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The development of an in silico profiler for mitochondrial toxicity

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Keywords

Structural alerts, mitochondrial toxicity, AOP, profiler, MIE
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Abstract

This study outlines the analysis of mitochondrial toxicity for a variety of pharmaceutical drugs extracted from Zhang et al. These chemicals were grouped into categories based upon structural similarity. Subsequently, mechanistic analysis was undertaken for each category to identify the Molecular Initiating Event driving mitochondrial toxicity. The mechanistic information elucidated during the analysis enabled mechanism-based structural alerts to be developed and combined together to form an \textit{in silico} profiler. This profiler is envisaged to be used to develop chemical categories based upon similar mechanisms as part of the Adverse Outcome Pathway paradigm. Additionally, the profiler could be utilised in screening large dataset in order to identify chemicals with the potential to induce mitochondrial toxicity.
Introduction

Over the past decade a number of changes have occurred in European Union chemicals policy that have led to an increase in efforts to develop alternative methods to traditional animal testing for risk assessment. These alternatives have been developed employing *in silico*, *in chemico* and *in vitro* methods focussing on replacing or reducing animals used in short-term toxicity tests. In order to be relevant, and useful, for regulatory assessment these alternatives should be based upon specific *in vivo* endpoints. Within recent years, interest has grown in developing a greater understanding of toxicity pathways. One such pathway approach is the Adverse Outcome Pathway (AOP) paradigm. An AOP is a framework that means to establish a mechanistic connection between an upstream Molecular Initiating Event (MIE) and a downstream adverse outcome relevant for risk assessment. The MIE is the critical event in the progression of an AOP as it provides insight into the initial interaction(s) between the chemical behaviour of the xenobiotic and the biological system that initiates the perturbation of the normal pathway. Elucidation of the mechanistic information relating to specific MIEs enables the identification of structural (and physico-chemical) features of chemicals that are responsible for the interaction with biological macromolecules, thus, facilitating the development of structural alerts. When utilised together, multiple structural alerts pertaining to the same MIE form the basis of an *in silico* profiler. The information within an *in silico* profiler can, in turn, be used to develop chemical categories centred on a common MIE (note that multiple MIEs can be initiated by a single chemical). This allows for read-across and data gap filling to be applied. The premise behind these structurally developed categories is that similar chemicals should have similar biological activities and therefore, should have the same MIE. Furthermore, the categories produced using *in silico* profilers can be supported by, and used to prioritise, additional
testing using *in vitro* and/or *in chemico* methods, within an integrated testing strategy. Such strategies can be used for hazard identification and risk assessment purposes, as well as being incorporated into *in silico* software tools such as the OECD QSAR Toolbox. \(^{10-11, 16-18}\)

A number of *in silico* profilers have been developed for a variety of organ-level toxicities, such as skin sensitisation, respiratory sensitisation, genotoxicity and hepatotoxicity.\(^{13-15, 19-21}\) However, very few profilers have dealt with toxicity induced by mitochondrial dysfunction.\(^{1,22,23}\) This is, in part, due to the number of mechanisms by which a chemical could induce mitochondrial dysfunction.\(^{24}\) An additional complication is that a single chemical might have the ability to induce more than one of these mechanisms, making it difficult to define a single MIE within the AOP paradigm. Over the past decade, interest in screening chemicals for an ability to induce mitochondrial dysfunction has increased.\(^{24,25}\) This is, in part, due to the withdrawal of a number of pharmaceuticals from the market after observed mitochondrial dysfunctions.\(^{26-31}\) Toxicity to mitochondria has led to such withdrawals as these are important organelles present within almost every cell type of the body, the exception being mature erythrocytes.\(^{32,33}\) The basic structure and function of mitochondria consists of two membranes, the outer and inner membrane, enclosing three compartments, the intermembrane space, the cristae and the mitochondrial matrix. The outer membrane is relatively smooth and permeable to molecules that are less than 5kDa in size. In contrast, the inner membrane contains multiple invaginations (cristae), is impermeable to all molecules except \(\text{O}_2\), \(\text{CO}_2\), and \(\text{H}_2\text{O}\) and contains each of the protein complexes within the electron transport chain, ATP synthase (Complex V) and various electron carriers. The mitochondria are responsible for a number of tasks vital to a cell’s normal functioning and survival. These tasks include the production of approximately 95% of the total amount of Adenosine Tri-Phosphate (ATP) generated by cells during oxidative phosphorylation. Oxidative phosphorylation is a process whereby energy from the transfer of electrons,
generated by the oxidation of Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide (FADH$_2$), along various complexes of the electron transport chain is used to pump protons across the inner mitochondrial membrane generating an electrochemical gradient. The electron transport chain comprises four complexes situated within the inner mitochondrial membrane. Complexes I and II are involved in the oxidation of NADH and FADH$_2$ respectively, providing the input of electrons into the respiratory chain. Complexes I, III and IV use the energy released from the transfer of electrons along the electron transport chain to pump protons out of the mitochondrial matrix into the intermembrane space.$^{25,31,34}$ Complex V, the terminal complex involved in oxidative phosphorylation, utilises the electrochemical gradient produced to transfer protons from the intermembrane space back into the mitochondrial matrix. The energy released from this action is used to phosphorylate adenosine diphosphate into ATP.

