑ Sodium (♂) ↑ Chloride (♂)
49	Meta-hydroquinone	2,6-Dihydroxyethylaminotoluene	100	316	↑ Bilirubin ↑ Urobilinogen ↓ Serum creatinine (♀)
50	Meta-hydroquinone	2-Methylresorcinol	100	200	↑ Clonic spasms ↑ Ptyalism ↑ Scratching movements ↑ Body weight (♂) ↑ Liver weight (♂) ↑ AST (♂) ↑ AAT (♂)
51	Aromatic azo	Basic Brown 16	50	150	↓ BWG (M)
52	Aromatic azo	Basic Brown 17	60	120	↑ Extramedullary haemopoiesis
53	Aromatic azo	Basic Red 76	20	60	↓ RBC (M) ↓ Haemoglobin ↓ Haematocrit (M) ↓ MCH concentration (F)
54	Aromatic azo	Disperse Black 9	100	No LO(A)EL	Nothing reported
55	Aromatic azo	Disperse Red 17	10	30	↑ Spleen weight
56	Aromatic azo	HC Yellow N^{o}. 7	10	40	↑ Kidney weight ↑ Bilateral discolouration of fundus ↑ Pytalism ↑ Tubular basophilia ↑ Blood phosphorous (F) ↓ Blood glucose (F) ↑ Blood sodium (M)