Chemical Profile

The development of an in silico profiler for mitochondrial toxicity

Mark D Nelms, Claire L Mellor, Mark Cronin, Judith C Madden, and Steve J Enoch

Chem. Res. Toxicol., **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.5b00275 • Publication Date (Web): 16 Sep 2015

The development of an in silico profiler for mitochondrial toxicity

Mark D Nelms*, Claire L Mellor*, Mark T.D Cronin, Judith C Madden, and Steven J Enoch[+]

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool. L3 3AF, UK

*Joint first authors

[+]Corresponding author

Dr Steve Enoch

E: s.j.enoch@ljmu.ac.uk

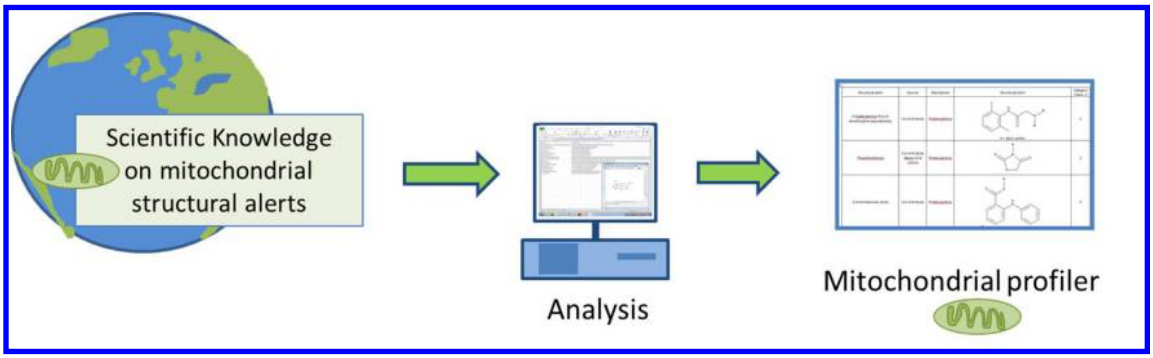
T: +44(0)151 231 2164

School of Pharmacy and Biomolecular Sciences
James Parsons Building
Liverpool John Moores University
Byrom Street
Liverpool
L3 3AF

Keywords

Structural alerts, mitochondrial toxicity, AOP, profiler, MIE

Table of Contents Graphic



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 *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.[1-7] 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[8]. 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.[9-12] 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.[13-16] 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, genotoxicityand 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 inter membrane 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 O_2, CO_2, and H_2O 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 inter membrane space.[25,31,34] Complex V, the terminal complex involved in oxidative phosphorylation, utilises the electrochemical gradient produced to transfer protons from the inter membrane 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.[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.[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.[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.[44,45] Inhibition of β-oxidation of mitochondrial fatty acids reduces the amount of NADH and $FADH_2$ available for oxidative phosphorylation that, in turn, reduces ATP production.[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.[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 *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*.[1] 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 S1in 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.[47] 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.[48] 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 *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 uggested that such chemicals affect mitochondrial metabolism by uncoupling oxidative phosphorylation.[31,39,49-51] 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.[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.[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

lower blood glucose levels within diabetic patients. Many of the thiazolidinediones have been suspected of initiating hepatotoxicity via mitochondrial dysfunction.[29] For example, troglitazone was withdrawn from the world market in 2000 due to hepatotoxicity observed in a number of patients.[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.[22,26,29,51] These drugs have been shown to inhibit the activity of mitochondrial complexes, the main target being Complex I.[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.[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.[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.[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.[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.[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.[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.[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.[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.[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.[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, perfluorooctanoic 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 species[23,67-69]. 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.[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[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[69-71]. It has been observed that perfluorodecanoic acid forms reactive oxygen species (ROS), hydrogen peroxide and peroxynitrite anion.[72] The presence of elevated ROS levels initiates oxidative stress within mitochondria. Oxidative stress has been shown to induce the membrane permeability transition (MPT).[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.[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_{12}, with the most marked increase in toxicity (a five- to ten- fold increase) occurring between C_6 and C_8 perfluoroalkyl acids and sulphates.[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.[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 twoprimary 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.[76-81] 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.[80,81] The increased oxidant stress may then cause the induction of the MPT by oxidation of the thiol sites on the membrane permeability transition pore.[79] 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.[82-84] Cytochrome c is also able to initiate downstream caspase activation events discussed below.[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.[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.[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.[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, methdilazine, 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.[53]