Previous research has shown that mitochondrial dysfunction may be induced by a range of chemicals and has been linked to a variety of organ toxicities within kidney, liver and nervous tissues.$^{26-31,35}$ The most susceptible tissues to mitochondrial dysfunction are those containing a higher concentration of mitochondria or those exposed to a higher concentration of chemical: such as the liver, kidneys and heart.$^{24,25,28,36}$ Five general mechanisms of mitochondrial dysfunction have been identified,$^{36,37}$ inhibitors of the electron transport chain and ATP synthase (Complex V), uncouplers of oxidative phosphorylation, opening of the membrane permeability transition pore, inhibition of fatty acid β-oxidation, and oxidation or inhibition of mitochondrial DNA.

As an example of the importance of mitochondrial toxicity approximately 35%, of more than 500 pharmaceutically relevant chemicals, have been shown to be directly involved in impairing normal mitochondrial functioning by inhibition of the electron transport chain and/or by uncoupling of oxidative phosphorylation.$^{38}$ Additionally, there are chemicals that
can induce mitochondrial toxicity via alternative mechanisms, such as inducing the membrane permeability transition, inhibition of β-oxidation of mitochondrial fatty acids, or interfering with mitochondrial DNA. Briefly stated, chemicals that inhibit the electron transport chain can do so by either direct binding to the complexes of the electron transport chain or ATP synthase or by acting as an alternative electron acceptor.\textsuperscript{27,36,37} The inhibition of electron flow along the electron transport chain by both of these mechanisms induces the formation of reactive oxygen species resulting in oxidative stress.\textsuperscript{27,36,37} Uncouplers of oxidative phosphorylation induce mitochondrial toxicity by shuttling protons into the mitochondrial matrix, via the inner mitochondrial membrane, bypassing ATP synthase. This assisted transport of protons back into the matrix dissipates the electrochemical potential, resulting in the loss of ATP production and, ultimately, cell death.\textsuperscript{27,31,36,37,39-43} Induction of the membrane permeability transition increases the permeability of the inner mitochondrial membrane to low molecular weight solutes (<1500Da), leading to a disruption of the electron transport chain, loss of membrane potential, and swelling of both the inner- and outer mitochondrial membranes.\textsuperscript{44,45} Inhibition of β-oxidation of mitochondrial fatty acids reduces the amount of NADH and FADH\textsubscript{2} available for oxidative phosphorylation that, in turn, reduces ATP production.\textsuperscript{46} Mitochondrial DNA encodes 13 components of the electron transport chain, damage that occurs to mitochondrial DNA can have a variety of downstream effects depending upon where it occurs.\textsuperscript{36,46} It should be noted, however, that there is the potential that multiple, competing, mechanisms could initiate mitochondrial toxicity observed for a single (group of) chemical(s), i.e. one chemical may induce several MIEs.

Given the importance of mitochondria within most cell systems, and the wide range of organ-level toxicities that may arise from mitochondrial dysfunction, the aim of the study was to identify structural alerts that could be used to form an \textit{in silico} profiler suitable for grouping chemicals into mechanism-based categories.
Methods

Data set

A set of 288 chemicals were extracted from Zhang et al. This article was chosen for use as it provides one of the largest freely available datasets, for which the chemical list has qualitative mitochondrial toxicity data associated with it. Within this data set 171 chemicals have been reported within the literature as inducing mitochondrial toxicity and were therefore considered to be mitochondrial toxicants. The chemicals with a negative result for mitochondrial toxicity were selected from the FDA-approved drug list, whereby the therapeutic action mechanism, of common and safe oral drugs, was not associated with a mechanism of drug-induced mitochondrial toxicity. Given the lack of supporting mechanistic information to confirm the presence, or absence, of mitochondrial toxicity additional analysis, was carried out as detailed below. The full dataset is available in the supplementary information. Positive and negative chemicals were taken as documented in Zhang et al (Table S1 in supporting information).

