AOP ID and Title:

AOP 130: Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity
Short Title: Inhibition of LPLA2, hepatotoxicity

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Status

Author status	OECD status	OECD project	SAAOP status
Under Development: Contributions and Comments Welcome	Under Development	1.39	Included in OECD Work Plan

Abstract

Drug-induced phospholipidosis (DIPL) is one of the lipid storage disorders, and some of the cationic amphiphilic drugs (CADs) lead to phospholipids accumulation in the lysosome. Some patients with phospholipidosis have been reported with steatosis, cirrhosis, and fibrosis but the mechanism of their association has not been clarified. CADs can easily pass through membranes due to their amphiphilic character and accumulate in the lysosome of the hepatocyte or alveolar macrophage. These drugs inhibit the activity of lysosomal phospholipase A2 by binding with enzyme or phospholipid, which causes phospholipids to accumulate in lysosome and form a lamellar body. Therefore, CADs led to phospholipidosis along with lysosomal dysfunction. Lysosomes are the predominant digestive organelle and it has the hydrolytic enzymes that degrade peptides, nucleic acids, carbohydrates, and lipids. Lysosomal dysfunction induced to lipid accumulation in the cytosol by inhibiting the lipid metabolism. And lysosomal membrane potential is changed due to accumulating CADs in lysosome and it induced the lysosomal cell death and mitochondrial dysfunction by secondary effects. To develop the AOP for phospholipid and hepatotoxicity, we survey the public literature. At present, direct evidence between phospholipidosis and hepatotoxicity is still lacking, but we try to explain using the AOP framework.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	828	Inhibition, Phospholipase A	Inhibition, Phospholipase A
2	KE	829	Damage, Lipid bilayer	Damage, Lipid bilayer
3	KE	831	Disturbance, Lysosomal function	Disturbance, Lysosomal function
	KE	177	N/A, Mitochondrial dysfunction 1	N/A, Mitochondrial dysfunction 1
5	KE	833	Occurrence, Cytoplasmic vacuolization (hepatocyte)	Occurrence, Cytoplasmic vacuolization (hepatocyte)
6	KE	835	Occurrence, Ballooning degeneration (hepatocyte)	Occurrence, Ballooning degeneration (hepatocyte)
7	KE	837	Occurrence, Cytoplasmic vacuolization (kupffer cell)	Occurrence, Cytoplasmic vacuolization (kupffer cell)
8	KE	838	Induction, Microvesicular fat	Induction, Microvesicular fat
9	KE	839	Formation, Mallory body	Formation, Mallory body
10	KE	836	Occurrence, Cytoplasmic vacuolization (Bile duct cell)	Occurrence, Cytoplasmic vacuolization (Bile duct cell)
11	AO	840	Formation, Liver fibrosis	Formation, Liver fibrosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Phospholipase A	adjacent	Damage, Lipid bilayer	High	
Inhibition, Phospholipase A	adjacent	Disturbance, Lysosomal function	High	
Inhibition, Phospholipase A	adjacent	N/A, Mitochondrial dysfunction 1	High	
Damage, Lipid bilayer	adjacent	Occurrence, Cytoplasmic vacuolization (hepatocyte)	Low	
Damage, Lipid bilayer	adjacent	Occurrence, Ballooning degeneration (hepatocyte)	Low	
Damage, Lipid bilayer	adjacent	Occurrence, Cytoplasmic vacuolization (Bile duct cell)	Low	
Damage, Lipid bilayer	adjacent	Occurrence, Cytoplasmic vacuolization (kupffer cell)	Low	
Disturbance, Lysosomal function	adjacent	Occurrence, Cytoplasmic vacuolization (hepatocyte)	Low	
Disturbance, Lysosomal function	adjacent	Occurrence, Ballooning degeneration (hepatocyte)	Low	
Disturbance, Lysosomal function	adjacent	Occurrence, Cytoplasmic vacuolization (Bile duct cell)	Low	
Disturbance, Lysosomal function	adjacent	Occurrence, Cytoplasmic vacuolization (kupffer cell)	Low	
N/A, Mitochondrial dysfunction 1	adjacent	Occurrence, Cytoplasmic vacuolization (hepatocyte)	Low	
N/A, Mitochondrial dysfunction 1	adjacent	Occurrence, Ballooning degeneration (hepatocyte)	Low	
N/A, Mitochondrial dysfunction 1	adjacent	Occurrence, Cytoplasmic vacuolization (Bile duct cell)	Low	
N/A, Mitochondrial dysfunction 1	adjacent	Occurrence, Cytoplasmic vacuolization (kupffer cell)	Low	
Occurrence, Cytoplasmic vacuolization (hepatocyte)	adjacent	Induction, Microvesicular fat	Low	
Occurrence, Cytoplasmic vacuolization (hepatocyte)	adjacent	Formation, Mallory body	Low	
Occurrence, Ballooning degeneration (hepatocyte)	adjacent	Induction, Microvesicular fat	Low	
Occurrence, Ballooning degeneration (hepatocyte)	adjacent	Formation, Mallory body	Low	
Occurrence, Cytoplasmic vacuolization (Bile duct cell)	adjacent	Induction, Microvesicular fat	Low	
Occurrence, Cytoplasmic vacuolization (Bile duct cell)	adjacent	Formation, Mallory body	Low	
Occurrence, Cytoplasmic vacuolization (kupffer cell)	adjacent	Induction, Microvesicular fat	Low	
Occurrence, Cytoplasmic vacuolization (kupffer cell)	adjacent	Formation, Mallory body	Low	
Induction, Microvesicular fat	adjacent	Formation, Liver fibrosis	High	
Formation, Mallory body	adjacent	Formation, Liver fibrosis	High	

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
1 to < 3 months	High
Adults	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Sprague-Dawley	Sprague-Dawley	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Sex Applicability

Sex	Evidence
Male	High
Unspecific	Low

References

Appendix 1

List of MIEs in this AOP

Event: 828: Inhibition, Phospholipase A

Short Name: Inhibition, Phospholipase A

Key Event Component

Process	Object	Action
phospholipase activity	phospholipase A1 member A (human)	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

List of Key Events in the AOP

Event: 829: Damage, Lipid bilayer

Short Name: Damage, Lipid bilayer

Key Event Component

Process	Object	Action
	Lipid bilayer of wall of lysosome	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 831: Disturbance, Lysosomal function

Short Name: Disturbance, Lysosomal function

Key Event Component

Process	Object	Action
	lysosome	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent
Aop:257 - Receptor mediated endocytosis and lysosomal overload leading to kidney toxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Event: 177: N/A, Mitochondrial dysfunction 1

Short Name: N/A, Mitochondrial dysfunction 1

Key Event Component

Process	Object	Action
	mitochondrion	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent

AOP ID and Name	Event Type
Aop:77 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1	KeyEvent
Aop:79 - Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure	KeyEvent
Aop:80 - Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure	KeyEvent
Aop:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KeyEvent
Aop:3 - Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:178 - Nicotinic acetylcholine receptor activation contributes to mitochondrial dysfunction and leads to colony loss/failure	KeyEvent
Aop:200 - Estrogen receptor activation leading to breast cancer	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:326 - Thermal stress leading to population decline (3)	MolecularInitiatingEvent
Aop:325 - Thermal stress leading to population decline (2)	MolecularInitiatingEvent
Aop:324 - Thermal stress leading to population decline (1)	MolecularInitiatingEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:437 - Inhibition of mitochondrial electron transport chain (ETC) complexes leading to kidney toxicity	KeyEvent
Aop:423 - Toxicological mechanisms of hepatocyte apoptosis through the PARP1 dependent cell death pathway	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to heart failure via increased myocardial oxidative stress	KeyEvent
Aop:480 - Mitochondrial complexes inhibition leading to heart failure via decreased ATP production	KeyEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	KeyEvent
Aop:509 - Nrf2 inhibition leading to vascular disrupting effects through activating apoptosis signal pathway and mitochondrial dysfunction	KeyEvent
Aop:511 - The AOP framework on ROS-mediated oxidative stress induced vascular disrupting effects	KeyEvent
Aop:256 - Inhibition of mitochondrial DNA polymerase gamma leading to kidney toxicity	KeyEvent
Aop:258 - Renal protein alkylation leading to kidney toxicity	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:205 - AOP from chemical insult to cell death	KeyEvent
Aop:335 - AOP for urothelial carcinogenesis due to chemical cytotoxicity by mitochondrial impairment	MolecularInitiatingEvent
Aop:362 - Immune mediated hepatitis	KeyEvent
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent
Aop:497 - ERa inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	KeyEvent
Aop:34 - LXR activation leading to hepatic steatosis	KeyEvent

AOP ID and Name	Event Type
Aop:447 - Kidney failure induced by inhibition of mitochondrial electron transfer chain through apoptosis, inflammation and oxidative stress pathways	KeyEvent
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent

Stressors

Name
Uranium
Nanoparticles and Micrometer Particles
Cadmium

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Mitochondrial dysfunction is a universal event occurring in cells of any species (Farooqui and Farooqui, 2012). Many invertebrate species (drosophila, C, elegans) are considered as potential models to study mitochondrial function. New data on marine invertebrates, such as molluscs and crustaceans and non-Drosophila species, are emerging (Martinez-Cruz et al., 2012). Mitochondrial dysfunction can be measured in animal models used for toxicity testing (Winklhofer and Haass, 2010; Waerzeggers et al., 2010) as well as in humans (Winklhofer and Haass, 2010).

Key Event Description

Mitochondrial dysfunction is a consequence of inhibition of the respiratory chain leading to oxidative stress.

Mitochondria can be found in all cells and are considered the most important cellular consumers of oxygen. Furthermore, mitochondria possess numerous redox enzymes capable of transferring single electrons to oxygen, generating the superoxide (O2-). Some mitochondrial enzymes that are involved in reactive oxygen species (ROS) generation include the electron-transport chain (ETC) complexes I, II and III; pyruvate dehydrogenase (PDH) and

glycerol-3-phosphate dehydrogenase (GPDH). The transfer of electrons to oxygen, generating superoxide, happens mainly when these redox carriers are charged enough with electrons and the potential energy for transfer is elevated, like in the case of high mitochondrial membrane potential. In contrast, ROS generation is decreased if there are not enough electrons and the potential energy for the transfer is not sufficient (reviewed in Lin and Beal, 2006).

Cells are also able to detoxify the generated ROS due to an extensive antioxidant defence system that includes superoxide dismutases, glutathione peroxidases, catalase, thioredoxins, and peroxiredoxins in various cell organelles (reviewed in Lin and Beal, 2006). It is worth mentioning that, as in the case of ROS generation, antioxidant defences are also closely related to the redox and energetic status of mitochondria. If mitochondria are structurally and functionally healthy, an antioxidant defence mechanism balances ROS generation, and there is not much available ROS production. However, in case of mitochondrial damage, the antioxidant defence capacity drops and ROS generation takes over. Once this happens, a vicious cycle starts and ROS can further damage mitochondria, leading to more free-radical generation and further loss of antioxidant capacity. During mitochondrial dysfunction the availability of ATP also decreases, which is considered necessary for repair mechanisms after ROS generation.

A number of proteins bound to the mitochondria or endoplasmic reticulum (ER), especially in the mitochondria-associated ER membrane (MAM), are playing an important role of communicators between these two organelles (reviewed Mei et al., 2013). ER stress induces mitochondrial dysfunction through regulation of Ca2+ signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca2+ at the MAM and increased Ca2+ uptake into the mitochondrial matrix, which induces Ca2+-dependent mitochondrial outer membrane permeabilization and apoptosis. At the same, ROS are produced by proteins in the ER oxidoreductin 1 (ERO1) family. ER stress activates ERO1 and leads to excessive production of ROS, which, in turn, inactivates SERCA and activates inositol-1,4,5- trisphosphate receptors (IP3R) via oxidation, resulting in elevated levels of cytosolic Ca2+, increased mitochondrial uptake of Ca2+, and ultimately mitochondrial dysfunction. Just as ER stress can lead to mitochondrial dysfunction, mitochondrial dysfunction also induces ER Stress (reviewed Mei et al., 2013). For example, nitric oxide disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca2+ flux which induce ER stress. Increased Ca2+ flux triggers loss of mitochondrial membrane potential (MMP), opening of mitochondrial permeability transition pore (mPTP), release of cytochrome c and apoptosis inducing factor (AIF), decreasing ATP synthesis and rendering the cells more vulnerable to both apoptosis and necrosis (Wang and Qin, 2010).

Metal-induced Mitochondrial Dysfunction

Mitochondria are an important site of Ca2+ regulation and storage, taking up Ca2+ ions electrophoretically from the cytosol through a Ca2+ uniporter, which can then accumulate in the mitochondria (Roos et al., 2012; Orrenius et al., 2015). Similarities between calcium and metals, such as cadmium and lead, makes the entrance and accumulation of these metals into the mitochondria via calcium metals possible by mode of molecular mimicry (Mathews et al., 2013; Adiele et al., 2012). The outer mitochondrial membrane also contains the divalent metal transporter (DMT1), which allows for mitochondrial uptake of divalent metals such as Fe and Mn. When cells are under heavy metal-induced stress, DMT has been shown to be overexpressed in the mitochondrial membrane, making the mitochondria targets of metal toxicity and accumulation.