Category 8: β-blockers

Alprenolol, propranolol and atenolol are a group of (non-)selective β-blockers used in the treatment of hypertension. As can be seen in Table 1 two chemicals, atenolol and propranolol, were reported within Zhang *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. [97,98] Chemicals that inhibit Complex V can do so by binding to one of two subunits (F_O/F_1) that comprises the ATP synthase enzyme, thus blocking the passage of protons back into the mitochondrial matrix[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 *et al* have described previously that propranolol binds to the Mg^{2+}-ATPase (F_0 subunit) of Complex V inhibiting state 3 respiration.[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.[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[97]. Additionally, results from Almotrefi suggest

that atenolol may interact with the more hydrophilic subunit (F_1) of Complex V, resulting in mitochondrial toxicity by stimulating the hydrolysis of ATP to ADP and inorganic phosphate.[97] 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).[22] 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 thiazolinedione 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. [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.[99] 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^{o} 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

1. Zhang H, Chen QY, Xiang ML, Ma CY, Huang Q, Yang SY (2009) In silico prediction of mitochondrial toxicity by using GA-CG-SVM approach. *Toxicol In Vitro 23*:134-140

2. EC (2006a) Corrigenda of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) *Official Journal of the European Union* EC 1907/2006

3. EC (2006b) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) *Official Journal of the European Union* EC 1907/2006

4. ECHA (2008) Guidance on information requirements and chemical safety assessment. Chapter R.6: QSARs and grouping of chemicals.

5. ECHA (2009) How to report read-across and categories:1-30

6. ECHA (2010) Practical guide 6: How to report read-across and categories.

7. EC(2003) Directive 2003/15/EC of the European Parliament and of the Council (7th Amendment) *Official Journal of the European Union*

8. Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM.(2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010. *Arch Toxicol 85*:367-485

9. Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung M.W, Johnson R.D, Mount D.R, Nichols J.W,. Russom C.L,. Schmieder P.K, Serrrano J.A,. Tietge J.E,. Villeneuve D.L (2010) Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem 29*:730-741

10. OECD (2012) Guidance on developing and assessing Adverse Outcome Pathways.

11. Vinken M (2013a) The adverse outcome pathway concept: a pragmatic tool in toxicology. *Toxicology 312*:158-165

12. Vinken M, Whelan M, and Rogiers V (2014) Adverse outcome pathways: hype or hope? *Arch Toxicol 88*:1-2

13. Enoch SJ, and Cronin MTD (2010) A review of the electrophilic reaction chemistry involved in covalent DNA binding.

Crit Rev Toxicol 40:728-748

14. Enoch SJ, Ellison CM, Schultz TW and Cronin MTD (2011) A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. *Crit Rev Toxicol 41*:783-802

15. Hewitt M, Enoch SJ, Madden JC, Przybylak KR, and Cronin MTD (2013) Hepatotoxicity: a scheme for generating chemical categories for read-across, structural alerts and insights into mechanism(s) of action. *Crit Rev Toxicol 43*:537-558

16. Przybylak KR, and Schultz TW (2013) Informing chemical categories through the development of Adverse Outcome Pathways. In: Cronin MTD, Madden, J. C., Enoch, S. J., and Roberts, D. W. (ed) Chemical Toxicity Prediction: Category Formation and Read-Across, vol 17. The Royal Society of Chemistry, Cambridge, UK

17. OECD QSAR Toolbox, www.qsartoolbox.org

18. Gutsell S, and Russell P (2013) The role of chemistry in developing understanding of adverse outcome pathways and their application in risk assessment. *Toxicol Res 2*:299

19. Enoch SJ, Madden JC, Cronin MTD (2008) Identification of mechanisms of toxic action for skin sensitisation using a SMARTS pattern based approach. *SAR QSAR Environ Res 19*:555-578