Category formation based upon structural similarity

All chemical structures were encoded into Simplified Molecular-Input Line-Entry System (SMILES) strings, neutralised and had salts removed. Each of the SMILES strings was extracted from the Royal Society of Chemistry’s ChemSpider website. Similarity calculations were implemented within the freely available Toxmatch software (v1.07) using the atom environment nearest neighbour approach, generating a data matrix with a Tanimoto similarity score for each chemical to all others within the data set. Subsequently, in-house code was implemented within Microsoft Excel that identified analogues with a similarity
index of 0.6 or greater; this was used in order to develop categories for the chemicals within the dataset with two, or more, analogues. Further analysis was undertaken upon those categories that met the following criteria: they contained three or more chemicals and at least one mitochondrial toxic chemical.

Mechanistic hypothesis and the development of alerts

Once categories had been developed using structural similarity a detailed search of the available literature was undertaken to elucidate the mechanistic information behind the molecular initiating event, along with other downstream key events, leading to the disruption of the mitochondria. This mechanistic information was subsequently utilised to support the definition of a structural alert suitable for grouping chemicals. These structural alerts were defined by identifying the common fragment present within each of the chemicals found to have positive mitochondrial toxicity according to literature information associated with them. Any additional information regarding the limits of the fragment found during the literature search, such as the requirement for an electron withdrawing group or the type of bond needed (e.g. a tertiary amine), was used to further refine the structural alert. The resulting alerts were subsequently defined as SMARTS patterns. A structural alert was only developed if information linking category members to mitochondrial toxicity was present within the scientific literature. The benefit of undertaking the analysis for each category is that it enabled the chemical space associated with a known, and tested, mechanism of mitochondrial toxicity to be identified. The development of chemical categories and identification of additional mechanistic information from the literature was crucial in addressing the limitations of the information in the original dataset. All structural alerts are available in the supplementary information and a KNIME workflow containing the structural alerts can be found in the COSMOS space which is a freely available tool (http://cosmosspace.cosmostox.eu/app/login).
Results and discussion

The aim of this study was to develop an \textit{in silico} profiler for mitochondrial toxicity based around clearly defined mechanistic information. This was achieved by grouping chemicals based upon their structural similarity, followed by a literature search to elucidate mechanistic information for the chemicals in categories associated with toxicity to mitochondria. Overall, 35 of the 288 chemicals were identified as belonging to categories containing mitochondrial toxic chemicals: local anaesthetics, antianginal, and antiarrhythmic; thiazolidinediones; nonsteroidal anti-inflammatory drugs; anthracycline antibiotics; perfluorinated chemicals; bile acids; phenothiazines; and β-blockers. A summary of the categories developed within this study is shown in Table 1. In total, eight categories were formed: two separate molecular initiating events for the perfluorinated chemical category were identified, whilst no structural alert for the β-blocker category could be defined. Subsequent mechanistic analysis showed the chemical within the eight categories covered five mechanisms of mitochondrial toxicity: inhibition of the electron transport chain, alternative electron acceptance, initiation of the death receptor pathway, uncoupling of oxidative phosphorylation and induction of the membrane permeability transition.

Table 1: Chemicals grouped into categories using structural similarity and their associated mitochondrial toxicity

Category 1: Local anaesthetics, antianginal and antiarrhythmic

The local anaesthetics category consisted of six analogues, four of which are anaesthetics, with ranolazine and tocainide being an antianginal and antiarrhythmic
respectively. All but one of the chemicals, tocainide, has been shown to exhibit toxicity towards mitochondria. The category is supported by a number of studies that have suggested that such chemicals affect mitochondrial metabolism by uncoupling oxidative phosphorylation.\textsuperscript{31,39,40-51} This uncoupling has been suggested to be mediated by both the protonophoric properties and the pKa of these chemicals. As the pKa is relatively similar to the intracellular pH, the level of protonated and deprotonated chemical is roughly at equilibrium. The presence of deprotonated chemical within the inner membrane space means that protons can be scavenged. Subsequently, the protonated chemical can combine with a hydrophobic anion to form a neutral ion-pair complex, which can then migrate across the inner mitochondrial membrane into the matrix, where the complex dissociates and the proton is released. Both the chemical and the hydrophobic anion then return to the inner membrane space, continuing the cycle. This assisted transport of protons back into the matrix dissipates the electrochemical potential, resulting in a loss of ATP production and ultimately cell death.\textsuperscript{31,39,41,42,51-53} It has been suggested that bupivacaine, and other highly lipophilic anaesthetics, can also act to uncouple oxidative phosphorylation via the mechanism outlined above without the need to complex with a lipophilic anion.\textsuperscript{49} The presence, and pKa, of the tertiary amine group is thought to be responsible for the ability of these chemicals to scavenge protons within the inner membrane space. Therefore, the lack of a tertiary amine group offers an explanation as to why no mitochondrial toxicity has been associated with tocainide.