Heavy metal exposure in aerobic organisms increases ROS formation through redox cycling, where metals with different valence states (Fe, Cu, Cr, etc.) directly produce ROS as they are reduced by cellular antioxidants and then react with oxygen (Shaki et al., 2012; Shaki et al., 2013; Pourahmad et al., 2006; Santos et al., 2007). The production of highly reactive hydroxyl radicals under mitochondrial oxidative stress and in the presence of transition metals occurs via the Fenton reaction or Haber-Weiss reaction (Hancock et al., 2001; Valko et al., 2005; Adam-Vizi et al., 2010). Metals and ROS are capable of damaging mitochondrial DNA as well as mechanisms of DNA repair and proliferation arrest (Valko et al., 2005). Metals and ROS have the potential to directly damage mitochondrial membranes and structure by binding to and oxidizing membrane lipids and proteins. This structural damage can collapse the MMP and lead to the opening of the MPTP (Orrenius et al., 2015; Roos et al., 2012; Pourahmad et al., 2006). Uranium and mercury, for example, have both been shown to directly inhibit the mitochondrial electron transport chain and interfere with ATP production (Shaki et al., 2012; Roos et al., 2012). Furthermore, as previously mentioned, metals have been shown to inhibit ROS-detoxifying enzymes. By binding to these enzymes, metals can inhibit their antioxidant functions, and cause an accumulation of ROS and increased synthesis of more antioxidant enzymes in order to combat the oxidative stress (Blajszczak and Bonini, 2017).

Summing up: Mitochondria play a pivotal role in cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). The production of ATP via oxidative phosphorylation is a vital mitochondrial function (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). The ATP is continuously required for signalling processes (e.g. Ca2+ signalling), maintenance of ionic gradients across membranes, and biosynthetic processes (e.g. protein synthesis, heme synthesis or lipid and phospholipid metabolism) (Kang and Pervaiz, 2012), and (Green, 1998; McBride et al., 2006). Inhibition of mitochondrial respiration contributes to various cellular stress responses, such as deregulation of cellular Ca2+ homeostasis (Graier et al., 2007) and ROS production (Nunnari and Suomalainen, 2012; reviewed Mei et al., 2013).). It is well established in the existing literature that mitochondrial dysfunction may result in: (a) an increased ROS production and a decreased ATP level, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) the reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) the loss of the mitochondrial membrane potential, (e) the loss of mitochondrial motility, causing a failure to re-localize to the sites with increased energy demands (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca2+ uptake, causing Ca2+ overload (reviewed in Lin and Beal, 2006; Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading to (i) the release of mitochondrial pro-death factors, including cytochrome c (Cyt. c), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013), which eventually leads to apoptotic, necrotic or autophagic cell death (Wang and Qin, 2010). Due to their structural and functional complexity, mitochondria present multiple targets for various compounds.

How it is Measured or Detected

Mitochondrial dysfunction can be detected using isolated mitochondria, intact cells or cells in culture as well as in vivo studies. Such assessment can be performed with a large range of methods (revised by Brand and Nicholls, 2011) for which some important examples are given. All approaches to assess mitochondrial dysfunction fall into two main categories: the first assesses the consequences of a loss-of-function, i.e. impaired functioning of the respiratory chain and processes linked to it. Some assay to assess this have been described for KE1, with the limitation that they are not specific for complex I. In the context of overall mitochondrial dysfunction, the same assays provide useful information, when performed under slightly different assay conditions (e.g. without addition of complex III and IV inhibitors). The second approach assesses a 'non-desirable gain-of-function', i.e. processes that are usually only present to a very small degree in healthy cells, and that are triggered in a cell, in which mitochondria fail.

I. Mitochondrial dysfunction assays assessing a loss-of function.

1. Cellular oxygen consumption.

See KE1 for details of oxygen consumption assays. The oxygen consumption parameter can be combined with other endpoints to derive more specific information on the efficacy of mitochondrial function. One approach measures the ADP-to-O ratio (the number of ADP molecules phosphorylated per oxygen atom reduced (Hinkle, 1995 and Hafner et al., 1990). The related P/O ratio is calculated from the amount of ADP added, divided by the amount of O_2 consumed while phosphorylating the added ADP (Ciapaite et al., 2005; Diepart et al., 2010; Hynes et al., 2006; James et al., 1995; von Heimburg et al., 2005).

2. Mitochondrial membrane potential (Δψm).

The mitochondrial membrane potential (Δψm) is the electric potential difference across the inner mitochondrial membrane. It requires a functioning respiratory chain in the absence of mechanisms that dissipate the proton gradient without coupling it to ATP production. The classical, and still most quantitative method uses a tetraphenylphosphonium ion (TPP+)-sensitive electrode on suspensions of isolated mitochondria. The Δψm can also be measured in live cells by fluorimetric methods. These are based on dyes which accumulate in mitochochondria because of Δψm. Frequently used are tetramethylrhodamineethylester (TMRE), tetramethylrhodaminemethyl ester (TMRM) (Petronilli et al., 1999) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1). Mitochondria with intact membrane potential concentrate JC-1, so that it forms red fluorescent aggregates, whereas de-energized mitochondria cannot concentrate JC-1 and the dilute dye fluoresces green (Barrientos et al., 1999). Assays using TMRE or TMRM measure only at one wavelength (red fluorescence), and depending on the assay setup, de-energized mitochondria become either less fluorescent (loss of the dye) or more fluorescent (attenuated dye quenching).

3. Enzymatic activity of the electron transport system (ETS).

Determination of ETS activity can be dene following Owens and King's assay (1975). The technique is based on a cell-free homogenate that is incubated with NADH to saturate the mitochondrial ETS and an artificial electron acceptor [l -(4 -iodophenyl) -3 - (4 -nitrophenyl) -5-phenylte trazolium chloride (INT)] to register the electron transmission rate. The oxygen consumption rate is calculated from the molar production rate of INT-formazan which is determined spectrophotometrically (Cammen et al., 1990).

4. ATP content.

For the evaluation of ATP levels, various commercially-available ATP assay kits are offered based on luciferin and luciferase activity. For isolated mitochondria various methods are available to continuously measure ATP with electrodes (Laudet 2005), with luminometric methods, or for obtaining more information on different nucleotide phosphate pools (e.g. Ciapaite et al., (2005).

II. Mitochondrial dysfunction assays assessing a gain-of function.

1. Mitochondrial permeability transition pore opening (PTP).

The opening of the PTP is associated with a permeabilization of mitochondrial membranes, so that different compounds and cellular constituents can change intracellular localization. This can be measured by assessment of the translocation of cytochrome c, adenylate kinase or AIF from mitochondria to the cytosol or nucleus. The translocation can be assessed biochemically in cell fractions, by imaging approaches in fixed cells or tissues or by life-cell imaging of GFP fusion proteins (Single 1998; Modjtahedi 2006). An alternative approach is to measure the accessibility of cobalt to the mitochondrial matrix in a calcein fluorescence quenching assay in live permeabilized cells (Petronilli et al., 1999).