20. Sakuratani Y, Zhang HQ, Nishikawa S, Yamazaki K, Yamada T, Yamada J, Gerova K, Chankov G, Mekenyan O, Hayashi M. l (2013) Hazard Evaluation Support System (HESS) for predicting repeated dose toxicity using toxicological categories. *SAR QSAR Environ Res 24*:351-363

21. Vinken M, Landesmann B, Goumenou M, Vinken S, Shah I, Jaeschke H, Willett C, Whelan M, Rogiers V. (2013b) Development of an adverse outcome pathway from drug-mediated bile salt export pump inhibition to cholestatic liver injury. *Toxicol Sci 136*:97-106

22. Naven RT, Swiss R, Klug-McLeod J, Will Y, and Greene N (2013) The development of structure-activity relationships for mitochondrial dysfunction:phosphorylation. *Toxicol Sci 131*:271-278

23. Wallace KB, Kissling GE, Melnick RL, Blystone CR (2013) Structure–activity relationships for perfluoroalkane-induced in vitrointerference with rat liver mitochondrial respiration. *Toxicol Lett 2*22:257-264

24. Nadanaciva S, and Will Y (2011) New insights in drug-induced mitochondrial toxicity. Curr Pharm Design 17:2100-2112

25. Dykens JA, and Will Y (2008b) Drug-induced Mitochondrial Dysfunction. John Wiley and Sons, Inc., Hoboken, New Jersey, USA

26. Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhäusl W, Fürnsinn

C.(2004) Thiazolidinediones, like metformin, inhibit respiratory complex I. A common mechanism contributing to their antibiabetic actions? *Diabetes 53*:1052-1059

27. Chan K, Truong D, Shangari N, and O'Brien PP (2005) Drug-induced mitochondrial toxicity. *Expert Opin Drug Met 1*:655-669

28. Dykens JA, and Will Y (2007a) The significance of mitochondrial toxicity testing in drug development Drug Discov Today 12:777-785

29. Dykens JA, Marroquin LD, and Will Y (2007b) Strategies to reduce late-stage drug attrition due to mitochondrial toxicity. *Expert Rev Mol Diagn 7*:161-175

30. Rolo AP, Palmeira CM, Holy JM, and Wallace KB (2004) Role of mitochondrial dysfunction in combined bile acid-induced cytotoxicity: The switch between apoptosis and necrosis. *Toxicol Sci 79*:196-204

31. Wallace KB, and Starkov AA (2000) Mitochondrial targets of drug toxicity. *Ann Rev Pharm Toxicol 40*:353-388

32. Cohen BH, and Gold DR (2001) Mitochondrial cytopathy in adults: What we know so far. *Clev Clin J Med 68*:625-642

33. Pieczenik SR, and Neustadt J (2007) Mitochondrial dysfunction and molecular pathways of disease. *Exp Mol Pathol 83*:84-92

34. Hatefi Y (1985) The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem 54*:1015-1069

35. Roberts D.W, Patlewicz G, Kern PS, Gerberick F, Kimber I, Dearman RJ, Ryan CA, Basketter DA, Aptula AO (2007) Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem Res Toxicol. (7)*:1019-30.

36. Amacher DE (2005) Drug-associated mitochondrial toxicity and its detection. *Curr Med Chem 12*:1829-1839

37. Krahenbuhl S (2001) Mitochondria: important target for drug toxicity? *J Hepatol 34*:334-336

38. Dykens JA (2008a) Preface. In: Dykens JA, and Will, Y. (ed) Drug-Induced Mitochondrial Dysfunction. John Wiley and Sons, Inc., Hoboken, New Jersey, USA

39. Cela O, Piccoli C, Scrima R, Quarato G, Marolla A, Cinnella G, Dambrosio M, Capitanio N. (2010) Bupivacaine uncouples the mitochondrial oxidative phosphorylation, inhibits respiratory chain complexes I and III and enhances ROS production: results of a study on cell cultures. *Mitochondrion 10*:487-496

40. Schonfeld P, Sztark F, Slimani M, Dabadie P, and Mazat JP (1992) Is bupivacaine a decoupler, a protonophore or a proton-leak-inducer. *FEBS Letters 304*:273-276