Category 2: anti-diabetic drugs

This category consists of three thiazolidinediones: pioglitazone, rosiglitazone, troglitazone: each of which were identified as inducing mitochondrial toxicity. Thiazolidinediones are the major orally administered drugs used in the treatment of Type 2 (non-insulin dependent) diabetes. These drugs are used to improve insulin sensitivity and

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lower blood glucose levels within diabetic patients. Many of the thiazolidinediones have been suspected of initiating hepatotoxicity via mitochondrial dysfunction.\textsuperscript{29} For example, troglitazone was withdrawn from the world market in 2000 due to hepatotoxicity observed in a number of patients.\textsuperscript{27,51} Research into the thiazolidinediones suggests the chemicals within this category elicit their mitochondrial dysfunction by inhibiting the electron transport chain and uncoupling oxidative phosphorylation.\textsuperscript{22,26,29,51} These drugs have been shown to inhibit the activity of mitochondrial complexes, the main target being Complex I.\textsuperscript{26,27,51,54} These chemicals decrease the membrane potential across the inner mitochondrial membrane which causes mitochondrial swelling and in turn induces mitochondrial permeability transition.\textsuperscript{54,55} Additionally, thiazolidinediones have been shown to uncouple oxidative phosphorylation in a manner similar to that described above for the chemicals within category one.\textsuperscript{22,26} It is thought that the properties that enable the thiazolidinediones to bind to the nuclear PPAR-gamma receptor confers the ability to bind to Complex I.\textsuperscript{26} Additionally, the heterocyclic properties of the ring system are thought to enable the thiazolidinedione to cycle between a protonated and deprotonated form conferring the ability to transport protons across the inner mitochondrial membrane thereby uncoupling oxidative phosphorylation.\textsuperscript{22}

Category 3: Nonsteroidal anti-inflammatory drugs

The third category comprises three chemicals: mefenamic acid, flufenamic acid, and tolfenamic acid: each of which has been identified as being able to induce mitochondrial toxicity. Each of these three chemicals are part of a group of nonsteroidal anti-inflammatory (NSAIDs). NSAIDs are some of the most widely used pharmaceutical drugs on the market that are used for their analgesic, antipyretic and anti-inflammatory properties to reduce and relieve symptoms for a variety of conditions. In order for the anti-inflammatory properties associated with NSAIDs to be present a carboxylic acid moiety is needed.\textsuperscript{51} The carboxylic acid moiety acts to inhibit cyclooxygenase activity, an enzyme responsible for the production
of mediators of the inflammatory response, thereby reducing the level of inflammatory signalling. Previous research substantiates the positive mitochondrial toxicity result for each chemical within this category. A variety of literature sources identify each of these chemicals as having the ability to uncouple oxidative phosphorylation via a similar mechanism as that described above for the lidocaine category.\textsuperscript{56-60} However, due to their lipophilicity, these chemicals do not necessarily need to be associated with a separate hydrophobic anion in order to translocate into the mitochondrial matrix. The carboxylic acid moiety, which is required for the anti-inflammatory properties of the NSAIDs, is believed to also be required to induce the uncoupling ability of this group of chemicals.\textsuperscript{51}