2. mtDNA damage as a biomarker of mitochondrial dysfunction.

Various quantitative polymerase chain reaction (QPCR)-based assays have been developed to detect changes of DNA structure and sequence in the mitochondrial genome. mtDNA damage can be detected in blood after low-level rotenone exposure, and the damage persists even after CI activity has returned to normal. With a more sustained rotenone exposure, mtDNA damage is also detected in skeletal muscle. These data support the idea that mtDNA damage in peripheral tissues in the rotenone model may provide a biomarker of past or ongoing mitochondrial toxin

exposure (Sanders et al., 2014a and 2014b).

3. Generation of ROS and resultant oxidative stress.

a. General approach. Electrons from the mitochondrial ETS may be transferred ‘erroneously’ to molecular oxygen to form superoxide anions. This type of side reaction can be strongly enhanced upon mitochondrial damage. As superoxide may form hydrogen peroxide, hydroxyl radicals or other reactive oxygen species, a large number of direct ROS assays and assays assessing the effects of ROS (indirect ROS assays) are available (Adam-Vizi, 2005; Fan and Li 2014). Direct assays are based on the chemical modification of fluorescent or luminescent reporters by ROS species. Indirect assays assess cellular metabolites, the concentration of which is changed in the presence of ROS (e.g. glutathione, malonaldehyde, isoprostanes,etc.) At the animal level the effects of oxidative stress are measured from biomarkers in the blood or urine.

b. Measurement of the cellular glutathione (GSH) status. GSH is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase (GSSH + NADPH + H+ à 2 GSH + NADP+). The ratio of GSH/GSSG is therefore a good indicator for the cellular NADH+/NADPH ratio (i.e. the redox potential). GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically with DTNB (Ellman’s reagent). As excess GSSG is rapidly exported from most cells to maintain a constant GSH/GSSG ratio, a reduction of total glutathione (GSH/GSSG) is often a good surrogate measure for oxidative stress.

c. Quantification of lipid peroxidation. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA)-reactive compounds such as malondialdehyde generated from the decomposition of cellular membrane lipid under oxidative stress (Pryor et al., 1976). This method is quite sensitive, but not highly specific. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be more specific for lipid peroxidation. A number of commercial ELISA kits have been developed for IsoPs, but interfering agents in samples requires partial purification before analysis. Alternatively, GC/MS may be used, as robust (specific) and sensitive method.

d. Detection of superoxide production. Generation of superoxide by inhibition of complex I and the methods for its detection are described by Grivennikova and Vinogradov (2014). A range of different methods is also described by BioTek (http://www.biotek.com/resources/articles/reactive-oxygen-species.html). The reduction of ferricytochrome c to ferrocytochrome c may be used to assess the rate of superoxide formation (McCord, 1968). Like in other superoxide assays, specificity can only be obtained by measurements in the absence and presence of superoxide dismutase. Chemiluminescent reactions have been used for their increased sensitivity. The most widely used chemiluminescent substrate is lucigenin. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical, and they become membrane impermeable after oxidation (trapping at site of formation). The best characterized of these probes are Hydro-Cy3 and Hydro-Cy5. generation of superoxide in mitochondria can be visualized using fluorescence microscopy with MitoSOX™ Red reagent (Life Technologies). MitoSOX™ Red reagent is a cationic derivative of dihydroethidium that permeates live cells and accumulates in mitochondria.

e. Detection of hydrogen peroxide (H_2O_2) production. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products in the presence of hydrogen peroxide (Zhou et al., 1997: Ruch et al., 1983). The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H_2O_2 form increasing amounts of fluorescent product (Tarpley et al., 2004).

Summing up, mitochondrial dysfunction can be measured by: • ROS production: superoxide (O2-), and hydroxyl radicals (OH−) • Nitrosative radical formation such as ONOO− or directly by: • Loss of mitochondrial membrane potential (MMP) • Opening of mitochondrial permeability transition pores (mPTP) • ATP synthesis • Increase in mitochondrial Ca2+ • Cytochrome c release • AIF (apoptosis inducing factor) release from mitochondria • Mitochondrial Complexes enzyme activity • Measurements of mitochondrial oxygen consumption • Ultrastructure of mitochondria using electron microscope and mitochondrial fragmentation measured by labelling with DsRed-Mito expression (Knott et al, 2008) Mitochondrial dysfunction-induced oxidative stress can be measured by: • Reactive carbonyls formations (proteins oxidation) • Increased 8-oxo-dG immunoreactivity (DNA oxidation) • Lipid peroxidation (formation of malondialdehyde (MDA) and 4- hydroxynonenal (HNE) • 3-nitrotyrosine (3-NT) formation, marker of protein nitration • Translocation of Bid and Bax to mitochondria • Measurement of intracellular free calcium concentration ([Ca2+]i): Cells are loaded with 4 µM fura-2/AM). • Ratio between reduced and oxidized form of glutathione (GSH depletion) (Promega assay, TB369; Radkowsky et al., 1986) • Neuronal nitric oxide synthase (nNOS) activation that is Ca2+-dependent. All above measurements can be performed as the assays for each readout are well established in the existing literature (e.g. Bal-Price and Brown, 2000; Bal-Price et al., 2002; Fujikawa, 2015; Walker et al., 1995). See also KE Oxidative Stress, Increase

Assay Type & Measured Content	**Description**	**Dose Range Studied**	**Assay Characteristics (Length/Ease of use/Accuracy)**