41. Spycher S, Smejtek P, Netzeva TI, and Escher BI (2008) Towards a class independent Quantitative Structure-Activity Relationship model for uncouplers of oxidative phosphorylation. *Chem Res Toxicol 21*:911-927

42. Sun X, and Garlid KD (1992) On the mechanism by which bupivacaine conducts protons across the membranes of mitochondria and liposomes. *J Biol Chem 267*:19147-19154

43. Terada H (1990a) Uncouplers of oxidative phosphorylation. *Environ Health Persp 87*:213-218

44. Kroemer G, Galluzzi L, and Brenner C (2007) Mitochondrial membrane permeabilization in cell death. *Physiol Rev 87*:99-163

45. Lemasters JJ, Theruvath TP, Zhong Z, and Nieminen AL (2009) Mitochondrial calcium and the permeability transition in cell death. *Biochim Biophys ACTA 1787*:1395-1401

46. Pessayre D, Berson A, and Fromenty B (2008) Features and mechanisms of drug-induced liver injury. In: Dykens JA, and Will, Y (ed) Drug-Induced Mitochondrial Dysfunction. John Wiley and Sons, Inc., Hoboken, New Jersey, USA

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

48. Daylight, www.daylight.com

49. Dabadie P, Bendriss P, Erny P, and Mazat JP (1987) Uncoupling effects of local anesthetics on rat liver mitochondria. *FEBS Letters 226*:77-82

50. Dippenaar JM (2007) Local anaesthetic toxicity. *SAJAA 13*:23-28

51. Mehta R, Chan K, Lee O, Tafazoli S, and O'Brien PJ (2008) Drug-associated mitochondrial toxicity. In: Dykens JA, and Will, Y (ed) Drug-Induced Mitochondrial Dysfunction. John Wiley and Sons, Inc., Hoboken, New Jersey, USA

52. Sztark F, Ouhabi R, Dabadie P, and Mazat JP (1997) Effects of the local aesthetic bupivacaine on mitochondria energy metabolism, change from uncoupling to decoupling depending on the respiration state. *Biochem Mol Biol Int 43*:997-1003

53. Terada H, Shima O, Yoshida K, and Shinohara Y (1990b) Effects of the local anaesthetic bupivacaine on oxidative phosphorylation in mitochondria. Change from decoupling to uncoupling by formation of a leakage type ion pathway specific from H+ in cooperation with hydrophobic anions. *J Biol Chem 265*:7837-7842

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

55. Masubuchi Y (2006) Metabolic and non-metabolic factors determining troglitazone hepatotoxicity: A review. Drug Metab *Pharmacok 21*:347-356

56. Boelsterli U (2002) Mechanisms of NSAID-induced hepatotoxicity: Focus on Nimesulide. *Drug Safety 25*:633-648

57. Masubuchi Y, Saito H, and Horie T (1998) Structural requirements for the hepatotoxicity of nonsteroidal anti-inflammatory drugs in isolated rat hepatocytes. *J Pharmacol Exp Ther 287*:208-213

58. Moreno-Sanchez R, Bravo C, Vasquez C, Ayala G, Silveira LH, and Martinez-Lavin M (1999) Inhibition and uncoupling of oxidative phosphorylation by Nonsteroidal Anti-Inflammatory Drugs. *Biochem Pharmacol 57*:743-752

59. Siraki AG, Chevaldina T, and O'Brien PJ (2005) Application of quantitative structure-toxicity relationships for acute NSAID cytotoxicity in rat hepatocytes. *Chem-Biol Interact 151*:177-191

60. Uyemura SA, Santos AC, Mingatto FE, Jordani MC, and Curti C (1997) Diclofenac sodium and mefenamic acid, potent inducers of the membrane permeability transition in renal cortex mitochondria. *Arch Biochem Biophys 342*:231-235