Category 4: Anthracycline antibiotics

Anthracycline antibiotics are a group of hydroxylated tetracycline quinones with a duanosamine sugar sidechain attached. One of the category members, doxorubicin, is one of the most widely used antineoplastic drugs within the U.S.\textsuperscript{61} Structural similarity identified three similar chemicals. A number of studies have shown that the anthracycline antibiotics cause mitochondrial dysfunction by acting as alternative electron acceptors interfering with, and inhibiting, the electron transport chain, leading to oxidative stress. This occurs because under normal physiological conditions anthracyclines are usually deprotonated and can permeate across the outer mitochondrial membrane. Once within the inner mitochondrial space these chemicals disrupt the electron transport chain by sequestering an electron from Complex I and are thus reduced to a semiquinone radical intermediate.\textsuperscript{61,62} These semiquinone radicals subsequently interact with molecular oxygen present within the mitochondria, producing reactive oxygen species (ROS), including hydroxyl and superoxide anion radicals. Downstream these ROS lead to a variety of effects such a mitochondrial permeability transition induction and oxidative damage of DNA, proteins and lipids.\textsuperscript{63-65} Due to the high level of similarity between the chemicals it can be assumed that the mechanism of
action is conserved throughout the category. It should also be noted that quinones are also used as antimalarials which are known mitochondrial toxicants.66

Category 5: Hypolipodemic drugs

Perfluorinated chemicals have been widely used in a variety of commercial and pharmaceutical products, such as flame retardants, surfactants and hypolipidemic drugs. These hypolipidemic drugs induce the proliferation of peroxisomes and thus increase β-oxidation of fatty acids. Three perfluorinated chemical analogues; perfluorodecanoic acid, perfluoroctanoic acid, perfluorooctane sulphonamide; were identified as having a high level of similarity. Despite the high levels of similarity between the chemicals. This highlights the need to undertake mechanistic analysis of the categories as structural similarity on its own is not enough. As is shown with this category slight variations in structure have the potential to induce different mechanistic pathways. Accordingly, information in the literature suggests that for this category there are two potential mechanisms by which the perfluorinated chemicals elicit their mitochondrial toxicity; uncoupling of oxidative phosphorylation and induction of the mitochondrial membrane permeability transition pore.

Perfluorooctane sulphonamide has been shown to uncouple oxidative phosphorylation in vitro via a protonophoric mechanism, similar to that described above, within various species23,67-69. In comparison to p-trifluromethoxyphenylhydrazone, one of the most potent uncouplers, perfluorooctane sulphonamide has been known to uncouple oxidative phosphorylation with a potency of a similar magnitude. It has been suggested that the pKa and ionisability of the amino acid moiety, in conjunction with the relatively high lipophilicity of the chemical, enables the shuttling of protons across the inner mitochondrial membrane into the matrix, dissipating the membrane potential.69 Starkov studied the structural activity of
perfluoroalkanes and their ability to elicit mitochondrial toxicity via disruption to oxidative phosphorylation.\textsuperscript{69} Their data identified that a protonated nitrogen atom with a favourable pKa is essential to the uncoupling action of perfluorooctane sulphonamides in mitochondria. In addition, perfluorooctane sulphonamide is one of a very limited number of uncoupling chemicals that does not contain a ring structure\textsuperscript{68}. In contrast, the perfluoroalkyl acids are believed to induce the mitochondrial membrane permeability transition at lower concentrations, whilst higher concentrations can uncouple oxidative phosphorylation\textsuperscript{69-71}. It has been observed that perfluorodecanoic acid forms reactive oxygen species (ROS), hydrogen peroxide and peroxynitrite anion.\textsuperscript{72} The presence of elevated ROS levels initiates oxidative stress within mitochondria. Oxidative stress has been shown to induce the membrane permeability transition (MPT).\textsuperscript{73,74} The MPT is an increase in permeability of the inner mitochondrial membrane to low molecular weight solutes (<1500 Daltons). The subsequent influx of solutes into the matrix instigates swelling of the inner and outer membranes causing disruption of the electron transport chain and a release of apoptotic proteins such as cytochrome c.\textsuperscript{23,72,75} The uncoupling action of the two perfluoroalkyl acids is similar to that described for category one. Two alerts were defined due to two distinct MIEs being identified (Table 2). Previous research has shown there to be an increase in toxicity concomitant to an increase in alkyl side chain length up to C\textsubscript{12}, with the most marked increase in toxicity (a five- to ten-fold increase) occurring between C\textsubscript{6} and C\textsubscript{8} perfluoroalkyl acids and sulphates.\textsuperscript{23} An unsubstituted amide fragment has been shown to be required in order for uncoupling by perfluorinated sulphonamides to occur: fully substituted sulphonamides, which lack the protonated amide moiety, were found to lack the ability to uncouple oxidative phosphorylation.\textsuperscript{69} The carboxylic acid moiety of the perfluoroalkyl acids chemicals is thought to be responsible for the uncoupling action of these chemicals at higher
concentrations. However, it is unclear which fragments are required in the induction of the
MPT.

