Defining Molecular Initiating Events in the Adverse Outcome Pathway Framework for Risk Assessment

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ABSTRACT

Consumer and environmental safety decisions are based on exposure and hazard data, interpreted using risk assessment approaches. The adverse outcome pathway (AOP) conceptual framework has been presented as a logical sequence of events or processes within biological systems which can be used to understand adverse effects and refine current risk assessment practices in ecotoxicology. This framework can also be applied to human toxicology and is explored, based around investigating the molecular initiating events (MIEs) of compounds. The precise definition of the MIE has yet to reach general acceptance. In this work we present a unified MIE definition:

an MIE is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome *via* a pathway. Case studies are presented and issues with current definitions are addressed. With the development of a unified MIE definition the field can look towards defining, classifying and characterizing more MIEs, and using knowledge of the chemistry of these processes to aid AOP research and toxicity risk assessment. We also present the role of MIE research in the development of *in vitro* and *in silico* toxicology and suggest how, by using a combination of biological and chemical approaches, MIEs can be identified and characterized despite a lack of detailed reports, even for some of the most studied molecules in toxicology.

INTRODUCTION

Toxicology risk assessment is vital to the safety of consumers, workers and the environment. Demands to produce assessments for an increasing number of compounds with fewer resources while cutting down on the use of animal testing has lead the field to look to non-animal alternatives including *in vitro* and *in silico* approaches. A large amount of the chemical and biological cause for toxicity remains unexplored as *in vivo* studies that focus more on apical endpoints at the highest levels of biological organization have historically been the preferred method for safety evaluation. A combination of new approaches and methods in biology and chemistry will lead to greater understanding of the processes connecting chemical exposure and adverse outcome. The adverse outcome pathway (AOP) framework for risk assessment brings this knowledge together in an attempt to develop predictive methods for human and environmental toxicology.[1]

AOPs were first outlined for environmental risk assessment by Ankley in 2010[2] (Figure 1). The AOP can be defined as a sequence of events from the exposure of an individual to a chemical through to an understanding of the adverse effect at the individual level (for human health) or population level (for ecotoxicology).[1] AOPs span multiple levels of biological organization, but always contain an initial molecular interaction between a compound and the organism that triggers subsequent effects at higher levels of biological organization. This interaction is the molecular initiating event (MIE).

Figure 1.

The chemistry of the MIE is important to understand when developing predictive methods for human and environmental toxicology. The chemistry of the molecule allows it to have specific MIEs. Because of this, links between chemical structure or chemical property and molecular initiating event will undoubtedly be stronger than links to toxicological endpoints, due to a smaller "jump" between chemical exposure and MIE. It is accepted that a single MIE could be the cause of multiple toxicological endpoints, or that a single endpoint may be the result of several MIEs. Several compounds with vastly different chemical properties could result in the same endpoint. Attempting to build structure activity relationships (SARs) and quantitative structure activity relationships (QSARs) around these molecules will be vastly simplified by examining groups of compounds based on understanding of their MIEs.

A large amount of work has been done to group chemicals in toxicology based on receptor binding,[3–6] one particular type of MIE. If a receptor has a single mechanism of binding, and hence is associated with a single MIE, we can confidently predict that a (Q)SAR based on this training set will be highly successful. However if the receptor is associated with several MIEs the

(Q)SAR will not be effective. By gaining knowledge of these MIEs we can work towards generating more useful sets of data.

By combining knowledge about the MIE a compound is likely to activate with receptor binding and dose response data, and an understanding of adverse outcomes downstream in the AOP, quantitative predictions for new compounds could be made.

The recent search for alternatives to animal based toxicity testing has been well documented.[7,8] The limitations of animal experiments have been discussed[9–12] and new methods are being investigated to replace, reduce and refine approaches.[13] The MIE/AOP framework looks for mechanistic understanding, rather than to directly replace existing assays. This understanding is based, as much as possible, on human relevant data rather than *in vitro* data generated on non-human cells or read across from animal data. In this way mechanistic insights will aid a combination of approaches that will be required to reduce reliance on animal methods.[14]

New technologies in the field of biology, giving greater knowledge of the exposure of chemicals in physiologically based pharmacokinetics (PBPK) modelling methods,[15,16] more understanding of the biological processes in the -omics technologies,[17–19] and a better sense of the holistic nature of biology through systems biology[20,21] give great promise to the advancement of risk assessment. As these methods reach their full potential their discoveries can be implemented into an AOP/MIE approach.

An MIE-based approach can assist the development of both *in silico* and *in vitro* methods through the mechanistic understanding of how assays work and what they tell us.[22,23] The value and emergence of (Q)SARs cannot be ignored when using *in silico* toxicology.[24] (Q)SARs have been developed as valuable tools for predicting acute aquatic toxicology and to classify aquatic toxicants, where little or no empirical data were available.[25,26] They were also used in the

development of the fathead minnow database to assign modes of action to chemicals.[27] In addition, QSAR based tools are already available to allow the prediction of metabolites formed from a parent chemical structure,[28] such as Meteor,[29] and MetaPrint 2D,[30] (Q)SAR tools have already found use in human toxicology, in the fields of mutagenicity and carcinogenicity.[31,32] Read across methods in hazard classification and the development of modelling potential beyond structural similarity represent recent approaches to developing (Q)SARs. The acceptance and use of even simple SAR techniques such as read-across and expert systems should lead to a reduction in the number of compounds needing to be tested using animals.[33] In toxicology (Q)SARs are currently used for screening rather than to provide leads for mechanisms.[34] Our vision for such (Q)SARs to be anchored to an MIE (or a series of MIEs) will relate chemical properties to activity more directly than ever before (Figure 2).

Figure 2.

Classification of compounds by mode of action (MOA) using *in vitro* methods is already possible.[35] Application of this technology to search by MIE would yield useful results to be fed into predictive methods and to identify AOPs. *In vitro* toxicology can gain from enhanced knowledge of MIEs as well, by aiding in the interpretation of results and enabling the targeting of testing.

Interest in the application of MOAs and AOPs in predictive toxicology have already begun to appear, including the use of mode of action for aquatic toxicity prediction[36] and the application of the skin sensitization AOP to a risk assessment.[37] MIEs add a new level of understanding to MOAs and AOPs that is gaining attention as basis for work into QSAR development,[38] category formation and read-across,[39] and molecular modelling leading to mechanistic understanding.[40]

THE MIE

As the key anchor for the AOP, and a commonly used term, an understanding of what an MIE is and how it should be defined is required. As AOPs become more prevalent, so will MIEs, particularly with the development of AOP maps and open source systems.[41]

Several definitions for the MIE have been suggested:

1) The initial point of chemical-biological interaction within the organism that starts the pathway[42] or AOP.[34]
2) Direct interaction of a chemical with specific biomolecules.[43]
3) The molecular level, chemical-induced perturbation of a biological system.[1]
4) Chemical interaction at a molecular target leading to a particular adverse outcome.[1]
5) The seminal interaction (e.g. DNA-binding, protein oxidation, or receptor/ligand interaction) of a chemical with a biological target.[1]

Having several competing definitions generates problems attempting to identify, classify and characterize MIEs. This raises the question: what exactly is an MIE?

Current MIE definitions vary greatly depending on the field from which they originate and, hence, the types of interactions they are intended to describe. Some definitions omit a link to a pathway or endpoint. Some push the approach towards adverse outcomes, cutting off the term from being used to describe therapeutic applications. Some do not distinguish between the first molecular interaction in a pathway and any subsequent interactions. By taking the best features of the current definitions and using our database of MIEs[44] drawn from existing literature we

suggest a unified definition to encompass all fields that should be useful to everybody using the concept of an MIE, and should promote discussion on the future of the area.

Our unified definition is:

> *A Molecular Initiating Event (MIE) is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway.*

As such, MIEs can encompass human and environmental toxicology, and are related directly to an outcome – adverse or otherwise. In the field of human toxicology, focus is on adverse outcomes, but we do not wish to exclude the use of MIEs in a therapeutic sense, as these may become toxicologically relevant for other chemicals or applications. For example a beneficial MIE fits well into pharmacological understanding as does an adverse MIE in overstimulation pharmacology. The distinction between adverse and therapeutic outcomes is less important than the applicability of the term MIE, as we expect the outcomes to be dependent on the dose.[45] Even though a number of MIEs are discussed, and can be associated with one compound, they will not necessarily operate at the same dose or site, and as such quantitative dose-response kinetics are relevant. The question of the dose required to cause adverse outcomes can be better answered once a greater number of MIEs are characterized, (Q)SARs have been developed, and chemical processes are better understood.[42] This definition includes covalent interactions, such as DNA binding, that are generally associated with the term, but also includes more subtle types of interactions with biosystems, such as proton tanslocation leading to mitochondrial uncoupling, and narcosis effects in basal cytotoxicity. Such interactions must be addressed from a toxicological standpoint, and their exclusion from an MIE database could lead to toxicities being missed in MIE based (Q)SAR approaches.

MIE EXAMPLES

Several compounds have been investigated, using toxicological databases and existing literature to elucidate their MIEs, and characterize them to gain mechanistic understanding of the pathway. These cases are presented to illustrate the value of MIEs in the realm of human toxicology. The examples highlight the value of the unified definition and show how previous definitions of an MIE may have been less appropriate.

To understand an MIE completely a lot of information is required. Information about chemicals that are associated with the MIE, structural features or properties of the chemical that causes its association, the types of interaction that occur between the chemical and biomolecule or biosystem, and the nature or structure of what the molecule interacts with. Obtaining all this information is very difficult, and partial information from different sources must be brought together when evaluating MIEs. Incompleteness in parts is to be expected as even the most well studied chemicals lack detailed reports of molecular interactions. However, insights can be gained without the entire picture. Here we report detailed investigations of acetaminophen, amiodarone, chlorpromazine, kojic acid, methotrexate and valproic acid (Figure 3). These structures have been chosen because they have diverse structures and activities, and because substantial amounts of data are available for all of them.

Figure 3

<u>Acetaminophen</u>

Acetaminophen (or paracetamol) is a widely used, mild analgesic. While safe at recommended doses, acute overdose of acetaminophen is well documented to cause potentially fatal liver failure. This risk is increased when combined with alcohol abuse.

Acetaminophen is metabolized in several ways, but the most potentially hazardous is the P450 oxidation by the enzyme CYP2E1 to form the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI).[46–48]

While NAPQI only accounts for a small amount of acetaminophen ingested it is the main source of toxicity. This metabolism occurs in the liver, and hence most toxicity is located here.

Glutathione, a scavenger of oxidizing species in the cell, binds to NAPQI covalently[46,48] - forming a non-hazardous metabolite that is excreted in urine.

When safe doses are exceeded the body's natural glutathione defense is depleted and excess NAPQI causes oxidative damage to cellular proteins,[46,48–50] lipids[48] and nucleic acids[49] as well as activating calpains.[51,52] NAPQI is also involved in generating reactive oxygen species (ROS),[49,50] chemically reactive molecules containing oxygen such as superoxides, peroxides and oxygen radicals, causing oxidative stress. These outcomes can lead to a mitochondrial permeability transition (MPT)[50,53–56] and can result in fatal hepatic damage.[50–52,57–61] This toxicity is a good example of why dose must be accounted for – as only when the glutathione defense is overcome does the dose becomes toxic.