61. Wallace KB (2003) Doxorubicin-induced cardiac mitochondrionopathy. *Pharmacol Toxicol 93*:105-115

62. Gerwirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol 57*:727-741

63. Kappus H (1986) Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. *Biochem Pharmacol 35*:1-6

64. Kim J-S, He L, and Lemasters JJ (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis.:463-470

65. Ohkuma Y, Hiraku Y, and Kawanishi S (2001) Sequence specific DNA damage induced by carcinogenic danthron and anthraquinone in the presence of Cu(II), cytochrome P450 reductase and NADPH. Free Radical *Res 34*:595-604

66. Nixon G.L, Pidathala C, Shone A.E, Antoine T, Fisher N, O'Neill P.M, Ward S.A, Biagini G.A (2013)Targeting the mitochondrial electron transport chain of Plasmodium falciparum: new strategies towards the development of improved antimalarials for the elimination era. *Future Med Chem. ;5*(13):1573-91. doi: 10.4155/fmc.13.121.

67. Schnellmann RG (1990a) The cellular effects of a unique pesticide sulfluramid (N-ethylperfluorooctane sulphonamide) on rabbit renal proximal tubules. *Toxicol in Vitro 4*:71-74

68. Schnellmann RG, and Manning RO (1990b) Perfluorooctane sulfonamide: a structurally novel uncoupler of oxidative phosphorylation. *Biochem Biophys ACTA 1016*:344-348

69. Starkov AA, and Wallace KB (2002) Structural determinants of fluorochemical-induced mitochondrial dysfunction. *Toxicol Sci 66*:244-252

70. Keller BJ, Yamanaka H, and Thurman RG (1992) Inhibition of mitochondrial respiration and oxygen-dependent hepatotoxicity by six structurally dissimilar peroxisomal proliferating agents. *Toxicology 71*:49-61

71. Langley AE (1990) Effects of perfluoro-n-decanoic acid on the respiratory activity of isolated rat liver mitochondria. *J Toxicol Environ Health 29*:329-336

72. Kleszczynski K, Stepnowski P, and Skladanowski AC (2009) Mechanism of cytotoxic action of perfluorinated acids II. Disruption of mitochondrial bioenergetics. *Toxicol Appl Pharmacol 235*:182-190

73. Battaglia V (2005) Oxidative Stress Is Responsible for Mitochondrial Permeability Transition Induction by Salicylate in Liver Mitochondria. *J Biol Chem 280*:33864-33872

74. Kowaltowski AJ, Castilho RF, Vercesi AE (2001) Mitochondrial permeability transition and oxidative stress. *FEBS Letters 495*:12-15

75. Kleszczynski K, and Skladanowski AC (2011) Mechanism of cytotoxic action of perfluorinated acids. III. Disturbance in Ca(2)+ homeostasis. *Toxicol Appl Pharmacol 251*:163-168

76. Krahenbuhl S, Talos C, Fischer S, and Reichen J (1994) Toxicity of bile acids on the electron transport chain of isolated rat liver mitochondria. *Hepatology 19*:471-479

77. Palmeira CM, and Rolo AP (2004) Mitochondrially-mediated toxicity of bile acids. *Toxicology 203*:1-15

78. Perez MJ (2009) Bile-acid-induced cell injury and protection. *World J Gastroentero 15*:1677

79. Sokol RJ, Straka MS, Dahl R, Devereaux MW, Yerushalmi B, Gumpricht E, Elkins N, Everson G. (2001) Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acids. *Pediatr Res 49*:519-531

80. Winklhofer-Roob BM, McKim JM, Jr, Devereaux MW, and Sokol RJ (1996) Characterization of site of reactive oxygen species (ROS) generation in mitochondria exposed to glycochenodeoxycholic acid [Abstract] Hepatology 24(part2):338A

81. Yerushalmi B, Dahl R, Devereaux MW, Gumpricht E, and Sokol RJ (2001) Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. *Hepatology 33*:616-626

82. Rodrigues CMP, Ma X, Linehan-Stieers C,Fan G, Kren BT, and Steer CJ (1999) Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarisation and channel formation. *Cell Death Differ 6*:842-854

83. Spivey JR, Bronk SF, and Gores GJ (1993) Glycochenodeoxycholate-induced lethal hepatocellular injury in rat hepatocytes. Role of ATP depletion and cytosolic free calcium. *J Clinical Invest 92*:17-24