Category 6: Bile acids

The bile acid category consists of two primary conjugate bile acids: glycocholic acid and
taurocholic acid; and three non-conjugated secondary bile acids: deoxycholic acid, chenodeoxycholic acid and lithocholic acid: all with a high level of similarity to the primary
bile acid; cholic acid. Bile acids are one of the main constituents of bile and are synthesised
from cholesterol by hepatocytes and are then converted into secondary bile acids via the
intestinal bacteria. They play a vital role in multiple functions within both the liver and
intestines, the main function being the sequestration of fats within micelles for excretion. Bile
acids have been shown to decrease the membrane potential of mitochondria, alongside a
decrease in state 3 respiration and an increase in state 4 respiration. The specific cellular
mechanism of bile acid-induced toxicity has not been elucidated. However, both the intrinsic
(mitochondrial) and extrinsic (death receptor) pathways have been implicated in the
disruption of normal mitochondrial function.

Intrinsic pathway

The intrinsic apoptotic pathway results in mitochondrial dysfunction due to an
increase in intracellular stress. Hydrophobic bile acids have been shown to inhibit the
electron transport chain by decreasing the activity of complexes I, III and IV, resulting in a
decrease in state 3 respiration and a concomitant generation of ROS. It has been proposed
that the inhibition of complex III leads to a subsequent electron leak through the ubiquinone-
complex III site and a concomitant ROS generation. The increased oxidant stress may
then cause the induction of the MPT by oxidation of the thiol sites on the membrane
permeability transition pore. Induction of the MPT triggers the release of cytochrome c,
thus, stimulating the translocation of Bax to the mitochondrial membrane, stimulating further release of cytochrome c.\textsuperscript{82-84} Cytochrome c is also able to initiate downstream caspase activation events discussed below.\textsuperscript{77,78,81,84,85}

**Extrinsic pathway**

Mitochondrial dysfunction and apoptosis initiated via the extrinsic pathway results from extracellular signals triggering downstream caspase activity. The oxidative stress generated by bile acids induces an increased presentation of Fas receptor within the plasma membrane, following phosphorylation of the Fas receptor by the epidermal growth factor receptor.\textsuperscript{78,86,87} Upon presentation of Fas within the plasma membrane Fas agonists can interact with the receptor, initiating the formation of the death-inducing signalling complex (DISC) and subsequent activation of caspase-8. In turn, caspase-8 activates caspases-3 and -7 triggering a caspase cascade that culminates in apoptosis.\textsuperscript{78,84-86,88} Additionally, caspase-8 can initiate the intrinsic pathway via proteolytic cleavage of Bid. Truncated Bid activates Bax and Bak proteins present on the mitochondria via oligomerisation and induction of MPT. The activated Bak and Bax proteins form channels within the mitochondria releasing additional cytochrome c. Cytosolic cytochrome c causes the assembly of apoptotic protease-activating factor-1 (APAF-1) and caspase-9, thus, activating caspase-9. Upon caspase-9 activation a proteolytic caspase cascade is initiated ultimately leading to cell death.\textsuperscript{84,85} The generation of ROS, induction of MPT and activation of the caspase cascade seem to be essential steps within both pathways to initiate mitochondrial perturbation and apoptosis. Therefore, it seems likely that both pathways work synergistically to induce mitochondrial dysfunction.

**Category 7: Antihistaminic, antipsychotic and antiemetic drugs**

Phenothiazines are a group of heterocyclic chemicals composed of a nitrogen and a sulphur atom joining two benzene rings. These chemicals are widely used in the treatment of
mental disorders, such as schizophrenia, psychosis, and anxiety, as well as conferring antihistaminic and antiemetic action. This category comprises seven chemicals: chlorpromazine, fluphenazine, mequitazine, methdilaizne, promethazine, thiethylperazine, and trimeprazine: five of which have been identified have been identified as being non-toxic, whilst the remaining two have been shown to be toxic (Table 1). It is important to rationalise the mixed toxicity results for the chemicals within this category. A number of studies in the literature report toxicity induced by chlorpromazine and fluphenazinethat corroborate the data in the current study (obtained from Zhang et al.)\(^7\,51\,89-92\).