The metabolism of acetaminophen to NAPQI is the initial mechanism behind acetaminophen hepatic toxicity, making it an appropriate MIE. The ability of acetaminophen to be oxidised in this way is responsible for its toxicity, making this an appropriate MIE from which (Q)SARs are developed. Our definition fits this as the initial interaction with the biomolecule CYP2E1 can be linked to liver failure via several AOPs. The interaction of NAPQI with glutathione, proteins,

nucleic acids or other biomolecules can be thought of as the MIE for NAPQI. However, with it not being the initial interaction this would be classed as another molecular interaction further along the AOP for acetaminophen. If the activations of a compound lack detailed study or the AOP is drawn differently, the MIE may change. For the purposes of this research, and to discover what it is about the administered drug that causes the adverse outcome, the initial oxidation is considered the MIE for acetaminophen.

Liver toxicity and acetaminophen are also being explored in other AOP pilot activities.[62]

Figure 4.

Amiodarone

Amiodarone is a class III antiarrhythmic agent used to treat atrial and ventricular arrhythmias by suppressing abnormal rhythms of the heart. Despite having several common side effects, amiodarone is used in cases where the arrhythmias are difficult to treat with other medication.

Amiodarone is well reported to cause a condition known as amiodarone-induced pulmonary toxicity (APT), a combination of factors affecting the lung leading to pulmonary fibrosis.[63–72] This condition can be thought of as a combination of several factors discussed below, including phospholipidosis, steatosis, oxidative stress and mitochondrial uncoupling. This shows how an adverse outcome may be due to a combination of MIEs

Amiodarone induces phospholipidosis via two distinct MIEs. The drug is a very potent (IC_{50} = 7.0 μmol[73]) inhibitor of phospholipases,[63,73–77] but the mechanism behind this is very poorly understood. Amiodarone also binds to the hydrophobic tail of phospholipids, making them resistant to breakdown.[74,75,78–80]

Amiodarone inhibits the cardiac enzyme carnitine palmitoyltransferase I (CPT-1)(IC_{50} = 228 μmol,[81] which is known to control fatty acid access to β-oxidation.[55,56,81–87] Inhibition is thought to be hydrophobic in nature, but is poorly understood.[55,56,81–87] Amiodarone also inhibits the microsomal triglyceride transfer protein (MTP), which secretes fatty acids from cells.[86,87] Again the mechanism is poorly understood. Both of these MIEs lead to fatty acid and triglyceride build up in the cells, and microvesicular steatosis.[55,56,81–83,85–88]

Amiodarone inhibits complexes I and II of the electron transport chain (ETC) via the coenzyme ubiquinone.[55,56,82–85,89,90] This is thought to be due to the formation of a charge-transfer complex between amiodarone and the coenzyme, supported by hydrophobic interactions.[85,89,90] Inhibition of the ETC leads to ATP deficiency,[84,90,91] and the generation of ROS, that cause damage to cellular proteins, lipids, nucleic acids[55,56,63,89,92–95] and the mitochondrial permeability transition (MPT).[55,56,92,96]

Amiodarone uncouples mitochondria due to its properties as a mild base (pK_a = 6.6[91]), and its ability to cross the inner mitochondrial membrane. Amiodarone is protonated at the tertiary amine and translocates the proton from the mitochondrial intermembrane space to the matrix, dissipating the proton gradient required for efficient ATP production.[55,56,82,89–91] This MIE can be highlighted as the interaction between Amiodarone and the biosystem contained within mitochondria, as it does not interact with a biomolecule to have this effect.

Amiodarone acts as an antiarrhythmic by inhibition of the human ether-a-go-go-related gene (hERG) channels.[55,97–102] The key structure for binding is a basic nitrogen flexibly attached to an aromatic ring.[97] The inclusion of hERG inhibition as an MIE highlights the importance of retaining therapeutic interactions as MIEs. Understanding the interactions between drugs and

their targets is valuable knowledge when searching for new drug compounds, and as such this should not be excluded.

Figure 5.

Chlorpromazine

Chlorpromazine is a dopamine antagonist and antipsychotic, used to treat schizophrenia. Chlorpromazine is known to exhibit a number of toxicities including inducing hepatotoxicity and cardiac toxicity.

Two metabolic pathways lead to toxic derivatives of chlorpromazine:

- Peroxidase catalysed formation of the sulfur cation radical form of chlorpromazine.[103]
- Ring hydroxylations by P450 processes at the 7[104–106] and 8[105] positions. This leads to further oxidations forming 7,8-dioxochlorpromazine.[105]

These toxic metabolites are neutralised by sulfoxidation - sulfoxidised chlorpromazine derivatives are non-toxic.[106]

Oxidative stress is brought on via the production of Reactive Oxygen Species (ROS) by the action of chlorpromazine metabolites.

7-hydroxychlorpromazine is able to covalently bind to glutathione, depleting stocks of the scavenger, causing oxidative stress,[104] much like NAPQI.

7-hydroxychlorpromazine is also able to bind to other proteins in the cell, causing oxidative damage.[104]

The chlorpromazine sulfur cation radical is a reactive species in itself. Co-oxidation of ascorbate, NADH and glutathione by the chlorpromazine sulfur cation radical, leads to oxidative stress.[103]

7,8-dihydroxychlorpromazine is able to generate ROS directly by reaction with molecular oxygen.[105]

While all of these metabolites cause oxidative stress, the MIEs for these processes are the metabolic interactions leading to the formation of the metabolites.

Chlorpromazine acts as an anti-psychotic via two MIEs. Chlorpromazine inhibits L-type calcium channels, preventing membrane depolarization and catecholamine secretion.[107,108] It also inhibits nicotinic receptors, another pathway for preventing catecholamine secretion[108]. Dopamine, norepinephrine and adrenaline are notable catecholamines. Increased levels of dopamine and norepinephrine are among the factors responsible for causing schizophrenia.

Chlorpromazine is known to cause phospholipidosis by inhibiting phospholipases.[74] Amiodarone causes the same AOP via phospholipase inhibition, and the MIE in this case is also poorly understood. The identification of several drugs activating the same MIE gives potential for structural comparison to identify features that may be responsible for poorly understood biological processes.

Chlorpromazine is known to inhibit hERG channels, leading to drug induced QT syndrome; lengthening of the QT interval.[107,109–111] This gives chlorpromazine proarrhythmic potential in the same vein as amiodarone, which also inhibits hERG channels. Unlike amiodarone, chlorpromazine is known to cause torsade du pointes, a potentially fatal arrhythmic condition.[107,109–111]

Structural similarities between chlorpromazine and amiodarone known to be involved (flexible basic nitrogen attached to an aromatic ring) give mechanistic promise for this inhibition to be a single MIE activated by both compounds.

Figure 6.

Kojic acid

Kojic acid is produced by several species of fungi, including Aspergillus oryzae, known as *Koji* in Japan. It is found in several Japanese consumables including sake and soy sauce, leading to high exposures in Japan.[112] It is best known as a mild skin-lightening agent, used in cosmetics, and as to preserve the color of foods.

Kojic acid is effective as a skin lightening agent via the inhibition of melanosis, the process by which the dark pigment melanin is formed.[112–117] The inhibition of tyrosinase is the primary action of the drug, via three MIEs:

- Chelation of kojic acid to the copper active site in tyrosinase.[115,118]
- Reduction of quinones to diphenols by kojic acid.[114,118]
- Kojic acid limiting the uptake of oxygen required for melanin formation.[112,114]

The type of inhibition that dominates changes across species,[113] although competitive inhibition dominates in humans.[115–117]

NF-kB is a protein complex in the skin which up-regulates the production of keratinocytes and melanocytes, cells responsible for the production of melanin. It is normally activated by UV radiation. NF-kB activity is suppressed by Kojic acid, down-regulating the production of melanin producing cells, causing lightening of the skin.[119,120] It is accepted that UV induced ROS activate NF-kB, and kojic acid neutralizes these species in the extracellular environment as the MIE.[121,122] It also chelates to iron, preventing the ROS being catalyzed to more hazardous free radicals, such as the hydroxyl radical.[122,123] This mirrors the action of chelation to copper in tyrosinase inhibition.

The ability to limit oxygen uptake, reduce quinones and diphenols, and neutralization of ROS as MIEs are more appropriately described as interactions with biosystems rather than biomolecules.

Figure 7.

Methotrexate

Methotrexate is an antimetabolite and anti-folate drug, used in the treatment of leukaemia. It is well known for its action on the folic acid cycle. It is structurally similar to folic acid, giving it anti-folate characteristics.

In the treatment of leukaemia with methotrexate, one of the most common and damaging side-effects is oral mucositis, a painful inflammation and ulceration of the mouth.

Methotrexate is a folate analogue. Structural similarities between methotrexate and folic acid dominate enzyme inhibitions. Inhibition of enzymes, such as dihydrofolate reductase (DHFR), leads to reduction of de novo biosynthesis of the nucleoside thymidine.[124–139] Thymidine is required for DNA synthesis. This gives methotrexate its anticancer activity. Inhibition of these enzymes also leads to a reduction in purine synthesis which in turn impacts on T cell activation, as a treatment for rheumatoid arthritis.[132] The majority of these inhibitions are competitive, due to folate similarities.[139]

Oral mucositis is thought to be caused by two MIEs. The generation of ROS associated with methotrexate treatment, and the activation of NF-kB by methotrexate.

Methotrexate is known to decrease the number of oxidative species scavengers in the body.[140,141] Methotrexate is reported to inhibit nicotinamide diphosphate (NADP) dependent dehydrogenase, leading to a deficiency of glutathione as NADP is a substrate for glutathione

production.[140] Methotrexate also inhibits polyamine producing enzymes, resulting in the depletion of polyamine ROS scavengers.[129] Oxidative stress leads to the damage of DNA, among other biomolecules, and cell apoptosis, which is credited to contribute to mucositis.[142]

The activation of NF-kB leads to the release of several cytokines inducing the formation of ulcers and tissue damage in the mouth leading to mucositis.[142]

Oral mucositis can lead to infection and bleeding and, from either of these, death.[142,143] Infection is especially high risk in patients receiving leukemia treatment, due to a low white blood cell count.[143]

Methotrexate is also reported to precipitate in the renal tubules of the kidneys, blocking them, especially in acidic urine. This leads to nephrotoxicity and overexpression of methotrexate.[124,144–146] This is another case of the compound interacting with a biosystem, rather than a biomolecule as the MIE.

Figure 8.

Valproic acid

Valproic acid is used as an anticonvulsant and mood-stabilizing drug. Valproic acid is also under investigation as a histone deacetylase inhibitor, a potential activity against HIV and cancers. Valproic acid exhibits reproductive and hepatic toxicity.