84. Yin X-M, and Ding WX (2003) Death receptor activation-induced hepatocyte apoptosis and liver injury. *Curr Mol Med 3*:491-508

85. Taylor RC, Cullen SP, and Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol 9*:231-241

86. Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA, Kaufmann SH, Gores GJ. l (1998) Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *J Clin Invest 103*:137-145

87. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkentin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB, Dent P.(2001) Deoxycholic Acid (DCA) causes ligand-independent activation of Epidermal Growth Factor Receptor (EGFR) and Fas receptor in primary hepatocytes: Inhibition of EGFR/Mitogen-activated protein kinase-signalling module enhances DCA-induced apoptosis. *Mol Biol Cell 12*:2609-2645

88. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, and Lemasters JJ (2002) Mechanisms of Hepatotoxicity. *Toxicol Sci 65*:166-176

89. Balijepalli S, Boyd MR, Ravindranath V (1999) Inhibition of mitochondrial complex I by haloperidol: the role of thiol oxidation. *Neuropharmacology 38*:567-577

90. Lucas-Heron B, Schmitt N, Ollivier B (1994) Mdx mouse skeletal muscle: Could a mitochondrial factor be responsible for the absence of progressive necrosis? *Neuroscience 129*:97-100

91. Nadanaciva S, Bernal A, Aggeler R, Capaldi R, and Will Y (2007a) Target identification of drug induced mitochondrial toxicity using immunocapture based OXPHOS activity assays. *Toxicol In Vitro 21*:902-911

92. Saito K, Kakei M, Uchimura S, Kashima T, and Tanaka H (1982) Toxic effects of chlorpromazine on red and white muscles in rats: an ultrastructural study. *Toxicol Appl Pharm 65*:347-353

93. Guth PS, and Sprites MA (1964) The phenothiazine tranquilizers: biochemical and biophysical actions. *Int Rev Neurobiol 7*:231-278

94. Eto K, Fukuda T, Araki Y, Inoue B, and Ogata M (1985) Effect of tricyclic drugs on mitochondrial membrane. *Acta Med Okayama 39*:289-295

95. Matsubara T, and Hagihara B (1968) Action mechanism of phenothiazine derivatives on mitochondrial respiration. *J Biochem 63*:156-164

96. Gallagher CH, Koch JH, and Mann DM (1965) The effect of phenothiazine on the metabolism of liver mitochondria. *Biochem Pharmacol 14*:789-797

97. Almotrefi A, and Dzimiri N (1992) Effect of beta-adrenoceptor blockers on mitochondrial ATPase activity in guinea pig heart preparations. *Eur J Pharmacol 215*:231-236

98. Wei Y-H, Lin TN, Hong CY, and Chiang B (1985) Inhibition of the mitochondrial Mg2+-ATPase by propranolol. *Biochem Pharmacol 34*:911-917

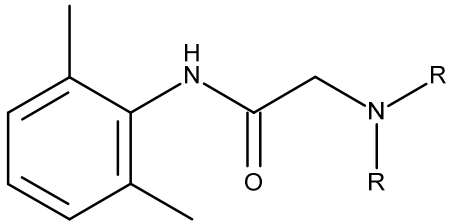
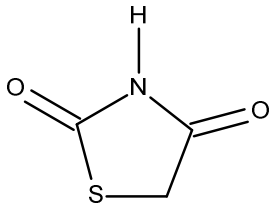
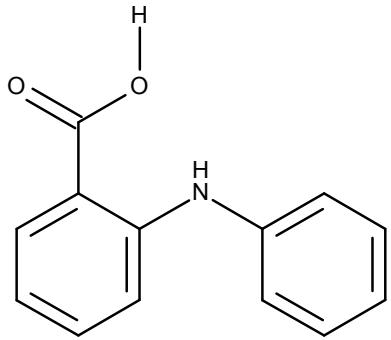
99. AOP-KB (2015) The OECD webpage describing AOPs. http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm. Accessed on 2nd September 2015