Rhodamine 123 Assay Measuring Mitochondrial membrane potential (MMP) and its collapse (Shaki et al., 2012)	Mitochondrial uptake of cationic fluorescent dye, rhodamine 123, is used for estimation of mitochondrial membrane potential. The fluorescence was monitored using Schimadzou RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively.	50, 100 and 500 µM of uranyl acetate	Short / easy Medium accurancy
TMRE fluorescence Assay Measuring Mitochondrial permeability transition pore (mPTP) opening (Huser et al., 1998)	Laser scanning confocal microscopy in combination with the potentiometric fluorescence dye tetramethylrhodamine ethyl ester to monitor relative changes in membrane potential in single isolated cardiac mitochondria. The cationic dye distributes across the membrane in a voltage-dependent manner. Therefore, the large potential gradient across the inner mitochondrial membrane results in the accumulation of the fluorescent dye within the matrix compartment. Rapid depolarizations are caused by the opening of the transition pore.	1 µM cyclosporin A	Short / easy Low accurancy
GSH / GSSG Determination Assay Measuring cellular glutathione (GSH) status; ratio of GSH/GSSG (Owen & Butterfield, 2010; Shaki et al., 2013)	GSH and GSSG levels are determinted biochemically with DTNB (Ellman's reagent). The developed yellow color was read at 412 nm on a spectrophotometer.	100 µM uranyl acetate	Short / easy Low accurancy
TBARS Assay Quantification of lipid peroxidation (Yuan et al., 2016)	MDA content, a product of lipid peroxidation, was measured using a thiobarbituric acid reactive substances (TBARS) assay. Briefly, the kidney cells were collected in 1 ml PBS buffer solution (pH 7.4) and sonicated. MDA reacts with thiobarbituric acid forming a colored product which can be measured at an absorbance of 532 nm.	200, 400, 800 µM uranyl acetate	Medium / medium High accurancy
Aequorin-based bioluminescence assay Increase in mitochondrial Ca^{2+} influx (Pozzan & Rudolf, 2009)	Together with GFP, the aequorin moiety acts as Ca^{2+} sensor *in vivo*, which delivers emission energy to the GFP acceptor molecule in a BRET (Bioluminescence Resonance Energy Transfer) process; the Ca2+ can then be visualized with fluorescence microscopy.		Short / easy Low accurancy
Western blot & immunostaining analyses Measuring cytochrome c release (Chen et al., 2000)	Examining the redistribution of Cyto c in cytosolic and mitochondrial cellular fractions. Cells are homogenized and centrifuged, then prepared for immunoblots. Cellular fractions were washed in PBS and lysed in 1% NP-40 buffer. Cellular proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes, probed using immunoblot analyses with antibodies specific to cyto c (6581A for Western and 65971A for immunostaining; Pharmingen)		Short / easy Medium accurancy

Quantikine Rat/Mouse Cytochrome c Immunoassay Measuring cytochrome c release (Shaki et al., 2012)	Cytochrome C release was measured a monoclonal antibody specific for rat/mouse cytochrome c was precoated onto the microplate. Seventy-five microliter of conjugate (containing mono-clonal antibody specific for cytochrome c conjugated to horseradish peroxidase). After 2 h of incubation, the substrate solution (100 µl) was added to each well and incubated for 30 min. After 100 µl of the stop solution was added to each well; the optical density of each well was determined by the aforementioned microplate spectrophotometer set to 450 nm.		Short / easy Low accurancy
Membrane potential and cell viability – Flow Cytometry Measuring cytochrome c release (Kruidering et al., 1997)	“Dc and viability were determined by analyzing the R123 and propidium iodide fluorescence intensity with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser, with the Lysis software program (Becton Dickinson). R123 is a cationic dye that accumulates in the negatively charged inner side of the mitochondria. When the potential drops, less R123 accumulates in the mitochondria, which results in a lower fluorescence signal. The potential was measured as follows: at the indicated times, a 500-ml sample of the cell suspension was taken and transferred to an Eppendorf minivial. To this sample, 100 ml of 6 mM R123 in buffer D was added. After incubation for 10 min at 37°C, the cell suspension was centrifuged for 5 min at 80 3 *g*. The cell pellet was resuspended in 200 ml of buffer D, containing 0.2 mM R123 and 10 mM propidium iodide, to prevent loss of R123 and to stain nonviable cells, respectively. The samples were transferred to FACScan tubes and analyzed immediately. Analysis was performed at a flow rate of 60 ml/min. R123 fluorescence was detected by the FL1 detector with an emission detection limit below 560 nm. Propidium iodide fluorescence was detected by the FL3 detector, with emission detection above 620 nm. Per sample 3,000 to 5,000 cells were counted (Van de Water *et al.*, 1993)”		Short / easy Medium accurancy

References

Adam-Vizi V. Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources. Antioxid Redox Signal. 2005, 7(9-10):1140-1149.

Adam-Vizi, V., & Starkov, A. A. (2010). Calcium and mitochondrial reactive oxygen species generation: How to read the facts. Journal of Alzheimer's Disease : JAD, 20 Suppl 2, S413-S426. doi:10.3233/JAD-2010-100465

Adiele, R. C., Stevens, D., & Kamunde, C. (2012). Differential inhibition of electron transport chain enzyme complexes by cadmium and calcium in isolated rainbow trout (oncorhynchus mykiss) hepatic mitochondria. Toxicological Sciences, 127(1), 110-119. doi:10.1093/toxsci/kfs091

Bal-Price A. and Guy C. Brown. Nitric-oxide-induced necrosis and apoptosis in PC12 cells mediated by mitochondria. J. Neurochemistry, 2000, 75: 1455-1464.

Bal-Price A, Matthias A, Brown GC., Stimulation of the NADPH oxidase in activated rat microglia removes nitric oxide but induces peroxynitrite production. J. Neurochem. 2002, 80: 73-80.

Barrientos A., and Moraes C.T. (1999) Titrating the Effects of Mitochondrial Complex I Impairment in the Cell Physiology. Vol. 274, No. 23, pp. 16188–16197.

Belyaeva, E. A., Sokolova, T. V., Emelyanova, L. V., & Zakharova, I. O. (2012). Mitochondrial electron transport chain in heavy metal-induced neurotoxicity : Effects of cadmium , mercury , and copper. Thescientificworld, 2012, 1-14. doi:10.1100/2012/136063

Blajszczak, C., & Bonini, M. G. (2017). Mitochondria targeting by environmental stressors: Implications for redox cellular signaling. Toxicology, 391, 84-89. doi:10.1016/j.tox.2017.07.013

Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J. 2011 Apr 15;435(2):297-312.

Braun RJ. (2012). Mitochondrion-mediated cell death: dissecting yeast apoptosis for a better understanding of neurodegeneration. Front Oncol 2:182.

Cammen M. Corwin, Susannah Christensen. John P. (1990) Electron transport system (ETS) activity as a measure of benthic macrofaunal metabolism MARINE ECOLOGY PROGRESS SERIES- (65) : 171-182.

Chen, Q., Gong, B., & Almasan, A. (2000). Distinct stages of cytochrome c release from mitochondria: Evidence for a feedback amplification loop linking caspase activation to mitochondrial dysfunction in genotoxic stress induced apoptosis. Cell Death and Differentiation, 7(2), 227-233. doi:10.1038/sj.cdd.4400629

Ciapaite, Lolita Van Eikenhorst, Gerco Bakker, Stephan J.L. Diamant, Michaela. Heine, Robert J Wagner, Marijke J. V. Westerhoff, Hans and Klaas Krab (2005) Modular Kinetic Analysis of the Adenine Nucleotide Translocator–Mediated Effects of Palmitoyl-CoA on the Oxidative Phosphorylation in Isolated Rat Liver Mitochondria Diabetes 54:4 944-951.