Valproic acid is teratogenic - it induces malformations to an embryo or fetus in the womb. This is primarily through the inhibition of class I histone deacetylases (HDACs).[147–157]

The inhibition of HDACs is suspected to occur via two MIEs:

- Binding of valproic acid to the catalytic center of the HDAC.[151,158]
- Down regulation of HDACs by proteosomal degradation.[147,151]

Valproic acid activates Wnt-dependent gene expression through HDAC inhibition leading to increased expression of β-catenin and Tcf/Lef, much like another HDAC inhibitor, trichostatin A.[149,156,157] The teratogenetic effects associated with valproic acid and trichostatin A are very similar.

Using derivative analysis, very specific structural requirements were discovered for valproic acid to exert its teratogenicity. An sp^3 hybridized carbon, attached to a free carboxylic acid, two alkyl chains and one hydrogen.[149,156,159–161]

Valproic acid acts as an anticonvulsant by increasing the concentration of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter.

This occurs through four MIEs:

- Increasing GABA production, through stimulation of glutamic acid decarboxylase.[149,156,162]
- And decreasing GABA catabolism, through:
    - inhibition of GABA transaminase.[149,150,156,162]
    - inhibition of succinate semialdehyde dehydrogenase.[149,150,156,162]
    - and inhibition of α-ketoglutarate dehydrogenase.[149]

Little mechanistic detail is available, but it is made clear that HDAC inhibition is not involved in anticonvulsant activity.[149]

Studies into structural derivatives have shown that valproic acid represents a compromise of chain length and branching, between strongly inducing GABA concentration increase and decreasing toxicity and drowsiness. Longer alkyl chain lengths, branching at the 3 carbon, and replacing the acid with amide all resulted in increased anticonvulsant activity but also greater

hepatotoxicity. Shorter alkyl chain lengths and making the drug linear or on a carbon ring decreased the anticonvulsant activity.[160]

Valproic acid causes hepatotoxicity through microvesicular steatosis,[163–165] and is also connected to Reye's syndrome.[165] All are related to fibrosis of liver cells. All are caused by the inhibition of the β-oxidation of fatty acids in the liver, which in turn is caused by three distinct MIEs:

- Depletion of coenzyme A (CoA), a coenzyme required for the oxidation of fatty acids.[165]
- Depletion of the biomolecule carnitine, which is required for the transportation of fatty acids to mitochondria for breakdown.[165]
- Direct enzyme inhibition of β-oxidation.[163–165]

Depletion of CoA is thought to occur via the formation of a CoA - valproate thioester.[165] The depletion of carnitine is poorly understood mechanistically, however we can speculate it may also have the ability to form an ester with valproic acid. These ester formations would represent the MIEs.

Direct inhibition of β-oxidation includes the enzyme CPT-1,[165] which is also inhibited by amiodarone via hydrophobic contacts. The prominence of hydrophobicity in the valproic acid structure would fit into the same MIE.

Structural derivative studies show valproic acid analogues to be more hepatotoxic with longer alkyl chains, and more branched alkyl chains - linking it to a hydrophobic mechanism.[160]

Structural derivative studies help to classify the MIEs for teratogenicity and hepatotoxicity for valproic acid, as the structural features required for each can be elucidated. The hydrophobic side chains are responsible for the inhibition of CPT-1 leading to hepatotoxicity, while the sp3

hybridized carbon attached to two alkyl groups, a hydrogen atom and a carboxylic acid is responsible for the inhibition of HDACs leading to teratogenicity.

Figure 9.

DISCUSSION

The aim of the unified MIE definition is to try and remove any bias towards particular areas of interest, making it applicable over fields of science including but not limited to toxicology. To provide a unified definition the current definitions must be harmonised. Firstly, any definition that strays from the initial molecular interaction is incorrect, as the first molecular interaction is the beginning of an AOP (definitions 2, 3 and 4). While the MIE has to be the initial interaction, it must also include the entirety of that interaction to allow chemical reactivity to be developed in a (Q)SAR (definition 1). The lack of an endpoint or pathway is also problematic, because without a measureable outcome an MIE has no verifiable purpose – it cannot predict or be related to a response (definitions 2, 3 and 5). Preferably, both a pathway and outcome should be mentioned (definitions 1 and 4). Research in toxicology usually focuses on adverse outcomes, but the term MIE is also useful for research into molecules with desirable effects. MIEs relating to therapeutic but potentially toxicologically-relevant effects such as hERG inhibition (amiodarone), melanosis inhibition (kojic acid), DHFR inhibition (methotrexate), and increase in GABA concentration (valproic acid) would be excluded from the definition if positive outcomes were disallowed. This would reduce the scope for identifying MIEs and negate their potential use in drug design (definitions 1 and 4). Finally a definition must not exclude interactions with molecules or systems that do not fit the description of biomolecules, molecular targets or biological targets (2, 4 and 5). This would exclude proton translocation (amiodarone), limiting

oxygen uptake, ROS neutralization, the reduction of quinones and diphenols (kojic acid), and the precipitation of a compound in the renal tubules (methotrexate). All of these outcomes have the potential to provide a measurable response, and so should be included in the definition. Our new definition addresses all of these issues, fits well into the AOP framework for risk assessment and is in agreement with other toxicity pathways research.[166]

CONCLUSION

We define an MIE as the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome *via* a pathway. This definition harmonises existing MIE definitions, and its use has been validated using MIEs extracted from chemical and biological databases and literature. This MIE definition works effectively across the many disciplines of human and environmental toxicology. The basis of a diverse set of initiating events reveals more about their nature than has been previously possible. As such no existing definition was as all-encompassing. Our unified MIE definition should help the field look towards defining, classifying and characterizing more MIEs, and using knowledge of the chemistry of these processes to aid AOP research and toxicity risk assessment.

Knowledge of MIEs has the potential to be very useful in the development of (Q)SARs, allowing a direct link between molecular properties and toxicological outcome. The elucidation of this knowledge can come in several forms. Detailed reports of MIEs are currently rather few, even for the most studied molecules, but a number of tools represent novel ways to draw them out. Databases of molecules with similar toxicological apical endpoints provide a start, as analysis of the chemical structures and properties of these molecules can provide insight into the number and possible activation of an associated MIE. Understanding of the biology of an active

site can provide similar assistance. Knowledge of the number and reactivity or binding associated with a protein can suggest the types of molecules that may interact with it. In these ways the MIE may be approached from either the biological or chemical perspective, as this interaction can be said to be the boundary between the chemistry of a molecule and its biological effect. A combined approach resulting in a database of MIEs would help to target existing risk assessment approaches to the endpoints of greatest concern (highest chance of activation). Commonly encountered MIEs can be identified to focus and prioritize further research to gain a greater understanding of how toxicity pathways are networked between compounds. With enough data an MIE database could provide predictive quantitative assessment of toxicity itself for new compounds.

As the AOP framework approach to toxicology gains momentum, the importance of a unified definition of MIEs grows. Our definition of a MIE includes all current AOP data and provides a platform for these exciting developments.AUTHOR INFORMATION

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ABBREVIATIONS

AOP, adverse outcome pathway; APT, amiodarone-induced pulmonary toxicity; CoA, coenzyme A; CPT-1, carnitine palmitoyltransferase I; DHFR, dihydrofolate reductase; ETC, electron transport chain; GABA, gamma-aminobutyric acid; HDAC, histone deacetylase; hERG, human ether-à-go-go-related gene; IC_{50}, half maximal inhibitory concentration; MIE, molecular initiating event; MOA, mode of action; MTP, mitochondrial transport protein; NADP, nicotinamide diphosphate; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PBPK, physiologically based pharmacokinetics; (Q)SAR, (quantitative) structure activity relationship; ROS, Reactive oxygen species;

REFERENCES

(1) OECD. Proposal for a Template, and Guidance on Developing and Assessing the Completeness of Adverse Outcome Pathways, Appendix I, Collection of Working Definitions. *http:/www.oecd.org/chemicalsafety/testingofchemicals/49963576.pdf*.

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

(3) ChEMBL database.

(4) Bender, A. (2010) Databases: Compound bioactivities go public. *Nat. Chem. Biol. 6*, 309–309.

(5) Wang, Y., Xiao, J., Suzek, T. O., Zhang, J., Wang, J., and Bryant, S. H. (2009) PubChem: a public information system for analyzing bioactivities of small molecules. *Nucleic Acids Res. 37*, W623–33.

(6) Seiler, K. P., George, G. a, Happ, M. P., Bodycombe, N. E., Carrinski, H. a, Norton, S., Brudz, S., Sullivan, J. P., Muhlich, J., Serrano, M., Ferraiolo, P., Tolliday, N. J., Schreiber, S. L., and Clemons, P. a. (2008) ChemBank: a small-molecule screening and cheminformatics resource database. *Nucleic Acids Res. 36*, D351–9.

(7) Committee on Toxicity Testing and Assessment of Environmental Agents and National Research Council. (2007) Toxicology Testing in the 21st Century: A Vision and a Strategy, p The National Academic Press.

(8) Hartung, T. (2009) Toxicology for the twenty-first century. *Nature 460*, 208–212.

(9) Leist, M., Hartung, T., and Nicotera, P. (2008) The Dawning of a New Age of Toxicology. *ALTEX 25*, 103–114.

(10) Hartung, T. (2008) Food for thought... on animal tests. *ALTEX 25*, 3–16.

(11) Schardein, J. L., Schwetz, B. A., and Kenel, M. F. (1985) Species Sensitivities and Prediction of Tetratogenic Potential. *Environ. Health Perspect. 61*, 55–67.

(12) Gottmann, E., Kramer, S., Pfahringer, B., and Helma, C. (2001) Data quality in predictive toxicology: reproducibility of rodent carcinogenicity experiments. *Environ. Health Perspect. 109*, 509–514.

(13) Smith, R. (2001) Animal research: the need for a middle ground. *Br. Med. J. 322*, 248–249.

(14) Hartung, T. (2009) A Toxicology for the 21st Century—Mapping the Road Ahead. *Toxicol. Sci. 109*, 18–23.

(15) Andersen, M. E., Clewell, H. J., Gargas, M. L., Smith, F. A., and Reitz, R. H. (1987) Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol. 87*, 185–205.

(16) Sexton, K., Reiter, L. W., and Zenick, H. (1995) Research to strengthen the scientific basis for health risk assessment: a survey of the context and rationale for mechanistically based methods and models. *Toxicology 102*, 3–20.

(17) Aardema, M. J., and MacGregor, J. T. (2002) Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. *Mutat. Res. 499*, 13–25.

(18) MacGregor, J. T. (2003) The future of regulatory toxicology: impact of the biotechnology revolution. *Toxicol. Sci. 75*, 236–248.

(19) Pognan, F. (2004) Genomics, proteomics and metabonomics in toxicology: hopefully not "fashionomics". *Pharmacogenomics 5*, 879–893.

(20) Kitano, H. (2002) Systems biology: a brief overview. *Science (80-.). 295*, 1662–1664.

(21) Henry, C. J. (2003) Evolution of Toxicology for Risk Assessment. *Int. J. Toxicol. 22*, 3–7.