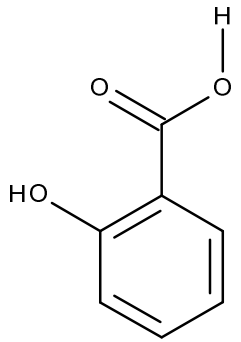
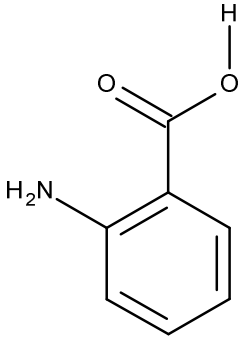
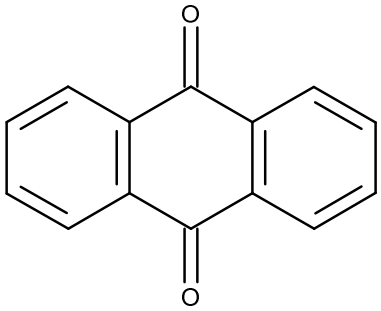
Tables

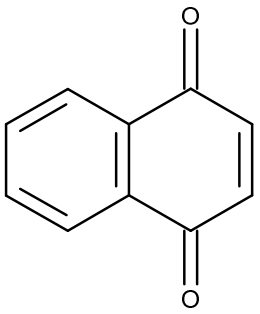
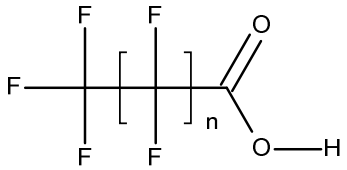
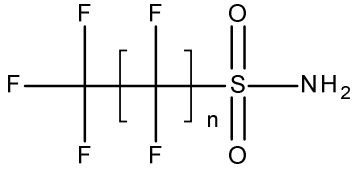
Table 1

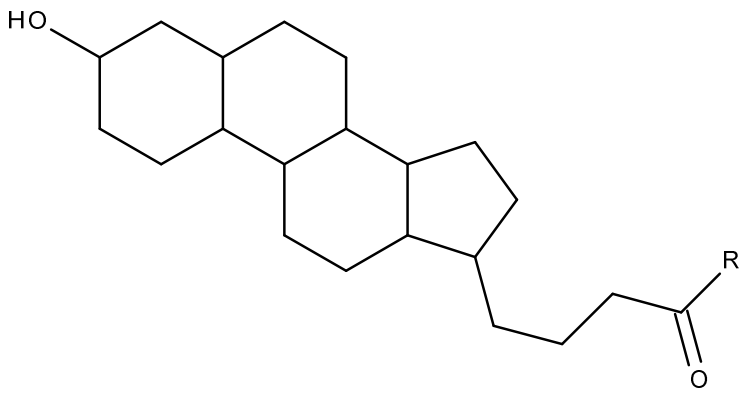
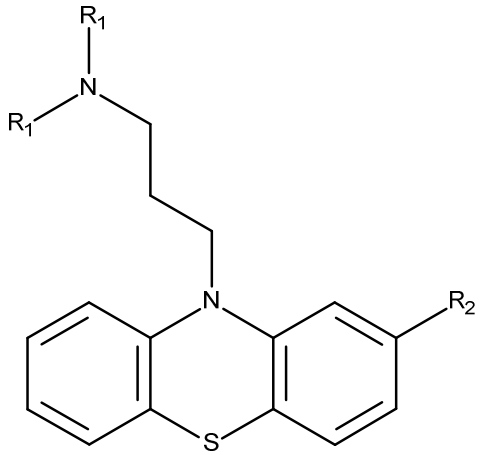
Category	Name	Mitochondrial toxicity
1 (Local anaesthetics, antianginal and antiarrhythmic)	Lidocaine	Positive
	Bupivacaine	Positive
	Etidocaine	Positive
	Ropivacaine	Positive
	Ranolazine	Positive
	Tocainide	Negative
2 (anti-diabetic drugs)	Rosiglitazone	Positive
	Pioglitazone	Positive
	Troglitazone	Positive
3 (Nonsteroidal anti-inflammatory drugs)	Mefenamic acid	Positive
	Flufenamic acid	Positive
	Tolfenamic acid	Positive
4 (Anthracycline antibiotics)	Daunorubicin	Positive
	Doxorubicin	Positive
	Epirubicin	Positive
	Idarubicin	Positive
5 (Hypolipodemic drugs)	Perfluorodecanoic acid	Positive
	Perfluorooctanoic acid	Positive
	Perfluorooctane-sulphonamide	Positive
6 (Bile acids)	Cholic acid	Positive
	Chenodeoxycholic acid	Positive
	Deoxycholic acid	Positive
	Glycocholic acid	Positive
	Lithocholic acid	Positive
	Taurocholic acid	Positive
7 (Antihistaminic, antipsychotic and antiemetic drugs)	Promethazine	Negative
	Chlorpromazine	Positive
	Fluphenazine	Positive
	Mequitazine	Negative
	Methdilazine	Negative
	Thiethylperazine	Negative
	Trimeprazine	Negative
8 (β-blockers)	Alprenolol	Negative
	Atenolol	Positive
	Propranolol	Positive