Correia SC, Santos RX, Perry G, Zhu X, Moreira PI, Smith MA. (2012). Mitochondrial importance in Alzheimer's, Huntington's and Parkinson's diseases. Adv Exp Med Biol 724:205 – 221.

Cozzolino M, Ferri A, Valle C, Carri MT. (2013). Mitochondria and ALS: implications from novel genes and pathways. Mol Cell Neurosci 55:44 – 49.

Diepart, C, Verrax, J Calderon, PU, Feron, O., Jordan, BF, Gallez, B (2010) Comparison of methods for measuring oxygen consumption in tumor cells in vitroAnalytical Biochemistry 396 (2010) 250–256.

Farooqui T. and . Farooqui, A. A (2012) Oxidative stress in Vertebrates and Invertebrate: molecular aspects of cell signalling. Wiley-Blackwell,Chapter 27, pp:377- 385.

Fan LM, Li JM. Evaluation of methods of detecting cell reactive oxygen species production for drug screening and cell cycle studies. J Pharmacol Toxicol Methods. 2014 Jul-Aug;70(1):40-7.

Fiskum G. Mitochondrial participation in ischemic and traumatic neural cell death. J Neurotrauma. 2000 Oct;17(10):843-55.

Friberg H, Wieloch T. (2002). Mitochondrial permeability transition in acute neurodegeneration. Biochimie 84:241–250.

Fujikawa DG, The Role of Excitotoxic Programmed Necrosis in Acute Brain Injury. Computational and Structural Biotechnology Journal, 2015, 13: 212–221.

Graier WF, Frieden M, Malli R. (2007). Mitochondria and Ca2+ signaling: old guests, new functions. Pflugers Arch 455:375–396.

Green DR. (1998). Apoptotic pathways: the roads to ruin. Cell 94:695-698.

Grivennikova VG, Vinogradov AD. Generation of superoxide by the mitochondrial Complex I. Biochim Biophys Acta. 2006, 1757(5-6):553-61.

Hafner RP, Brown GC, Brand MD: Analysis of the control of respiration rate, phosphorylation rate, proton leak rate and protonmotive force in isolated mitochondria using the 'top-down' approach of metabolic control theory. Eur J Biochem188 :313 –319,1990.

Hancock, J. T., Desikan, R., & Neill, S. J. (2001). Role of reactive oxygen species in cell signalling pathways. Biochemical Society Transactions, 29(Pt 2), 345-350. doi:10.1042/0300-5127:0290345 [doi]

Hao, Y., Huang, J., Liu, C., Li, H., Liu, J., Zeng, Y., . . . Li, R. (2016). Differential protein expression in metallothionein protection from depleted uranium-induced nephrotoxicity. Scientific Reports, doi:10.1038/srep38942

Hao, Y., Ren, J., Liu, C., Li, H., Liu, J., Yang, Z., . . . Su, Y. (2014). Zinc protects human kidney cells from depleted uranium induced apoptosis. Basic & Clinical Pharmacology & Toxicology, 114, 271-280. doi:10.1111/bcpt.12167

Hinkle PC (1995) Measurement of ADP/O ratios. In Bioenergetics: A Practical Approach. Brown GC, Cooper CE, Eds. Oxford, U.K., IRL Press, p.5 –6.

Huerta-García, E., Perez-Arizti, J. A., Marquez-Ramirez, S. G., Delgado-Buenrostro, N. L., Chirino, Y. I., Iglesias, G. G., & Lopez-Marure, R. (2014). Titanium dioxide nanoparticles induce strong oxidative stress and mitochondrial damage in glial cells. Free Radical Biology and Medicine, 73, 84-94. doi:10.1016/j.freeradbiomed.2014.04.026

Hüser, J., Rechenmacher, C. E., & Blatter, L. A. (1998). Imaging the permeability pore transition in single mitochondria. Biophysical Journal, 74(4), 2129-2137. doi:10.1016/S0006-3495(98)77920-2

Hynes, J.. Marroquin, L.D Ogurtsov, V.I. Christiansen, K.N. Stevens, G.J. Papkovsky, D.B. Will, Y. (2006)) Investigation of drug-induced mitochondrial toxicity using fluorescence-based oxygen-sensitive probes, Toxicol. Sci. 92 186–200.

James, P.E. Jackson, S.K.. Grinberg, O.Y Swartz, H.M. (1995) The effects of endotoxin on oxygen consumption of various cell types in vitro: an EPR oximetry study, Free Radic. Biol. Med. 18 (1995) 641–647.

Kang J, Pervaiz S. (2012). Mitochondria: Redox Metabolism and Dysfunction. Biochem Res Int 2012:896751.

Kann O, Kovács R. (2007). Mitochondria and neuronal activity. Am J Physiol Cell Physiol 292:C641-576.

Karlsson, H. L., Gustafsson, J., Cronholm, P., & Möller, L. (2009). Size-dependent toxicity of metal oxide particles—A comparison between nano- and micrometer size. Toxicology Letters, 188(2), 112-118. doi:10.1016/j.toxlet.2009.03.014

Knott Andrew B., Guy Perkins, Robert Schwarzenbacher & Ella Bossy-Wetzel. Mitochondrial fragmentation in neurodegeneration. Nature Reviews Neuroscience, 2008, 229: 505-518.

Kruidering, M., Van De Water, B., De Heer, E., Mulder, G. J., & Nagelkerke, J. F. (1997). Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: Mitochondrial dysfunction by inhibition of complexes I to IV of the respiratory

chain. The Journal of Pharmacology and Experimental Therapeutics, 280(2), 638-649.

Llaudet E, Hatz S, Droniou M, Dale N. Microelectrode biosensor for real-time measurement of ATP in biological tissue. Anal Chem. 2005, 77(10):3267-73.

Lee HC, Wei YH. (2012). Mitochondria and aging. Adv Exp Med Biol 942:311-327.

Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem.2003;278:8516–8525.

Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 2006. 443:787-795.

Martin LJ. (2011). Mitochondrial pathobiology in ALS. J Bioenerg Biomembr 43:569 – 579.

Martinez-Cruz, Oliviert Sanchez-Paz, Arturo Garcia-Carreño, Fernando Jimenez-Gutierrez, Laura Ma. de los Angeles Navarrete del Toro and Adriana Muhlia-Almazan. Invertebrates Mitochondrial Function and Energetic Challenges (www.intechopen.com), Bioenergetics, Edited by Dr Kevin Clark, ISBN 978-953-51-0090-4, Publisher InTech, 2012, 181-218.

Mathews, C. K., Holde, K. E. van, Appling, D. R., & Anthony-Cahill, S. J. (2013). Biochemistry (4th ed.). Toronto: Pearson.

McBride HM, Neuspiel M, Wasiak S. (2006). Mitochondria: more than just a powerhouse. Curr Biol 16:R551–560.