(22) Teubner, W., Mehling, A., Schuster, P. X., Guth, K., Worth, A., Burton, J., van Ravenzwaay, B., and Landsiedel, R. (2013) Computer models versus reality: how well do in

silico models currently predict the sensitization potential of a substance. *Regul. Toxicol. Pharmacol. 67*, 468–485.

(23) Westerink, R. H. S. (2013) Do we really want to REACH out to in vitro? *Neurotoxicology 39*, 169–172.

(24) Cherkasov, A., Muratov, E. N., Fourches, D., Varnek, A., Baskin, I. I., Cronin, M., Dearden, J., Gramatica, P., Martin, Y. C., Todeschini, R., Consonni, V., Kuz'min, V. E., Cramer, R., Benigni, R., Yang, C., Rathman, J., Terfloth, L., Gasteiger, J., Richard, A., and Tropsha, A. (2014) QSAR Modeling: Where Have You Been? Where Are You Going To? *J. Med. Chem.*

(25) Auer, C. M., Nabholz, J. V., and Karl, P. (1990) Mode of Action and the Assessment of Chemical Hazards in the Presence of Limited Data: Use of Structure-Activity Relationships (SAR) under TSCA, Section 5. *Environ. Health Perspect. 87*, 183–197.

(26) Verhaar, H. J. M., van Leeuwen, C. J., and Hermans, J. L. M. (1992) Classifying environmental pollutants. 1: Structure-activity relationships for prediction of aquatic toxicity. *Chemosphere 25*, 471–491.

(27) Russom, C. L., Bradbury, S. P., Broderius, S. J., Hammermeister, D. E., and Drummond, R. A. (1997) Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (Pimephales promelas). *Environ. Toxicol. Chem. 16*, 948–967.

(28) Piechota, P., Cronin, M. T. D., Hewitt, M., and Madden, J. C. (2013) Pragmatic approaches to using computational methods to predict xenobiotic metabolism. *J. Chem. Inf. Model. 53*, 1282–1293.

(29) Marchant, C. A., Briggs, K. A., and Long, A. (2008) In Silico Tools for Sharing Data and Knowledge on Toxicity and Metabolism: Derek for Windows, Meteor, and Vitic. *Toxicol. Mech. Methods 18*, 177–187.

(30) Carlsson, L., Spjuth, O., Adams, S., Glen, R. C., and Boyer, S. (2010) Use of historic metabolic biotransformation data as a means of anticipating metabolic sites using MetaPrint2D and Bioclipse. *BMC Bioinformatics 11*, 1–7.

(31) Benfenati, E., Benigni, R., Demarini, D. M., Helma, C., Kirkland, D., Martin, T. M., Mazzatorta, P., Ouédraogo-Arras, G., Richard, a M., Schilter, B., Schoonen, W. G. E. J., Snyder, R. D., and Yang, C. (2009) Predictive models for carcinogenicity and mutagenicity: frameworks, state-of-the-art, and perspectives. *J. Environ. Sci. Heal. 27*, 57–90.

(32) Bakhtyari, N. G., Raitano, G., Benfenatif, E., Martin, T., and Young, D. (2013) Comparison of In Silico Models for prediction of Mutagenicity. *J. Environ. Sci. Heal. 31*, 45–66.

(33) Höfer, T., Gerner, I., Gundert-Remy, U., Liebsch, M., Schulte, A., Spielmann, H., Vogel, R., and Wettig, K. (2004) Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation. *Arch. Toxicol. 78*, 549–564.

(34) 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. (Camb). 2*, 299.

(35) Van den Hof, W. F. P. M., Coonen, M. L. J., van Herwijnen, M., Brauers, K., Wodzig, W. K. W. H., van Delft, Joost, H. M., and Kleinjans, J. C. S. (2013) Classification of Hepatotoxicants Using HepG2 Cells: A Proof of Principle Study. *Chem. Res. Toxicol.*

(36) Martin, T. M., Grulke, C. M., Young, D. M., Russom, C. L., Wang, N. Y., Jackson, C. R., and Barron, M. G. (2013) Prediction of aquatic toxicity mode of action using linear discriminant and random forest models. *J. Chem. Inf. Model. 53*, 2229–2239.

(37) Maxwell, G., MacKay, C., Cubberley, R., Davies, M., Gellatly, N., Glavin, S., Gouin, T., Jacquoilleot, S., Moore, C., Pendlington, R., Saib, O., Sheffield, D., Stark, R., and Summerfield, V. (2014) Applying the skin sensitisation adverse outcome pathway (AOP) to quantitative risk assessment. *Toxicol. Vitr. 28*, 8–12.

(38) Yang, C., Arvidson, K., Richard, A., Worth, A., Tarkhov, A., Ringeissen, S., Marusczyk, J., Gasteiger, J., Rathman, J., and Schwab, C. (2014) CHEMOTYPES AND CHEMOTYPER: A new structural representation standard to include atomic/bond properties into structural alerts for toxicity effects and mechanisms.

(39) Nelms, M. D., Ates, G., Madden, J. C., Vinken, M., Cronin, M. T. D., Rogiers, V., and Enoch, S. J. (2014) Proposal of an in silico profiler for categorisation of repeat dose toxicity data of hair dyes. *Arch. Toxicol.*

(40) Tsakovska, I., Al Sharif, M., Alov, P., Diukendjieva, A., Fioravanzo, E., Cronin, M. T. D., and Pajeva, I. (2014) Molecular modelling study of the PPARγ receptor in relation to the mode of action/adverse outcome pathway framework for liver steatosis. *Int. J. Mol. Sci. 15*, 7651–7666.

(41) European Commission Institute for Health and Consumer Protection. AOP Wiki.

(42) OECD. (2011) Report of the Workshop on Using Mechanistic information in Forming Chemical Categories. OECD Environment, Health and Safety Publications Series on Testing and Assessment No.138, p ENV/JM/MONO(2011)8.

(43) Villeneuve, D. L., and Garcia-Reyero, N. (2011) Vision & strategy: Predictive Ecotoxicology in the 21st century. *Environ. Toxicol. Chem. 30*, 1–8.

(44) Allen, T. E. H., Goodman, J. M., Gutsell, S., and Russell, P. (2013) MIE Database, Unpublished Data.

(45) Andersen, M. E., Dennison, J. E., Thomas, R. S., and Conolly, R. B. (2005) New directions in incidence-dose modeling. *Trends Biotechnol. 23*, 122–127.

(46) Black, M. (1984) Acetaminophen Hepatotoxicity. *Annu. Rev. Med. 35*, 577–593.

(47) Manyike, P. T., Kharasch, E. D., Kalhorn, T. F., and Slattery, J. T. (2000) Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. *Clin. Pharmacol. Ther. 67*, 275–282.

(48) Albano, E., Rundgren, M., Harvison, P. J., Nelson, S. D., and Moldeus, P. (1985) Mechanisms of N-Acetyl-p-benzoquinone imine cytotoxicity. *Mol. Pharmacol. 28*, 306–311.

(49) McGill, M. R., Yan, H.-M., Ramachandran, A., Murray, G. J., Rollins, D. E., and Jaeschke, H. (2011) HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity. *Hepatology 53*, 974–982.

(50) Jaeschke, H., McGill, M. R., Williams, C. D., and Ramachandran, A. (2011) Current issues with acetaminophen hepatotoxicity - a clinically relevant model to test the efficacy of natural products. *Life Sci. 88*, 737–745.

(51) Liu, X., Van Vleet, T., and Schnellmann, R. G. (2004) The role of calpain in oncotic cell death. *Annu. Rev. Pharmacol. Toxicol. 44*, 349–370.

(52) Jaeschke, H., and Bajt, M. L. (2006) Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol. Sci. 89*, 31–41.

(53) Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W. A., Han, D., and Kaplowitz, N. (2008) Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J. Biol. Chem. 283*, 13565–13577.

(54) Adams, M. L., Pierce, R. H., Vail, M. E., White, C. C., Tonge, R. P., Kavanagh, T. J., Fausto, N., Nelson, S. D., and Bruschi, S. A. (2001) Enhanced acetaminophen hepatotoxicity in transgenic mice overexpressing BCL-2. *Mol. Pharmacol. 60*, 907–915.

(55) Waldhauser, K. M., Török, M., Ha, H.-R., Thomet, U., Konrad, D., Brecht, K., Follath, F., and Krähenbühl, S. (2006) Hepatocellular toxicity and pharmacological effect of amiodarone and amiodarone derivatives. *J. Pharmacol. Exp. Ther. 319*, 1413–1423.

(56) Kaufmann, P., Török, M., Hänni, A., Roberts, P., Gasser, R., and Krähenbühl, S. (2005) Mechanisms of benzarone and benzbromarone-induced hepatic toxicity. *Hepatology 41*, 925–935.

(57) Wendel, A., Feuerstein, S., and Konz, K.-H. (1979) Acute Paracetamol intoxication of Starved Mice leads to Lipid Peroxidation in vivo. *Biochem. Pharmacol. 28*, 2051–2055.

(58) Limaye, P. B., Apte, U. M., Shankar, K., Bucci, T. J., Warbritton, A., and Mehendale, H. M. (2003) Calpain released from dying hepatocytes mediates progression of acute liver injury induced by model hepatotoxicants. *Toxicol. Appl. Pharmacol. 191*, 211–226.

(59) McGill, M. R., Sharpe, M. R., Williams, C. D., Taha, M., Curry, S. C., and Jaeschke, H. (2012) The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice

involves mitochondrial damage and nuclear DNA fragmentation. *J. Clin. Invest. 122*, 1574–1583.

(60) Van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R., and Vandenabeele, P. (2001) Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ. 8*, 1136–1142.

(61) Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prévost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., and Kroemer, G. (2000) Two distinct pathways leading to nuclear apoptosis. *J. Exp. Med. 192*, 571–580.

(62) Willett, C., Rae, J. C., Goyak, K. O., Minsavage, G., Westmoreland, C., Andersen, M., Avigan, M., Duche, D., Hartung, T., Jaeschke, H., Kleensang, A., Landesmann, B., Toole, C., Rowan, A., Schultz, T., Seed, J., Senior, J., Shah, I., Subramanian, K., Vinken, M., and Watkins, P. (2014) Building Shared Experience to Advance Practical Application of Pathway-Based Toxicology: Liver Toxicity Mode-of-Action. *ALTEX*, pp 1–22.

(63) Reasor, M. J., and Kacew, S. (1996) An Evaluation of Possible Mechanisms Underlying Amiodarone-Induced Pulmonary Toxicity. *Exp. Biol. Med. 212*, 297–304.

(64) Range, F. T., Hilker, E., Breithardt, G., Buerke, B., and Lebiedz, P. (2013) Amiodarone-Induced Pulmonary Toxicity-A Fatal Case Report and Literature Review. *Cardiovasc. Drugs Ther. 27*, 247–254.

(65) Martin, W. J., and Rosenow, C. (1988) Amiodarone pulmonary toxicity recognition and pathogenesis (part 2). *Chest 93*, 1242–1248.