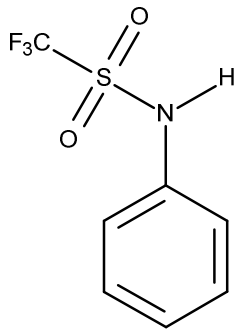
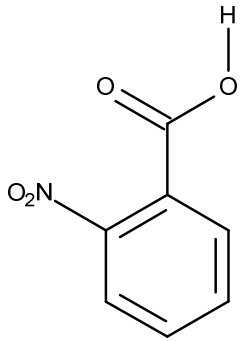
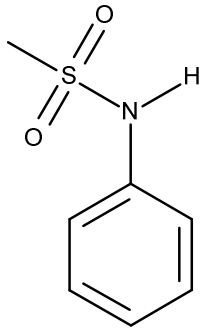
Table 2

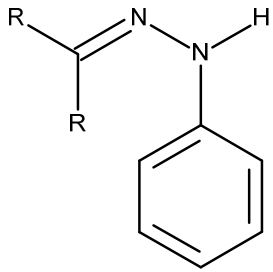
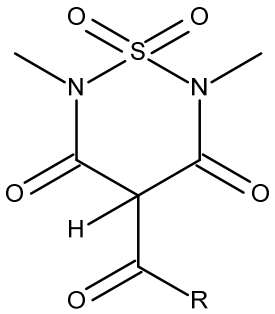
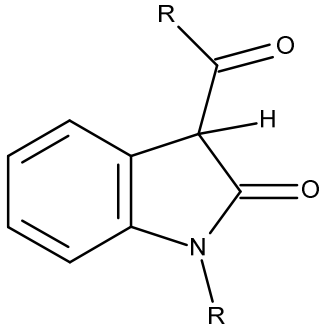
Structural alert	Source	Mechanism	Structural Alert	Category (Table 1)
2-(Dialkylamino)-N-(2,6-dimethylphenylacetamides	Current study	Protonophore	 R = alkyl carbon	1
Thiazolidinediones	Current study [22]	Protonophore		2
2-Anilinobenzoic acids	Current study	Protonophore		3

Salicylates	[22]	Protonophore		expands 3
Anthranilic acids	[22]	Protonophore		expands 3
Anthracene-9,10-diones	Current study	Redox cycling		4

Naphthoquinones	[22]	Redox cycling		expands 4
Perfluorinated-carboxcylic-acids	Current study	Protonophore	 n = 5-11	5a
Perfluorinated-sulphonamides	Current study	Protonophore	 n = 5-11	5b

Bile acids	Current study	Unclear	 R = alkyl carbon	6
Phenothiazines	Current study	Inhibition of Complexes I-IV	 $R_1 = CH_2, CH_3$ $R_2 = Cl, CF_3$	7

Fluoromethylsulphanilides	[22]	Protonophore		
Nitrophenols	[22]	Protonophore		
Nitrosulphonanilides	[22]	Protonophore		

Phenylhydrazones	[22]	Protonophore	 R = any carbon	
Thiadiazinedione dioxides	[22]	Protonophore	 R = any carbon	
Acylindolones	[22]	Protonophore	 R = alkyl carbon	