McCord, J.M. and I. Fidovich (1968) The Reduction of Cytochrome C by Milk Xanthine Oxidase. J. Biol. Chem. 243:5733-5760.

Mei Y, Thompson MD, Cohen RA, Tong X. (2013) Endoplasmic Reticulum Stress and Related Pathological Processes. J Pharmacol Biomed Anal.. 1:100-107.

Miccadei, S., & Floridi, A. (1993). Sites of inhibition of mitochondrial electron transport by cadmium. Elsevier Scientific Publishers Ireland Ltd., 89, 159-167.Xu, X. M., & Møller, S. G. (2010). ROS removal by DJ-1: Arabidopsis as a new model to understand Parkinson's Disease. Plant signaling & behavior, 5(8), 1034–1036. doi:10.4161/psb.5.8.12298

Modjtahedi N, Giordanetto F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. Trends Cell Biol. 2006 May;16(5):264-72.

Nunnari J, Suomalainen A. (2012). Mitochondria: in sickness and in health. Cell 148:1145–1159. Hajnóczky G, Csordás G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, Yi M. (2006). Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium 40:553-560.

Oliviert Martinez-Cruz, Arturo Sanchez-Paz, Fernando Garcia-Carreño, Laura Jimenez-Gutierrez, Ma. de los Angeles Navarrete del Toro and Adriana Muhlia-Almazan. Invertebrates Mitochondrial Function and Energetic Challenges (www.intechopen.com), Bioenergetics, Edited by Dr Kevin Clark, ISBN 978-953-51-0090-4, Publisher InTech, 2012, 181-218.

Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2015). Calcium and mitochondria in the regulation of cell death. Biochemical and Biophysical Research Communications, 460(1), 72-81. doi:10.1016/j.bbrc.2015.01.137

Owen, J. B., & Butterfield, D. A. (2010). Measurement of oxidized/reduced glutathione ratio. Methods in Molecular Biology (Clifton, N.J.), 648, 269-277. doi:10.1007/978-1-60761-756-3_18 [doi]

Owens R.G. and King F.D. The measurement of respiratory lectron-transport system activity in marine zooplankton. Mar. Biol. 1975, 30:27-36.

Pan, Y., Leifer, A., Ruau, D., Neuss, S., Bonrnemann, J., Schmid, G., . . . Jahnen-Dechent, W. (2009). Gold nanoparticles of diameter 1.4 nm trigger necrosis by oxidative stress and mitochondrial damage. Small, 5(8), 2067-2076. doi:10.1002/smll.200900466

Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, Di Lisa F: Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Biophys J 1999, 76:725-734.

Pourahmad, J., Ghashang, M., Ettehadi, H. A., & Ghalandari, R. (2006). A search for cellular and molecular mechanisms involved in depleted uranium (DU) toxicity. Environmental Toxicology, 21(4), 349-354. doi:10.1002/tox.20196

Pozzan, T., & Rudolf, R. (2009). Measurements of mitochondrial calcium in vivo. Biochimica Et Biophysica Acta (BBA) - Bioenergetics, 1787(11), 1317-1323. doi:https://doi.org/10.1016/j.bbabio.2008.11.012

Promega GSH-Glo Glutathione Assay Technical Bulletin, TB369, Promega Corporation, Madison, WI.

Pryor, W.A., J.P. Stanley, and E. Blair. (1976) Autoxidation of polyunsaturated fatty acids: II. A Suggested mechanism for the Formation of TBA-reactive materials from prostaglandin-like Endoperoxides. Lipids, 11:370-379.

Radkowsky, A.E. and E.M. Kosower (1986) Bimanes 17. (Haloalkyl)-1,5-diazabicyclo[3.3.O]octadienediones (halo-9,10-dioxabimanes): reactivity toward the tripeptide thiol, glutathione, J. Am. Chem. Soc 108:4527-4531.

Roos, D., Seeger, R., Puntel, R., & Vargas Barbosa, N. (2012). Role of calcium and mitochondria in MeHg-mediated cytotoxicity. Journal of Biomedicine and Biotechnology, 2012, 1-15. doi:10.1155/2012/248764

Ruch, W., P.H. Cooper, and M. Baggiollini (1983) Assay of H2O2 production by macrophages and neutrophils with Homovanillic acid and horseradish peroxidase. J. Immunol Methods 63:347-357.

Sanders LH, McCoy J, Hu X, Mastroberardino PG, Dickinson BC, Chang CJ, Chu CT, Van Houten B, Greenamyre JT. (2014a). Mitochondrial DNA damage: molecular marker of vulnerable nigral neurons in Parkinson's disease. Neurobiol Dis. 70:214-23.

Sanders LH, Howlett EH2, McCoy J, Greenamyre JT. (2014b) Mitochondrial DNA damage as a peripheral biomarker for mitochondrial toxin exposure in rats. Toxicol Sci. Dec;142(2):395-402.

Santos, N. A. G., Catão, C. S., Martins, N. M., Curti, C., Bianchi, M. L. P., & Santos, A. C. (2007). Cisplatin-induced nephrotoxicity is associated with oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis in rat kidney mitochondria. Archives of Toxicology, 81(7), 495-504. doi:10.1007/s00204-006-0173-2

Shaki, F., Hosseini, M. J., Ghazi-Khansari, M., & Pourahmad, J. (2012). Toxicity of depleted uranium on isolated rat kidney mitochondria. Biochimica Et Biophysica Acta - General Subjects, 1820(12), 1940-1950. doi:10.1016/j.bbagen.2012.08.015

Shaki, F., Hosseini, M., Ghazi-Khansari, M., & Pourahmad, J. (2013). Depleted uranium induces disruption of energy homeostasis and oxidative stress in isolated rat brain mitochondria. Metallomics, 5(6), 736-744. doi:10.1039/c3mt00019b

Single B, Leist M, Nicotera P. Simultaneous release of adenylate kinase and cytochrome c in cell death. Cell Death Differ. 1998 Dec;5(12):1001-3.

Tahira Farooqui and Akhlaq A. Farooqui. (2012) Oxidative stress in Vertebrates and Invertebrate: molecular aspects of cell signalling. Wiley-Blackwell,Chapter 27, pp:377- 385.

Tarpley, M.M., D.A. Wink, and M.B. Grisham (2004) Methods for detection of reactive Metabolites of Oxygen and Nitrogen: in vitro and in vivo considerations. Am . J. Physiol Regul Integr Comp Physiol. 286:R431-R444.

Valko, M., Morris, H., & Cronin, M. T. (2005). Metals, toxicity and oxidative stress. Current Medicinal Chemistry, 12(10), 1161-1208. doi:10.2174/0929867053764635 [doi]

von Heimburg, D. Hemmrich, K. Zachariah S.,. Staiger, H Pallua, N.(2005) Oxygen consumption in undifferentiated versus differentiated adipogenic mesenchymal precursor cells, Respir. Physiol. Neurobiol. 146 (2005) 107–116.