(66) Liu, F. L., Cohen, R. D., Downar, E., Butany, J. W., Edelson, J. D., and Rebuck, A. S. (1986) Amiodarone pulmonary toxicity: functional and ultrastructural evaluation. *Thorax 41*, 100–105.

(67) Dusman, R. E., Stanton, M. S., Miles, W. M., Klein, L. S., Zipes, D. P., Fineberg, N. S., and Heger, J. J. (1990) Clinical features of amiodarone-induced pulmonary toxicity. *Circulation 82*, 51–59.

(68) Rakita, L., Sobol, S. M., Mostow, N., and Vrobel, T. (1983) Amiodarone pulmonary toxicity. *Am. Heart J. 106*, 906–916.

(69) Martin, W. J., and Rosenow, E. C. (1988) Amiodarone pulmonary toxicity recognition and pathogenesis (part 1). *Chest 93*, 1067–1075.

(70) Choi, I.-S., Kim, B.-S., Cho, K.-S., Park, J.-C., Jang, M.-H., Shin, M.-C., Jung, S.-B., Chung, J.-H., and Kim, C.-J. (2002) Amiodarone induces apoptosis in L-132 human lung epithelial cell line. *Toxicol. Lett. 132*, 47–55.

(71) Bargout, R., Jankov, A., Dincer, E., Wang, R., Komodromos, T., Ibarra-Sunga, O., Filippatos, G., and Uhal, B. D. (2000) Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. *Am. J. Physiol. - Lung Cell. Mol. Physiol. 278*, 1039–1044.

(72) Di Matola, T., D'Ascoli, F., Fenzi, G., Rossi, G., Martino, E., Bogazzi, F., and Vitale, M. (2000) Amiodarone induces cytochrome c release and apoptosis through an iodine-independent mechanism. *J. Clin. Endocrinol. Metab. 85*, 4323–4330.

(73) Heath, M. F., Costa-Jussà, F. R., Jacobs, J. M., and Jacobson, W. (1985) The induction of pulmonary phospholipidosis and the inhibition of lysosomal phospholipases by amiodarone. *Br. J. Exp. Pathol. 66*, 391–397.

(74) Anderson, N., and Borlak, J. (2006) Drug-Induced Phospholipidosis. *FEBS Lett. 580*, 5533–5540.

(75) Kodavanti, U. P., and Mehendale, H. M. (1990) Cationic Amphiphilic Drugs and Phospholipid Storage Disorder. *Pharmacol. Rev. 42*, 327–354.

(76) Reasor, M. J., and Kacew, S. (2001) Drug-Induced Phospholipidosis: Are There Functional Consequences? *Exp. Biol. Med. 226*, 825–830.

(77) Sawada, H., Takami, K., and Asahi, S. (2005) A toxicogenomic approach to drug-induced phospholipidosis: analysis of its induction mechanism and establishment of a novel in vitro screening system. *Toxicol. Sci. 83*, 282–292.

(78) Joshi, U. M., Kodavanti, P. R. S., Coudert, B., Dwyer, T. M., and Mehendale, H. M. (1988) Types of Interaction of Amphiphilic Phospholipid Vesicles. *J. Pharmacol. Exp. Ther. 246*, 150–157.

(79) Joshi, U. M., Rao, P., Kodavanti, S., Lockard, V. G., and Mehendale, H. M. (1989) Fluorescence studies on binding of amphiphilic drugs to isolated lamellar bodies: relevance to phospholipidosis. *Biochim. Biophys. Acta 1004*, 309–320.

(80) Halliwell, W. H. (1997) Cationic Amphiphilic Drug-Induced Phospholipidosis. *Toxicol. Pathol. 25*, 53–60.

(81) Kennedy, J. A., Unger, S. A., and Horowitz, J. D. (1996) Inhibition of carnitine palmitoyltransferase-1 in rat heart and liver by perhexiline and amiodarone. *Biochem. Pharmacol. 52*, 273–280.

(82) Spaniol, M., Bracher, R., Ha, H. R., Follath, F., and Krähenbühl, S. (2001) Toxicity of amiodarone and amiodarone analogues on isolated rat liver mitochondria. *J. Hepatol. 35*, 628–636.

(83) Fromenty, B., and Pessayre, D. (1995) Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. *Pharmacol. Ther. 67*, 101–154.

(84) Grattagliano, I., Bonfrate, L., Diogo, C. V, Wang, H. H., Wang, D. Q. H., and Portincasa, P. (2009) Biochemical mechanisms in drug-induced liver injury: Certainties and doubts. *World J. Gastroenterol. 15*, 4865–4876.

(85) Fromenty, B., Fisch, C., Labbe, G., Degott, C., Deschamps, D., Berson, A., Letteron, P., and Pessayre, D. (1990) Amiodarone inhibits the mitochondrial beta-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. *J. Pharmacol. Exp. Ther. 255*, 1371–1376.

(86) Teresa Donato, M., Martínez-Romero, A., Jiménez, N., Negro, A., Herrera, G., Castell, J. V, O'Connor, J.-E., and Gómez-Lechón, M. J. (2009) Cytometric analysis for drug-induced steatosis in HepG2 cells. *Chem. Biol. Interact. 181*, 417–423.

(87) Lettéron, P., Sutton, A., Mansouri, A., Fromenty, B., and Pessayre, D. (2003) Inhibition of microsomal triglyceride transfer protein: another mechanism for drug-induced steatosis in mice. *Hepatology 38*, 133–140.

(88) Hautekeete, M. L., Degott, C., and Benhamou, J. P. (1990) Microvesicular steatosis of the liver. *Acta Clin. Belg. 45*, 311–326.

(89) Serviddio, G., Bellanti, F., Giudetti, A. M., Gnoni, G. V., Capitanio, N., Tamborra, R., Romano, A. D., Quinto, M., Blonda, M., Vendemiale, G., and Altomare, E. (2011) Mitochondrial oxidative stress and respiratory chain dysfunction account for liver toxicity during amiodarone but not dronedarone administration. *Free Radic. Biol. Med. 51*, 2234–2242.

(90) Nicolescu, A. C., Ji, Y., Comeau, J. L., Hill, B. C., Takahashi, T., Brien, J. F., Racz, W. J., and Massey, T. E. (2008) Direct mitochondrial dysfunction precedes reactive oxygen species production in amiodarone-induced toxicity in human peripheral lung epithelial HPL1A cells. *Toxicol. Appl. Pharmacol. 227*, 370–379.

(91) Fromenty, B., Fisch, C., Berson, A., Letteron, P., Larrey, D., and Pessayre, D. (1990) Dual effect of amiodarone on mitochondrial respiration. Initial protonophoric uncoupling effect followed by inhibition of the respiratory chain at the levels of complex I and complex II. *J. Pharmacol. Exp. Ther. 255*, 1377–1384.

(92) Green, D. R., and Reed, J. C. (1998) Mitochondria and Apoptosis. *Science (80-.). 281*, 1309–1312.

(93) Eguchi, Y., Shimizu, S., and Tsujimoto, Y. (1997) Intracellular ATP Levels Determine Cell Death Fate by Apoptosis or Necrosis. *Cancer Res. 57*, 1835–1840.

(94) Golli-Bennour, E. El, Bouslimi, A., Zouaoui, O., Nouira, S., Achour, A., and Bacha, H. (2012) Cytotoxicity effects of amiodarone on cultured cells. *Exp. Toxicol. Pathol. 64*, 425–430.

(95) Valcheva-Kuzmanova, S. V, Stavreva, G. T., Dancheva, V. Y., Terziev, L. G., Shopova, V. L., and Stoyanova, A. M. (2012) Effect of Aronia Melanocapa fruit juice on the activity of

anioxidant enzymes in a rat model of amiodarone-induced pneumotoxicity. *J. Biomed. Clin. Res. 5*, 97–103.

(96) Zamzami, N., and Kroemer, G. (2003) Apoptosis: Mitochondrial Membrane Permeabilization - The (W)hole Story? *Curr. Biol. 13*, 71–73.

(97) Waldhauser, K. M., Brecht, K., Hebeisen, S., Ha, H. R., Konrad, D., Bur, D., and Krähenbühl, S. (2008) Interaction with the hERG channel and cytotoxicity of amiodarone and amiodarone analogues. *Br. J. Pharmacol. 155*, 585–595.

(98) Singh, B. N. (1996) Antiarrhythmic actions of amiodarone: a profile of a paradoxical agent. *Am. J. Cardiol. 78*, 41–53.

(99) Sanguinetti, M. C., and Tristani-Firouzi, M. (2006) hERG potassium channels and cardiac arrhythmia. *Nature 440*, 463–469.

(100) Zhang, Y. H., Cheng, H., Alexeenko, V. A., Dempsey, C. E., and Hancox, J. C. (2010) Characterization of recombinant hERG K(+) channel inhibition by the active metabolite of amiodarone desethyl-amiodarone. *J. Electrocardiol. 43*, 440–448.

(101) Rosenbaum, M. B., Chiale, P. A., Ryba, D., and Elizari, M. V. (1974) Control of tacyarrhythmias with Wolf-Parkinson-White syndrome by amiodaroen hydrochloride. *Am. J. Cardiol. 34*, 215–223.

(102) Rosenbaum, M. B., Chiale, P. A., Halpern, M. S., Nau, G. J., Przybylski, J., Levi, R. J., Lázzari, J. O., and Elizari, M. V. (1976) Clinical efficacy of amiodarone as an antiarrhythmic agent. *Am. J. Cardiol. 38*, 934–944.

(103) Eghbal, M. A., Tafazoli, S., Pennefather, P., and O'Brien, P. J. (2004) Peroxidase catalysed formation of cytotoxic prooxidant phenothiazine free radicals at physiological pH. *Chem. Biol. Interact. 151*, 43–51.

(104) Wen, B., and Zhou, M. (2009) Metabolic activation of the phenothiazine antipsychotics chlorpromazine and thioridazine to electrophilic iminoquinone species in human liver microsomes and recombinant P450s. *Chem. Biol. Interact. 181*, 220–226.

(105) Neptune, M., and McCreery, R. L. (1978) Chemical and electrochemical oxidation of 7-hydroxychlorpromazine. *J. Med. Chem. 21*, 362–368.

(106) Watson, R. G., Olomu, A., Clements, D., Waring, R. H., Mitchell, S., and Elias, E. (1988) A proposed mechanism for chlorpromazine jaundice--defective hepatic sulphoxidation combined with rapid hydroxylation. *J. Hepatol. 7*, 72–78.

(107) Thomas, D., Wu, K., Kathöfer, S., Katus, H. A., Schoels, W., Kiehn, J., and Karle, C. A. (2003) The antipsychotic drug chlorpromazine inhibits HERG potassium channels. *Br. J. Pharmacol. 139*, 567–574.

(108) Lee, I. S., Park, T. J., Suh, B. C., Kim, Y. S., Rhee, I. J., and Kim, K. T. (1999) Chlorpromazine-induced inhibition of catecholamine secretion by a differential blockade of nicotinic receptors and L-type Ca2+ channels in rat pheochromocytoma cells. *Biochem. Pharmacol. 58*, 1017–1024.