Both chlorpromazine and fluphenazine have been observed to inhibit mitochondrial respiration within brain and liver tissues\(^93\) This toxicity was induced by binding to, and inhibiting, Complex I of the electron transport chain.\(^24\,27\,51\) Further investigation revealed that chlorpromazine is also capable of impairing mitochondrial function by inhibiting Complex IV and acting as an uncoupler of oxidative phosphorylation.\(^51\,94\,95\) It has been found that the addition of the chlorine atom increases and alters the mechanism by which mitochondrial toxicity occurs, i.e. chlorpromazine acts as an uncoupler of oxidative phosphorylation at low concentrations and an electron transport chain inhibitor at higher concentrations.\(^94\) Each chemical within this category contains a phenothiazine fragment. This class of drugs were found to cause toxicity towards mitochondria by inhibiting oxidative phosphorylation within liver mitochondria\(^96\). Due to this conserved fragment it can be hypothesised that the other chemicals within this category elicit their toxicity via a similar mechanism. Research into promethazine has shown that it can impede both state 3 and state 4 respiration and intramitochondrial potassium ion compartmentalisation at high and low concentrations.\(^94\,95\) Work carried out within Terada demonstrated that an acid dissociable group, bulky hydrophobic moiety and strong electron-withdrawing group are essential for the induction of uncoupling this conclusion can support why this class of drugs may act as uncouplers.
Further investigation into chlorpromazine reveals that this chemical elicits its electron transport chain inhibitor action by inhibiting Complex V of the electron transport chain. Based upon information in the literature both the tertiary amine moiety and the phenothiazine fragment with an associated electron-withdrawing group are required in order to initiate uncoupling of oxidative phosphorylation.\textsuperscript{53}

Category 8: \(\beta\)-blockers

Alprenolol, propranolol and atenolol are a group of (non-)selective \(\beta\)-blockers used in the treatment of hypertension. As can be seen in Table 1 two chemicals, atenolol and propranolol, were reported within Zhang \textit{et al} as inducing mitochondrial toxicity, whilst the remaining chemical, alprenolol, was reported as being negative for mitochondrial toxicity. Propranolol has been seen to inhibit, via non-competitive binding, Complex V of the respiratory chain.\textsuperscript{97,98} Chemicals that inhibit Complex V can do so by binding to one of two subunits (\(F_0/F_1\)) that comprises the ATP synthase enzyme, thus blocking the passage of protons back into the mitochondrial matrix\textsuperscript{98}. Together the membrane-bound \(F_0\) and matrix protruding \(F_1\) subunits are responsible for catalysing both the synthesis and hydrolysis of ATP. Wei \textit{et al} have described previously that propranolol binds to the \(\text{Mg}^{2+}\text{-ATPase (F}_0\text{ subunit)}\) of Complex V inhibiting state 3 respiration.\textsuperscript{98} It has also been seen that the potency of ATPase inhibition induced by propranolol is of the same order of magnitude as its ability to inhibit other membrane-bound enzymes.\textsuperscript{97} Therefore, this inhibitory effect induced by propranolol is due to its membrane stabilising activity and its ability to bind to the lipophilic \(F_0\) subunit of Complex V. In contrast, atenolol, a relatively more hydrophilic drug, has been shown to act via stimulating Complex V activity. The decrease in lipophilicity and, therefore, a decrease in ability to penetrate and interact with membrane macromolecules is pertinent to the decrease in inhibitory potency of atenolol\textsuperscript{97}. Additionally, results from Almotrefi suggest
that atenolol may interact with the more hydrophilic subunit (F₁) of Complex V, resulting in mitochondrial toxicity by stimulating the hydrolysis of ATP to ADP and inorganic phosphate. Further research into how propranolol and atenolol is required in order to establish a clear structure-activity relationship for these structurally similar, β-blockers, given that they appear to have differing mechanisms of mitochondrial toxicity.

*Development of an in silico profiler for mitochondrial toxicity*

The ability to predict organ-level toxicity will become increasingly important to the long term goal of replacing animal use in determining a Lowest Observed (Adverse) Effect Level (LO(A)EL). Traditionally, LO(A)ELs are identified after undertaking a 28- or 90-day repeated dose study, with the lowest dose initiating a treatment related adverse effect in an organ(s) producing the LO(A)EL value. However, as no animal testing is permissible for cosmetic ingredients in Europe alternatives are required. As discussed previously this has led to increased interest in the understanding of toxicity pathways and in the development of AOPs. As such the structural alerts that have been developed in this study are intended for use in chemical risk assessment within the AOP paradigm. Within the AOP paradigm one of the roles of computational toxicology is to define the chemistry associated with key MIEs as an *in silico* profiler. To this end the mechanistic chemistry outlined in the current study was combined with that previously published by Naven et al enabling 17 structural alerts to be defined (Table 2). Briefly, Naven and co-workers analysed a database of more than 2000 compounds resulting in the identification of eleven structural alerts associated with the uncoupling of oxidative phosphorylation (which was defined by an increase in basal respiration). The two sets of structural alerts are, in the most part, complimentary with only the thiazolidinedione alert being identified in both studies. In addition, the majority of the
combined set of alerts identified relate to protonophoric mechanisms, with only two alerts for redox cycling (naphthoquinones and anthracene-9,10-diones) and only a single alert related to the inhibition of Complexes I-IV (phenothiazines). There is a clear need for additional structure-activity studies to define the chemical space for both these mechanisms, especially for chemicals capable of inhibiting Complexes I-IV. It should be noted that due to the limitations of the data available this study does not aim to create a profiler that would be used for pharmaceutical risk assessment, this study has highlighted the need for more mitochondrial chemical testing.

