



LJMU Research Online

Cronin, MTD, Enoch, SJ, Mellor, CL, Przybylak, KR, Richarz, AN and Madden, JC

In Silico Prediction of Organ Level Toxicity: Linking Chemistry to Adverse Effects

<http://researchonline.ljmu.ac.uk/id/eprint/6208/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Cronin, MTD, Enoch, SJ, Mellor, CL, Przybylak, KR, Richarz, AN and Madden, JC (2017) In Silico Prediction of Organ Level Toxicity: Linking Chemistry to Adverse Effects. Toxicological Research, 33 (3). pp. 173-182. ISSN 1976-8257

LJMU has developed [LJMU Research Online](#) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>



***In Silico* Prediction of Organ Level Toxicity: Linking Chemistry to Adverse Effects**

**Mark T.D. Cronin, Steven J. Enoch, Claire L. Mellor, Katarzyna R. Przybylak,
Andrea-Nicole Richarz and Judith C. Madden**

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, England

In silico methods to predict toxicity include the use of (Quantitative) Structure-Activity Relationships ((Q)SARs) as well as grouping (category formation) allowing for read-across. A challenging area for *in silico* modelling is the prediction of chronic toxicity and the No Observed (Adverse) Effect Level (NO(A)EL) in particular. A proposed solution to the prediction of chronic toxicity is to consider organ level effects, as opposed to modelling the NO(A)EL itself. This review has focussed on the use of structural alerts to identify potential liver toxicants. *In silico* profilers, or groups of structural alerts, have been developed based on mechanisms of action and informed by current knowledge of Adverse Outcome Pathways. These profilers are robust and can be coded computationally to allow for prediction. However, they do not cover all mechanisms or modes of liver toxicity and recommendations for the improvement of these approaches are given.

Key words: Adverse outcome pathways, Read-across, Structural alert, Liver toxicity, Hepatotoxicity, Quantitative structure-activity relationship (QSAR)

THE CURRENT PARADIGM FOR *IN SILICO* MODELLING: THE SUCCESS (OR OTHERWISE) OF (QUANTITATIVE) STRUCTURE-ACTIVITY RELATIONSHIPS ((Q)SARs) TO PREDICT TOXICITY

There are a many so-called *in silico* or computational approaches to predict the toxicity of chemicals (1,2). These attempt to relate the physico-chemical or structural properties of a molecule to its toxic effect. They include, amongst other methods, the use of (quantitative) structure-activity relationships ((Q)SARs) as well as grouping or category formation which allows for read-across. These methods have a number of applications from screening libraries of compounds in product development through to full risk assessment. They also enable toxicologists and risk assessors to replace and reduce animal testing. However, these methods

Correspondence to: Mark T.D. Cronin, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, England
E-mail: m.t.cronin@ljmu.ac.uk

This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

are not always reliable and must be assessed on their individual merit for the compound and context in question. Indeed, they may not be appropriate for some toxicity endpoints in some circumstances. In order to understand when they may be successfully used, it is essential to describe and assess the relative strengths and weaknesses of the various *in silico* approaches. The aim of this paper is to provide an overview of the shortfalls of the current *in silico* approaches to predict toxicity and illustrate how they may be improved for “difficult-to-predict” endpoints - in this case repeated dose toxicity at the organ level, focussing on the liver.

In order to understand the difficulties of using *in silico* approaches for toxicity prediction, and for which endpoints they are most appropriate, it is worthwhile to consider when they are likely to provide robust models. In particular, (Q)SARs work optimally when a steady-state, or equilibrium, is achieved; this explains their utility for endpoints such as acute fish toxicity (where an equilibrium is normally observed (3)), and, with an understanding of the caveats such as metabolism, degradation and volatility, bioaccumulation (4). SARs, which can be defined in terms of structural alerts, are very amenable to provide predictive approaches to endpoints where there is a direct interaction between the chemical and the biological system, such as the formation of a covalent bond in the disruption of DNA

(leading to mutagenicity) (5) or an immunoprotein (leading to skin sensitisation) (6).

In general “traditional” QSARs provide a correlative approach between an effect (usually defined in terms of a regulatory endpoint) and properties of a molecule. Therefore, when a trivial relationship occurs, this provides a model. However, many adverse effects following exposure to chemicals are a result of disruption or perturbation to pathways leading complex organ level toxicity (which are increasingly being described through the use of Adverse Outcome Pathways (AOPs)) (7,8). There may be many triggers to such toxicity and many interactions that result in these effects. They are often dependent on various absorption, distribution, metabolism and excretion (ADME) effects as well as dose, duration and type of exposure and even lifestage of the organism. Due to the complexity of the results of complex tests e.g. for repeated dose, reproductive and development toxicity, “traditional” (Q)SAR techniques have struggled to provide meaningful and robust models. In terms of regulatory endpoints, this has meant that there are a number of toxicological endpoints that are currently, at best, only poorly predicted by (Q)SAR techniques; these include chronic, reproductive and developmental toxicity, as well as non-genotoxic carcinogenicity (9). Of these more difficult toxicity endpoints for modelling, there are often complex and interacting mechanisms that bring about the effect.