(109) Gupta, A., Lawrence, A. T., Krishnan, K., Kavinsky, C. J., and Trohman, R. G. (2007) Current concepts in the mechanisms and management of drug-induced QT prolongation and torsade de pointes. *Am. Heart J. 153*, 891–899.

(110) Reilly, J. G., Ayis, S. A., Ferrier, I. N., Jones, S. J., and Thomas, S. H. L. (2000) QTc-interval abnormalities and psychotropic drug therapy in psychiatric patients. *Lancet 355*, 1048–1052.

(111) Glassman, A. H., and Bigger, J. T. (2001) Antipsychotic Drugs□: Prolonged QTc Interval, Torsade de Pointes, and Sudden Death. *Am. J. Psychiatry 158*, 1774–1782.

(112) Burdock, G. A., Soni, M. G., and Carabin, I. G. (2001) Evaluation of health aspects of kojic acid in food. *Regul. Toxicol. Pharmacol. 33*, 80–101.

(113) Chen, J. S., Wei, C., Rolle, R. S., Otwell, W. S., Balaban, M. O., and Marshall, M. R. (1991) Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidases. *J. Agric. Food Chem. 39*, 1396–1401.

(114) Chen, J. S., Wei, C., and Marshall, M. R. (1991) Inhibition Mechanism of Kojic Acid on Polyphenol Oxidase. *J. Agric. Food Chem. 39*, 9–13.

(115) Maeda, K., and Fukuda, M. (1991) In vitro effectiveness of several whitening cosmetic components in human melanocytes. *J. Soc. Cosmet. Chem. 42*, 361–368.

(116) Cotellessa, C., Peris, K., Onorati, M. T., Fargnoli, M. C., and Chimenti, S. (1999) The use of chemical peelings in the treatment of different cutaneous hyperpigmentations. *Dermatologic Surg. 25*, 450–454.

(117) Kim, H., Choi, H.-R., Kim, D.-S., and Park, K.-C. (2012) Topical hypopigmenting agents for pigmentary disorders and their mechanisms of action. *Ann. Dermatol. 24*, 1–6.

(118) Kahn, V., Ben-Shalom, N., and Zakin, V. (1997) Effect of Kojic Acid on the Oxidation of N -Acetyldopamine by Mushroom Tyrosinase. *J. Agric. Food Chem. 45*, 4460–4465.

(119) Moon, K., Ahn, K. S., Lee, J., and Kim, Y. S. (2001) Kojic Acid , a Potential Inhibitor of NF-KB Activation in Transfec- tant Human HaCaT and SCC-13 Cells. *Arch. Pharm. Res. 24*, 307–311.

(120) Ahn, K. S., Moon, K.-Y., Lee, J., and Kim, Y. S. (2003) Downregulation of NF-κB activation in human keratinocytes by melanogenic inhibitors. *J. Dermatol. Sci. 31*, 193–201.

(121) Niwa, Y., and Akamatsu, H. (1991) Kojic acid scavenges free radicals while potentiating leukocyte functions including free radical generation. *Inflammation 15*, 303–315.

(122) Mitani, H., Koshiishi, I., Sumita, T., and Imanari, T. (2001) Prevention of the photodamage in the hairless mouse dorsal skin by kojic acid as an iron chelator. *Eur. J. Pharmacol. 411*, 169–174.

(123) McBryde, W. A. E., and Atkinson, G. F. (1961) Spectrophotometric study of the reaction between Iron (III) and Kojic acid. *Can. J. Chem. 39*, 510–525.

(124) Fotoohi, A. K., and Albertioni, F. (2008) Mechanisms of antifolate resistance and methotrexate efficacy in leukemia cells. *Leuk. Lymphoma 49*, 410–426.

(125) Braun, J., and Rau, R. (2009) An update on methotrexate. *Curr. Opin. Rheumatol. 21*, 216–223.

(126) Kremer, J. M. (2008) Methotrexate treatment of rheumatic diseases: can we do better? *Arthritis Rheum. 58*, 3279–3282.

(127) Jolivert, J., and Chabner, B. A. (1983) Intracellular Pharmacokinetics of Methotrexate Polyglutamates in Human Breast Cancer Cells. *J. Clin. Invest. 72*, 773–778.

(128) McGuire, J. J., and Bertino, J. R. (1981) Enzymatic synthesis and function of folylpolyglutamates. *Mol. Cell. Biochem. 38*, 19–48.

(129) Wessels, J. A. M., Huizinga, T. W. J., and Guchelaar, H.-J. (2008) Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. *Rheumatology 47*, 249–255.

(130) Allegra, C. J., Hoang, K., Yeh, G. C., Drake, J. C., and Baram, J. (1987) Evidence for direct inhibition of de novo purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate. *J. Biol. Chem. 262*, 13520–13526.

(131) Van Triest, B., Pinedo, H. M., Giaccone, G., and Peters, G. J. (2000) Downstream molecular determinants of response to 5-fluorouracil and antifolate thymidylate synthase inhibitors. *Ann. Oncol. 11*, 385–391.

(132) Liu, D.-Y., Lon, H.-K., Wang, Y.-L., DuBois, D. C., Almon, R. R., and Jusko, W. J. (2013) Pharmacokinetics, pharmacodynamics and toxicities of methotrexate in healthy and collagen-induced arthritic rats. *Biopharm. Drug Dispos. 34*, 203–214.

(133) Baggott, J. E., Vaughn, W. H., and Hudson, B. B. (1986) Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5'-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide. *Biochem. J. 236*, 193–200.

(134) Chu, E., Drake, J. C., Boarman, D., Baram, J., and Allegra, C. J. (1990) Mechanism of thymidylate synthase inhibition by methotrexate in human neoplastic cell lines and normal human myeloid progenitor cells. *J. Biol. Chem. 265*, 8470–8478.

(135) Matthews, R. G., and Baugh, C. M. (1980) Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroylpolyglutamate substrates and with dihydropteroylpolyglutamate inhibitors. *Biochemistry 19*, 2040–2045.

(136) Baram, J., Chabner, B. A., Drake, J. C., Fitzhugh, A. L., Sholar, P. W., and Allegra, C. J. (1988) Identification and biochemical properties of 10-formyldihydrofolate, a novel folate found in methotrexate-treated cells. *J. Biol. Chem. 263*, 7105–7111.

(137) Bunni, M., Doig, M. T., Donato, H., Kesavan, V., and Priest, D. G. (1988) Role of Methylenetetrahydrofolate Depletion in Methotrexate-mediated Intracellular Thymidylate Synthesis Inhibition in Cultured L1210 Cells. *Cancer Res. 48*, 3398–3404.

(138) Baggott, J. E., and Krumdieck, C. L. (1979) Folylpoly-gamma-glutamates as Cosubstrates of 10-Formyltetrahydrofolate:5'-Phosphoribosyl-5-amino-4-imidazolecarboxamide Formyltrasferase. *Biochemistry 18*, 1036–1041.

(139) Morrison, P. F., and Allegra, C. J. (1989) Folate cycle kinetics in human breast cancer cells. *J. Biol. Chem. 264*, 10552–10566.

(140) Uraz, S., Tahan, V., Aygun, C., Eren, F., Unluguzel, G., Yuksel, M., Senturk, O., Avsar, E., Haklar, G., Celikel, C., Hulagu, S., and Tozun, N. (2008) Role of ursodeoxycholic acid in prevention of methotrexate-induced liver toxicity. *Dig. Dis. Sci. 53*, 1071–1077.

(141) Jahovic, N., Cevik, H., Sehirli, A. O., Yeğen, B. C., and Sener, G. (2003) Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. *J. Pineal Res. 34*, 282–287.

(142) Sonis, S. T., Elting, L. S., Keefe, D., Peterson, D. E., Schubert, M., Hauer-Jensen, M., Bekele, B. N., Raber-Durlacher, J., Donnelly, J. P., and Rubenstein, E. B. (2004) Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. *Cancer 100*, 1995–2025.

(143) Elting, L. S., Cooksley, C., Chambers, M., Cantor, S. B., Manzullo, E., and Rubenstein, E. B. (2003) The burdens of cancer therapy. Clinical and economic outcomes of chemotherapy-induced mucositis. *Cancer 98*, 1531–1539.

(144) Van den Bongard, D., Mathôt, R., Boogerd, W., Schornagel, J., Soesan, M., Schellens, J., and Beijnen, J. (2001) Successful rescue with leucovorin and thymidine in a patient with high-dose methotrexate induced acute renal failure. *Cancer Chemother. Pharmacol. 47*, 537–540.

(145) Ahmed, Y. A. A. R., and Hasan, Y. (2013) Prevention and Management of High Dose Methotrexate Toxicity. *J. Cancer Sci. Ther. 5*, 106–112.

(146) Widemann, B. C., Balis, F. M., Kempf-Bielack, B., Bielack, S., Pratt, C. B., Ferrari, S., Bacci, G., Craft, A. W., and Adamson, P. C. (2004) High-dose methotrexate-induced nephrotoxicity in patients with osteosarcoma. *Cancer 100*, 2222–2232.

(147) Khan, N., Jeffers, M., Kumar, S., Hackett, C., Boldog, F., Khramtsov, N., Qian, X., Mills, E., Berghs, S. C., Carey, N., Finn, P. W., Collins, L. S., Tumber, A., Ritchie, J. W., Jensen, P. B., Lichenstein, H. S., and Sehested, M. (2007) Determination of the class and isoform selectivity of small molecule histone deacetylase inhibitors. *Biochem. J. 409*, 581–589.

(148) Detich, N., Bovenzi, V., and Szyf, M. (2003) Valproate induces replication-independent active DNA demethylation. *J. Biol. Chem. 278*, 27586–27592.

(149) Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., and Klein, P. S. (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J. Biol. Chem. 276*, 36734–36741.

(150) Gurvich, N., Tsygankova, O. M., Meinkoth, J. L., and Klein, P. S. (2004) Histone Deacetylase Is a Target of Valproic Acid-Mediated Cellular Differentiation. *Cancer Res. 64*, 1079–1086.

(151) Krämer, O. H., Zhu, P., Ostendorff, H. P., Golebiewski, M., Tiefenbach, J., Peters, M. a, Brill, B., Groner, B., Bach, I., Heinzel, T., and Göttlicher, M. (2003) The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J. 22*, 3411–3420.

(152) Garcia-Manero, G., Kantarjian, H. M., Sanchez-Gonzalez, B., Yang, H., Rosner, G., Verstovsek, S., Rytting, M., Wierda, W. G., Ravandi, F., Koller, C., Xiao, L., Faderl, S., Estrov, Z., Cortes, J., O'brien, S., Estey, E., Bueso-Ramos, C., Fiorentino, J., Jabbour, E., and Issa, J.-P. (2006) Phase 1/2 study of the combination of 5-aza-2'-deoxycytidine with valproic acid in patients with leukemia. *Blood 108*, 3271–3279.

(153) Soriano, A. O., Yang, H., Faderl, S., Estrov, Z., Giles, F., Ravandi, F., Cortes, J., Wierda, W. G., Ouzounian, S., Quezada, A., Pierce, S., Estey, E. H., Issa, J.-P. J., Kantarjian, H. M., and Garcia-Manero, G. (2007) Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. *Blood 110*, 2302–2308.