Waerzeggers, Yannic Monfared, Parisa Viel, Thomas Winkeler, Alexandra Jacobs, Andreas H. (2010) Mouse models in neurological disorders: Applications of non-invasive imaging, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, Volume 1802, Issue 10, Pages 819-839.

Walker JE, Skehel JM, Buchanan SK. (1995) Structural analysis of NADH: ubiquinone oxidoreductase from bovine heart mitochondria. Methods Enzymol.;260:14–34.

Wang A, Costello S, Cockburn M, Zhang X, Bronstein J, Ritz B. (2011). Parkinson’s disease risk from ambient exposure to pesticides. Eur J Epidemiol 26:547-555.

Wang, L., Li, J., Li, J., & Liu, Z. (2009). Effects of lead and/or cadmium on the oxidative damage of rat kidney cortex mitochondria. Biol.Trace Elem.Res., 137, 69-78. doi:10.1007/s12011-009-8560-1

Wang Y., and Qin ZH., Molecular and cellular mechanisms of excitotoxic neuronal death, Apoptosis, 2010, 15:1382-1402.

Wieloch T. (2001). Mitochondrial Involvement in Acute Neurodegeneration 52:247–254.

Winklhofer, K. Haass,C (2010) Mitochondrial dysfunction in Parkinson's disease, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1802: 29-44.

Yuan, Y., Zheng, J., Zhao, T., Tang, X., & Hu, N. (2016). Uranium-induced rat kidney cell cytotoxicity is mediated by decreased endogenous hydrogen sulfide (H2S) generation involved in reduced Nrf2 levels. Toxicology Research, 5(2), 660-673. doi:10.1039/C5TX00432B

Zhang, H., Chang, Z., Mehmood, K., Abbas, R. Z., Nabi, F., Rehman, M. U., . . . Zhou, D. (2018). Nano copper induces apoptosis in PK-15 cells via a mitochondria-mediated pathway. Biological Trace Element Research, 181(1), 62-70. doi:10.1007/s12011-017-1024-0

Zhou, M., Z.Diwu, Panchuk-Voloshina, N. and R.P. Haughland (1997), A Stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: application in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal. Biochem 253:162-168.

Event: 833: Occurrence, Cytoplasmic vacuolization (hepatocyte)

Short Name: Occurrence, Cytoplasmic vacuolization (hepatocyte)

Key Event Component

Process	Object	Action
autophagic cell death		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Event: 835: Occurrence, Ballooning degeneration (hepatocyte)

Short Name: Occurrence, Ballooning degeneration (hepatocyte)

Key Event Component

Process	Object	Action
autophagic cell death		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Event: 837: Occurrence, Cytoplasmic vacuolization (kupffer cell)

Short Name: Occurrence, Cytoplasmic vacuolization (kupffer cell)

Key Event Component

Process	Object	Action
autophagic cell death		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
Kupffer cell

Event: 838: Induction, Microvesicular fat

Short Name: Induction, Microvesicular fat

Key Event Component

Process	Object	Action
Microvesicular hepatic steatosis		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Event: 839: Formation, Mallory body

Short Name: Formation, Mallory body

Key Event Component

Process	Object	Action

Process	Object	Action
Mallory bodies		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 836: Occurrence, Cytoplasmic vacuolization (Bile duct cell)

Short Name: Occurrence, Cytoplasmic vacuolization (Bile duct cell)

Key Event Component

Process	Object	Action
autophagic cell death		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cholangiocyte

List of Adverse Outcomes in this AOP

Event: 840: Formation, Liver fibrosis

Short Name: Formation, Liver fibrosis

Key Event Component

Process	Object	Action
liver fibrosis	liver	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	AdverseOutcome

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
liver

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 836: Inhibition, Phospholipase A leads to Damage, Lipid bilayer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	High	

Relationship: 837: Inhibition, Phospholipase A leads to Disturbance, Lysosomal function

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	High	

Relationship: 3154: Inhibition, Phospholipase A leads to N/A, Mitochondrial dysfunction 1

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	High	

Relationship: 839: Damage, Lipid bilayer leads to Occurrence, Cytoplasmic vacuolization (hepatocyte)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 840: Damage, Lipid bilayer leads to Occurrence, Ballooning degeneration (hepatocyte)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 841: Damage, Lipid bilayer leads to Occurrence, Cytoplasmic vacuolization (Bile duct cell)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 842: Damage, Lipid bilayer leads to Occurrence, Cytoplasmic vacuolization (kupffer cell)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 843: Disturbance, Lysosomal function leads to Occurrence, Cytoplasmic vacuolization (hepatocyte)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 844: Disturbance, Lysosomal function leads to Occurrence, Ballooning degeneration (hepatocyte)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 845: Disturbance, Lysosomal function leads to Occurrence, Cytoplasmic vacuolization (Bile duct cell)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 846: Disturbance, Lysosomal function leads to Occurrence, Cytoplasmic vacuolization (kupffer cell)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 3155: N/A, Mitochondrial dysfunction 1 leads to Occurrence, Cytoplasmic vacuolization (hepatocyte)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 3156: N/A, Mitochondrial dysfunction 1 leads to Occurrence, Ballooning degeneration (hepatocyte)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 3158: N/A, Mitochondrial dysfunction 1 leads to Occurrence, Cytoplasmic vacuolization (Bile duct cell)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 3157: N/A, Mitochondrial dysfunction 1 leads to Occurrence, Cytoplasmic vacuolization (kupffer cell)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 851: Occurrence, Cytoplasmic vacuolization (hepatocyte) leads to Induction,

Microvesicular fat

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 852: Occurrence, Cytoplasmic vacuolization (hepatocyte) leads to Formation, Mallory body

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 853: Occurrence, Ballooning degeneration (hepatocyte) leads to Induction, Microvesicular fat

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 854: Occurrence, Ballooning degeneration (hepatocyte) leads to Formation, Mallory body

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 855: Occurrence, Cytoplasmic vacuolization (Bile duct cell) leads to Induction, Microvesicular fat

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 856: Occurrence, Cytoplasmic vacuolization (Bile duct cell) leads to Formation, Mallory body

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 857: Occurrence, Cytoplasmic vacuolization (kupffer cell) leads to Induction, Microvesicular fat

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 858: Occurrence, Cytoplasmic vacuolization (kupffer cell) leads to Formation, Mallory body

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	Low	

Relationship: 859: Induction, Microvesicular fat leads to Formation, Liver fibrosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	High	

Relationship: 860: Formation, Mallory body leads to Formation, Liver fibrosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	adjacent	High	