IN SILICO MODELS FOR REPEATED DOSE TOXICITY - PREDICTING NO OBSERVED (ADVERSE) EFFECT LEVELS (NO(A)ELs)

Due to the complexity of the phenomenon, the test design and protocol, and the endpoint required for (regulatory) risk assessment purposes, predicting the toxicity of chemicals following low dose repeated exposure, with *in silico* methods, remains a great challenge. There are many reasons for the difficulty in modelling effects brought about by low dose repeated applications of a chemical. Many of these effects are different to those brought about by acute exposure; in order to understand and model such toxicity reference to, and understanding of, the mechanism of action is required. There are potentially many differences between the (toxic) effects of chemicals at high, acute, doses as compared to low repeated doses. A high, acute, dose may lead to lethality by a single, and sometimes well defined, mechanism and/or AOP - however currently the AOP is currently often unknown. Prolonged exposure to low doses may lead to a multitude of adverse effects being the results of different mechanisms, or AOP networks (many which may currently be unknown) (10). A proportion of mechanisms have as the initiating step a weak receptor interaction, the nature, quantification and relevance of which may be poorly understood. The perturbation of biochemical pathways, and their assessment and prediction, of these so-called adverse events

has become the focus (at least partially) of what is currently recognised as “21st Century Toxicology” (11) whilst it is noted that many, possibly the vast majority of, chemicals will cause toxicity via unspecific effects (12-14). This provides a strong clue, or guiding hand, for modelling, in other words the models should be based around the individual adverse effect rather than the regulatory endpoint.

Whilst it has become apparent that modelling would be more successful if based on individual adverse effects, it is true to say that there are many current QSARs that attempt to predict the “outcomes” from *in vivo* repeated dose toxicity tests. The reason for this is that such data are perceived as being useful to risk assessment and have been easily retrievable from historical databases. For instance, taking chronic mammalian toxicity as an example, the outcome is often interpreted as a no observable (adverse) effect level (NO(A)EL) for a substance (please note in this manuscript the term NO(A)EL is intended to include both the no observable effect level (NOEL) and no observable adverse effect level (NOAEL)). Rather than this being a definable effect, such as a concentration that causes a 50% effect (EC50) to a specific organ, NO(A)ELs are the concentration at which no (adverse) effect is seen and rely on expert interpretation of study findings. As such the derivation and elucidation of the actual NO(A)EL, e.g. for risk assessment purposes, is dependent on the doses tested and what are seen as being important and relevant adverse effects etc. Thus, whilst it is useful for risk assessment purposes, the modelling of a relatively arbitrarily derived values such as a NO(A)EL may potentially pose many problems. Determination of the NO(A)EL from chronic toxicity testing follows examination of all relevant organs (for changes and alterations compared to the control) as well as clinical chemistry, behaviour etc. For many chemicals, the NO(A)EL is dependent on organ level toxicity i.e. the organ(s) which is/are affected by the lowest dose. In terms of predicting NO(A)ELs, this means that if the NO(A)EL value is as a result of organ level toxicity (acknowledging that the NO(A)EL value may be a result of many other effects), it could be considered to be a prediction of organ level effects. In terms of the strengths of the QSAR approach, it is unlikely the NO(A)EL value represents any type of steady-state equilibrium and is often an artefact of the test design and doses tested (benchmark dose may be more appropriate for modelling however, but has been seldom evaluated). Neither will the QSAR comply with a strict interpretation of the first of the OECD Principles for the Validation of QSARs in terms of it being a defined endpoint (15).

Whilst NO(A)ELs are difficult values to model and hence predict, we are rapidly approaching a time when there will be widely available mammalian chronic toxicity data and use must be made of these data. These data will be publicly available, or available on restricted or for commercial use. The databases include, amongst others, RepDose (16), Tox-

RefDb (17), HESS (18), eTox (19), LeadScope (<http://www.leadscope.com/>) and COSMOS (20; <https://www.mn-am.com/projects/cosmosdatasharepoint/>) as well as regulatory data available within the OECD QSAR Toolbox (<http://www.qsartoolbox.org/>), eChemPortal (<http://www.echemportal.org/>) and AMBIT (http://cefic-lri.org/lri_toolbox/ambit/). Because of this greater access to data, often of unknown quality, reliability and/or relevance, we must develop strategies to model NO(A)ELs efficiently, in a manner suitable for regulatory use and other risk assessment scenarios. Thus, in terms of modelling a NO(A)ELs, it may be necessary to identify and model the doses that bring about individual organ level effects rather than attempting to predict the response level in an *in vivo* test.

Key to modelling the NO(A)EL values will be understanding the value itself and how it has been derived i.e. which organ level (or other) effect has been chosen as being sufficiently significant to be described as toxicity. The modeller should not lose sight of the fact that the NO(A)EL value is a result of a complex *in vivo* study which requires dosing of animals at a number of levels, aiming to observe no (adverse) effect to a number of organs. The results, normally in the form of a detailed report require expert interpretation and analysis to determine the NO(A)EL. Some recent work has attempted to make this process transparent with regard to the derivation of Thresholds of Toxicological Concern (TTC) (21) - whilst not intended for modelling, such approaches may be of interest to modellers.