(154) Braiteh, F., Soriano, A. O., Garcia-Manero, G., Hong, D., Johnson, M. M., Silva, L. D. P., Yang, H., Alexander, S., Wolff, J., and Kurzrock, R. (2008) Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. *Clin. Cancer Res. 14*, 6296–6301.

(155) Yang, H., Hoshino, K., Sanchez-Gonzalez, B., Kantarjian, H., and Garcia-Manero, G. (2005) Antileukemia activity of the combination of 5-aza-2'-deoxycytidine with valproic acid. *Leuk. Res. 29*, 739–748.

(156) Gurvich, N., and Klein, P. S. (2002) Lithium and valproic acid: parallels and contrasts in diverse signaling contexts. *Pharmacol. Ther. 96*, 45–66.

(157) Blaheta, R. A., and Cinatl Jr, J. (2002) Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med. Res. Rev. 22*, 492–511.

(158) Göttlicher, M., Minucci, S., Zhu, P., Krämer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and Heinzel, T. (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J. 20*, 6969–6978.

(159) Lampen, A., Siehler, S., Ellerbeck, U., and Go, M. (1999) New Molecular Bioassays for the Estimation of the Teratogenic Potency of Valproic Acid Derivatives in Vitro: Activation of the Peroxisomal Proliferator-Activated Receptor (PPAR-delta). *Toxicol. Appl. Pharmacol. 160*, 238–249.

(160) Loscher, W., and Nau, H. (1985) Pharmacological evaluation of various metabolites and analogues of valproic acid. *Neuropharmacology 24*, 427–435.

(161) Nau, H., and Loxher, W. (1986) Pharmacologic Evaluation of Various Metabolites and Analogs of Valproic Acid□: Teratogenic Potencies in Mice. *Fundam. Appl. Toxicol. 6*, 669–676.

(162) Löscher, W. (1999) Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action. *Prog. Neurobiol. 58*, 31–59.

(163) Massart, J., Begriche, K., Buron, N., Porceddu, M., Borgne-Sanchez, A., and Fromenty, B. (2013) Drug-Induced Inhibition of Mitochondrial Fatty Acid Oxidation and Steatosis. *Curr. Pathobiol. Rep. 1*, 147–157.

(164) Kassahun, K., Farrell, K., and Abbott, F. (1991) Identification and characterization of the glutathione and N-acetylcysteine conjugates of (E)-2-propyl-2,4-pentadienoic acid, a toxic metabolite of valproic acid, in rats and humans. *Drug Metab. Dispos. 19*, 525–535.

(165) Silva, M. F. B., Aires, C. C. P., Luis, P. B. M., Ruiter, J. P. N., IJlst, L., Duran, M., Wanders, R. J. A., and Tavares de Almeida, I. (2008) Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: a review. *J. Inherit. Metab. Dis. 31*, 205–216.

(166) Kleensang, A., Maertens, A., Rosenberg, M., Fitzpatrick, S., Lamb, J., Auerbach, S., Brennan, R., Crofton, K. M., Gordon, B., Fornace, A. J., Gaido, K., Gerhold, D., Haw, R., Henney, A., Ma'ayan, A., McBride, M., Monti, S., Ochs, M. F., Pandey, A., Sharan, R., Stierum, R., Tugendreich, S., Willett, C., Wittwehr, C., Xia, J., Patton, G. W., Arvidson, K., Bouhifd, M., Hogberg, H. T., Luechtefeld, T., Smirnova, L., Zhao, L., Adeleye, Y., Kanehisa, M., Carmichael, P., Andersen, M. E., and Hartung, T. (2014) t4 workshop report: Pathways of Toxicity. *ALTEX 31*, 53–61.

FIGURES

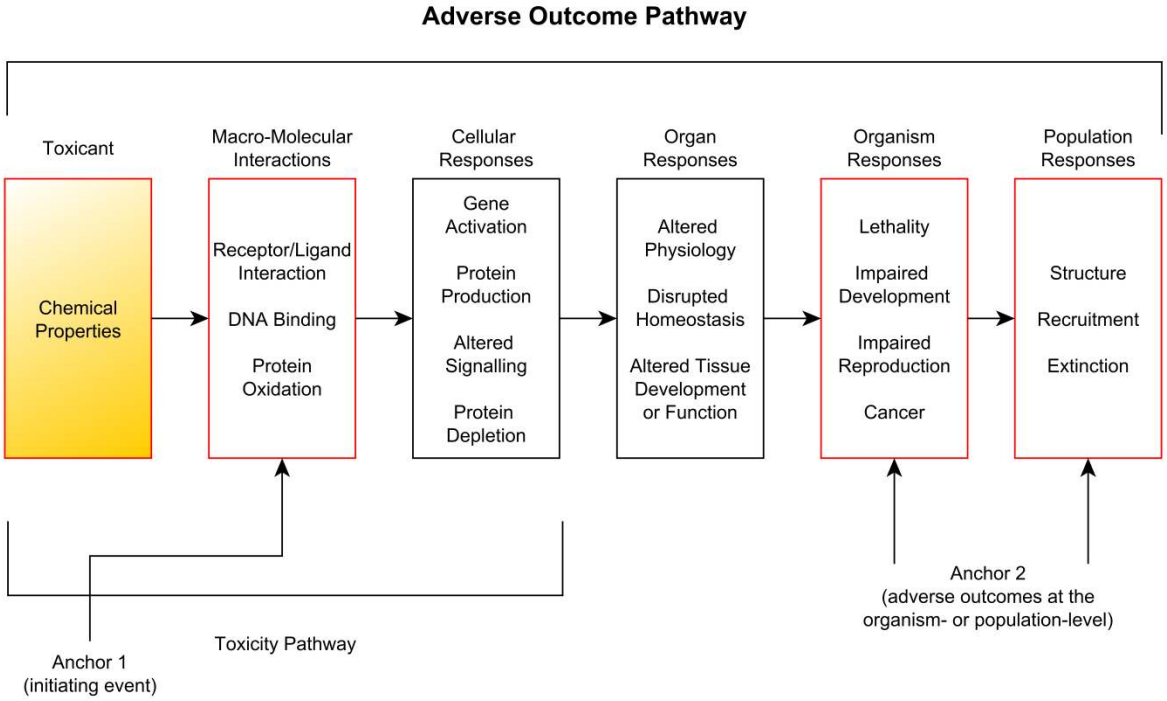

Figure 1. Ankley's conceptual diagram of an adverse outcome pathway (AOP), including the molecular initiating event (MIE). Image adapted from Ankley 2010.[2]

Figure 2. Framework for a (quantitative) structure activity relationship ((Q)SAR) approach based around MIEs. The (Q)SAR relates molecular properties to molecular initiating event (MIE), and the adverse outcome pathway (AOP) infers an adverse outcome from the MIE.

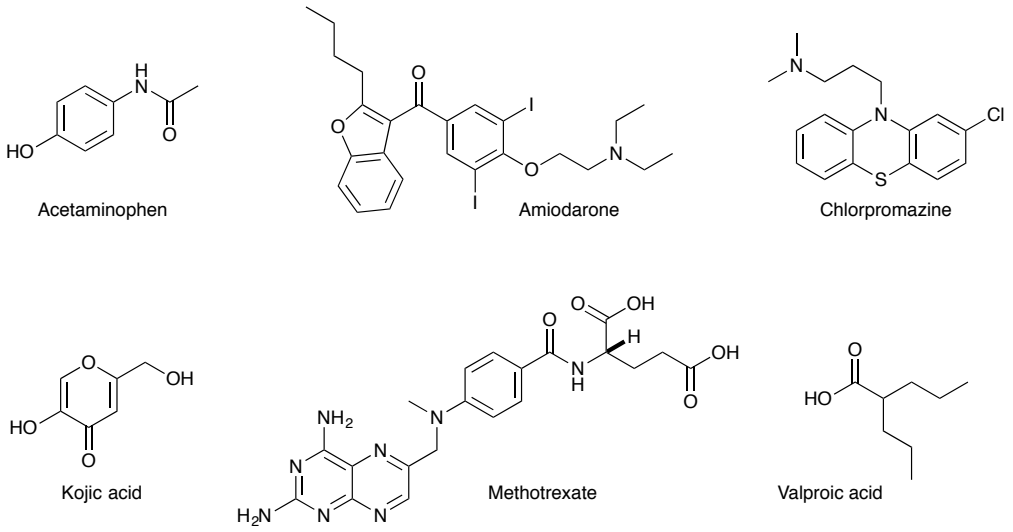

Figure 3. The six molecules for which detailed studies of the MIEs are described here.

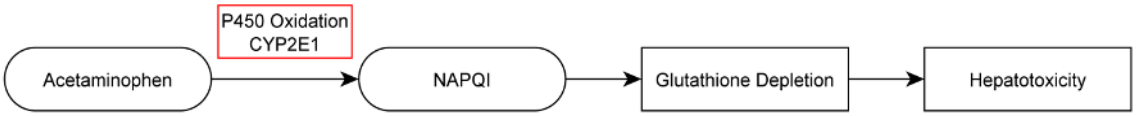

Figure 4. Representation of an adverse outcome pathway (AOP) for acetaminophen-induced hepatotoxicity. The molecular initiating event (MIE) is labelled in red. NAPQI = *N*-acetyl-*p*-benzoquinone imine.