Table 2: Summary of the structural alerts identified in the current study and from the work of Naven et al. For the structural alerts acting as protonophores the acidic hydrogen is as explicitly drawn.\textsuperscript{22}
AOP development

Recently the development of AOPs has become an integral part of 21st century toxicology, with the availability of the OECD AOP knowledgebase making AOP development a worldwide focus.\(^9\) In order for mitochondria-mediated AOPs to be developed, further investigation into the organ(s) affected is required; this was, however, beyond the scope of the current study. The main outcome from the current study is that the structural alerts defined enable chemicals to be grouped into mechanistically-based categories based around the knowledge of a number of key MIEs for mitochondrial toxicity. The resulting categories can thus be used for either prioritisation of chemicals for further in vitro testing or, where sufficient in vivo data exist, for read-across predictions of organ-level toxicity (from, for example, repeat dose toxicity testing). In terms of predicting organ-level toxicity in the future it is likely that additional steps in the AOP will need to be investigated within in vitro assays using a range of organ specific cell lines. For example, the use of primary human renal proximal tubule epithelial cells in the MTT assays to investigate nephrotoxicity due to mitochondrial dysfunction. This will enable a mechanistically-based weight of evidence to be constructed based around the AOP and allow the formation of AOPs based on mitochondrial dysfunction. Currently, chemistry-based grouping methods such as those outlined above offer the most immediate solution to risk assessment without using animals.

Conclusions

The aim of this study was to develop an in silico profiler for mitochondrial toxicity based around clearly defined mechanistic information utilising structural similarity and chemical category formation. The analysis resulted in the development of eight chemical categories and the definition of eight structural alerts. Importantly, these structural alerts were derived using mechanistic information in the available literature to elucidate knowledge of a
number of key Molecular Initiating Events that disrupt the normal functioning of mitochondria. The structural alerts developed within this study have been combined with those from the literature to create a single profiler useful for grouping chemicals into categories, thus, enabling predictions to be made regarding mitochondrial toxicity. It is envisaged that this profiler will help in the formation of a mitochondrial dysfunction AOP.

Funding Information

The funding from the European Community’s Seventh Framework Program (FP7/2007-2013) COSMOS Project under grant agreement n° 266835 and from Cosmetics Europe is gratefully acknowledged.

Supporting Information Available

The supporting information contains the Zang et al(2010) data set and the structural alerts produced within this study. This material is available free of charge via the internet at http://pubs.acs.org.

Abbreviations

Adverse outcome pathway, AOP; Molecular initiating event, MIE; Simplified molecular-input line-entry system, SMILES; Non-steroidal anti-inflammatory drugs, NSAIDs; Reactive oxygen species, ROS; Membrane permeability transition, MPT; Death-inducing signalling complex, DISC; Apoptotic protease-activating factor-1, APAF-1; Lowest observed (adverse) effect level, LO(A)EL.
References


OECD QSAR Toolbox, www.qsartoolbox.org


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47. ChemSpider http://www.chemspider.com/


54. Nadanaciva S, Dykens JA, Bernal A, Capaldi RA, and Will Y (2007b) Mitochondrial impairment by PPAR agonists and
statins identified via immunocaptured OXPHOS complex activities and respiration. *Toxicol Appl Pharm* 223:277-287


### Table 1

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<tr>
<th>Category</th>
<th>Name</th>
<th>Mitochondrial toxicity</th>
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<tr>
<td>1 (Local anaesthetics, antianginal and antiarrhythmic)</td>
<td>Lidocaine</td>
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<td>Etidocaine</td>
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<td>Ropivacaine</td>
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<td>Tocainide</td>
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Table 2

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