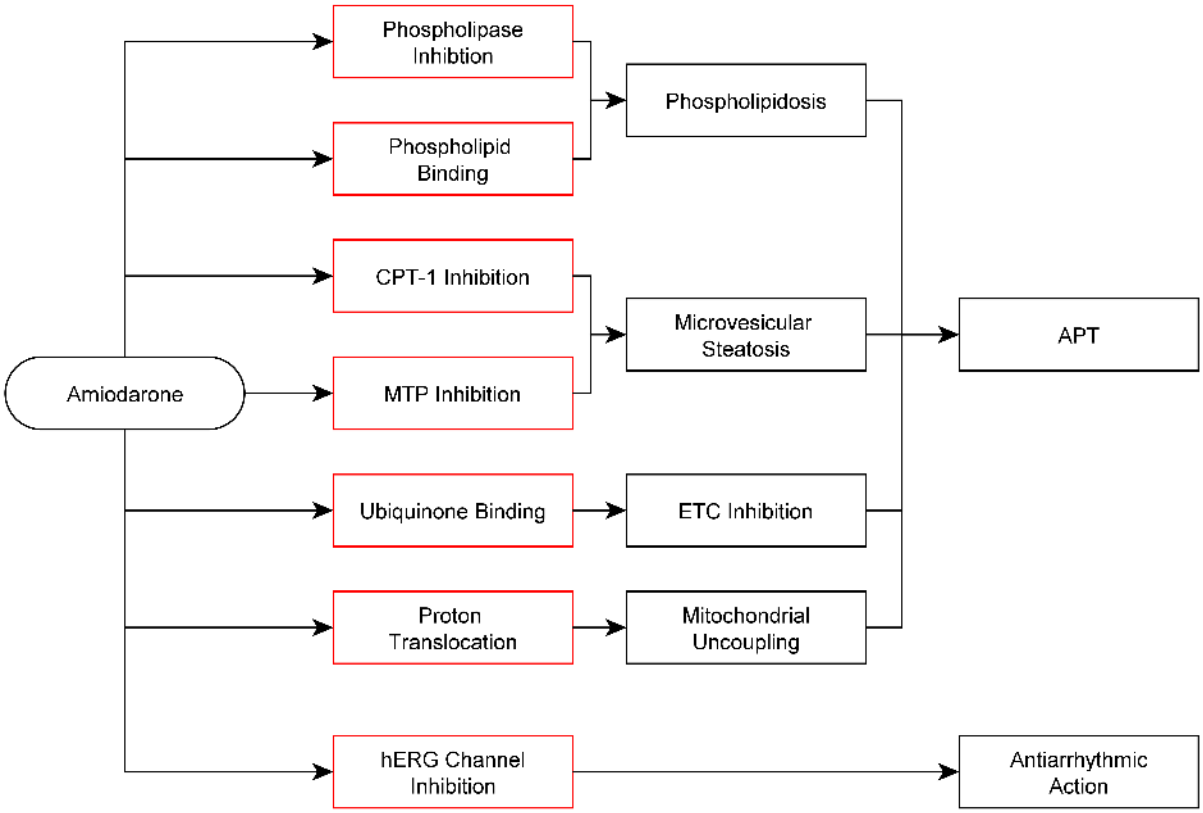

Figure 5. Representation of a molecular initiating event (MIE) map for amiodarone, including pulmonary toxicity and antiarrhythmic action. MIEs are labelled in red. CPT-1 = carnitine palmitoyltransferase I; MTP = mitochondrial transport protein; hERG = human ether-à-go-go-related gene; ETC = electron transport chain; APT = amiodarone-induced pulmonary toxicity.

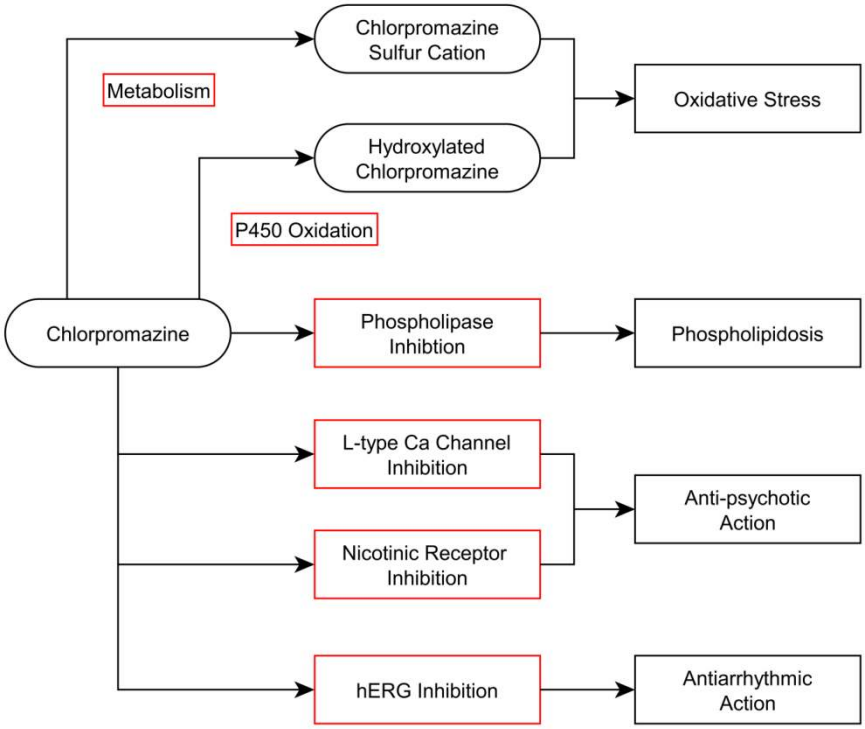

Figure 6. Representation of a molecular initiating event (MIE) map for chlorpromazine, including oxidative stress, phospholipidosis, anti-psychotic action and antiarrhythmic action. MIEs are labelled in red. hERG = human ether-à-go-go-related gene.

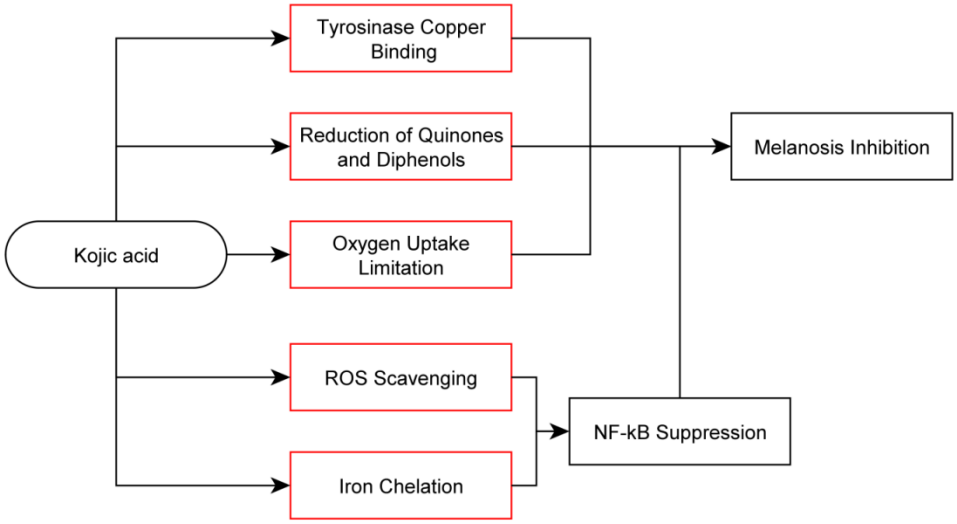

Figure 7. Representation of a molecular initiating event (MIE) map for kojic acid-induced skin-lightening. MIEs are labelled in red. NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells.

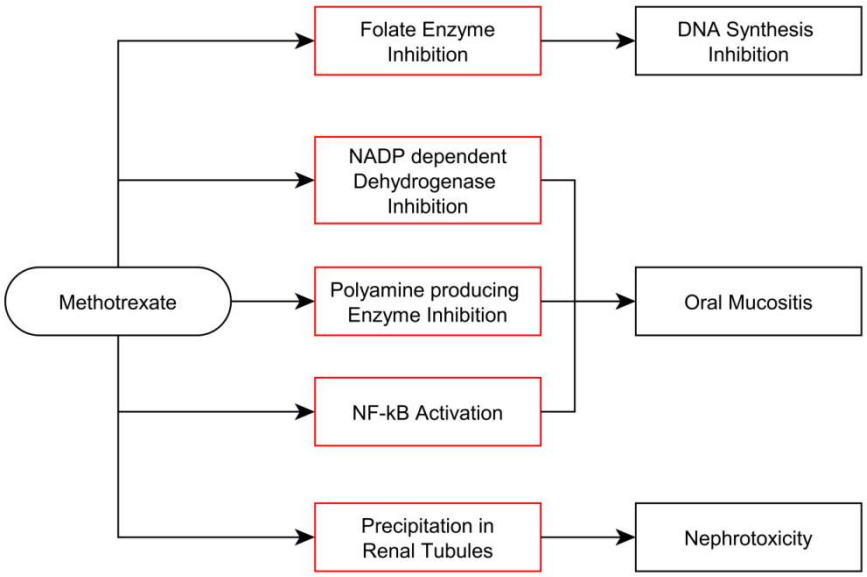

Figure 8. Representation of a molecular initiating event (MIE) map for methotrexate, including DNA synthesis inhibition, oral mucositis and nephrotoxicity. MIEs are labelled in red. NADP = nicotinamide adenine dinucleotide phosphate; NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells.

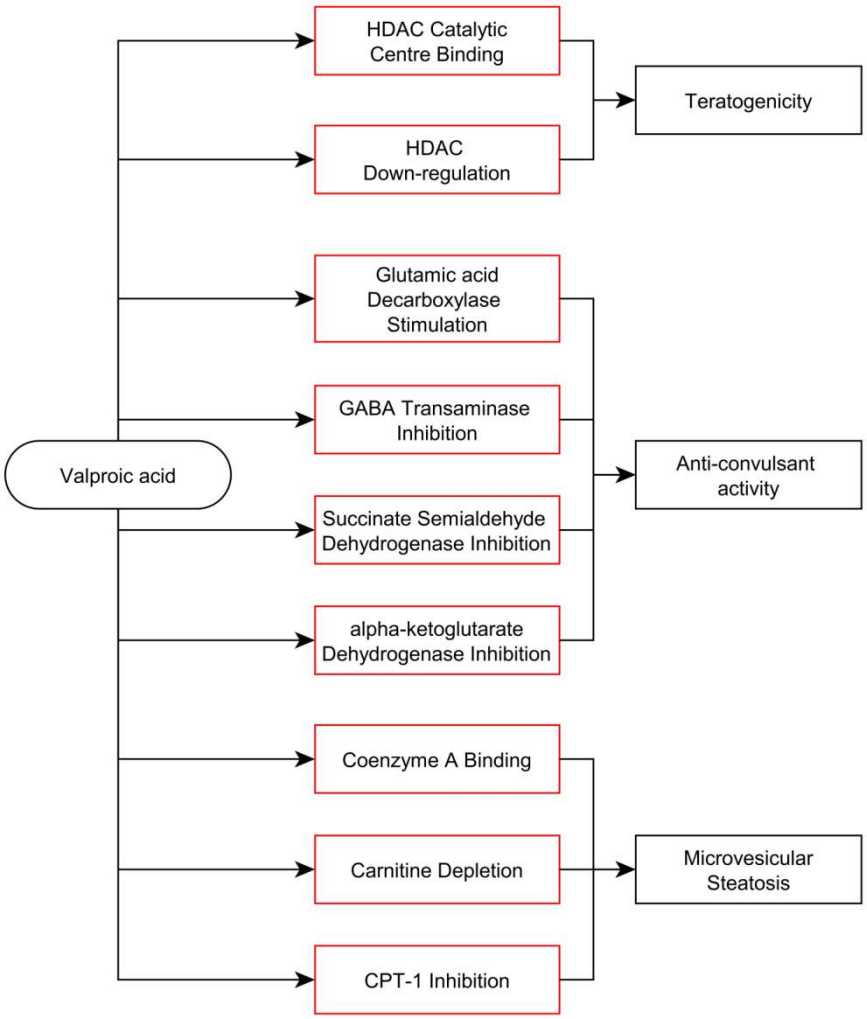

Figure 9. Representation of a molecular initiating event (MIE) map for valproic acid, including teratogenicity, anti-convulsant activity, and microvesicular steatosis. MIEs are labelled in red. HDAC = histone deacetylase; GABA = gamma-aminobutyric acid; CPT-1 = carnitine palmitoyltransferase I.

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