Despite the difficulties in predicting NO(A)ELs, there have been a number of QSAR models (22), however there has been no coherent or consistent assessment of their performance. More recent approaches are based around the grouping of chemicals and application of read-across (23,24). These recent approaches include the use of new, and freely available, expert systems for grouping chemicals according to chronic toxicity - e.g. the OECD QSAR Toolbox, the HESS system, AMBIT and associated database (see previous links). Whilst there may be no obvious overlap between the more established models and the new systems, it is, of course, entirely possible that the expert system approaches are implicitly identifying "similar" chemicals and using those to make the predictions of chronic toxicity. This process is at the heart of grouping and read-across which will be described in more detail below.

IN SILICO MODELLING OF LIVER TOXICITY

The liver is a key organ in terms of toxicology and crucial in interpreting repeated dose toxicity (25-27). Obviously the liver has a vital physiological role and is prone to toxicity due to high, first-pass, blood flow which increases the likelihood of toxicants reaching a significant concentration. There is a range of direct, indirect and idiosyncratic effects that chemicals can cause in the liver, some of which

are described in more detail below. The possibilities for toxicity (and its modelling) are complicated by the often contradictory effects of metabolism in the liver (28). The liver accounts for a significant proportion of *in vivo* metabolism. It must be remembered that a high metabolic capacity that produces a large number of novel metabolites is both beneficial (in terms of detoxification and excretion) and harmful in terms of toxicification. The situation is made even more complex due to the naturally occurring defence mechanisms in the liver. Therefore, whilst some compounds may have the ability to be reactive in the liver, no toxicity is seen due to the protection offered by these systems. Commercially, toxicity to the liver is very important and has many consequences. Whilst it is especially significant to the pharmaceutical industry - where the term coined is Drug Induced Liver Injury (DILI) - all industrial sectors need to be careful of the harmful effects of compounds to the liver (28).

There are a number of problems in the computational modelling of liver toxicity; these are centred around the complexity of the endpoint, data and the current suite of modelling techniques available (29). Specifically the problems can be summarised as follows:

- Toxicity data for modelling - whilst there are guidelines for the standardised reporting of preclinical outcomes, it must be remembered that there is no specific *in vivo* "test" for liver toxicity, as would be associated with other endpoints e.g. skin sensitisation or irritation. Therefore the modeller is reliant on other forms of data e.g. histopathological observations from *in vivo* testing or reports of adverse drug reactions from clinical use etc. It is also noted that there is inherent variability in all *in vivo* studies (25,27). The net result is that such data that may be available are not from consistent assays, may not reflect potency and will be of variable quality. In addition, the presentation of the data may not be in a form suitable for modelling i.e. they may be held within study reports and not readily available in databases.

- The datasets available for consideration are noted below, however, their chemical space is often biased towards pharmaceutical active ingredients and may not be representative of chemical space for other types of compounds e.g. cosmetics or industrial chemicals.

- As noted above, there is a plethora of modes and/or mechanisms of action that bring about liver toxicity. This inevitably complicates modelling if compounds with different mechanisms of action are lumped together. Whilst it may be preferable to develop models on a mechanistic basis, there is currently no easy method to classify compounds into particular mechanisms. The situation with modes of action is complicated by the sheer number of mechanisms and the fact they may be inter-related, dependent on dose duration and level, the age and nutritional status of the organism, genetic susceptibility etc.

- The current (Q)SAR approaches to modelling do not take into account the complexity of relevant issues such as

metabolism (either toxification or detoxification) or the defence mechanisms naturally present in the liver. As such, they run the risk of oversimplifying a complex toxicological event.

Whilst there are undoubtedly concerns over the quantity and quality of data relating to liver toxicity, there are some significant areas where data could be utilised. Sources of liver toxicity data are reviewed and summarised by Przybylak and Cronin (29). There are several distinct and usable (albeit of variable meaning and quality) sources of data. For instance information on liver toxicity has long been available in the literature and clinical reports on the adverse effects of drugs. Whilst these data are available (including the significant datasets noted below), as noted above, they are seldom compiled in a format suitable for modelling i.e. with checked structures, downloadable etc. There are, of course, a number of biomarkers for liver toxicity e.g. ALT, ADH etc (30). These may provide usable information, although not wholly mechanistically based. Other, more reasonable (in terms of modelling) examples of potentially usable hepatotoxicity data exist, for instance the United States Food and Drug Administration (US FDA) Adverse Effects Reporting System (FAERS), which contains information for pharmaceuticals as well as human hepatotoxicity data gathered from spontaneous, voluntary reporting adverse effects ([https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrug Effects/ucm083765.htm](https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/ucm083765.htm)). Zhu and Kruhlak (31) developed a bespoke human hepatotoxicity database for the purposes of modelling. This section is not intended to be a full review of available liver toxicity data (refer to (29) for a more detailed description). The key to modelling liver toxicity is not only to obtain more data, but to develop an efficient strategy to use the data that are easily available to create mechanistically relevant models.

There are a wide variety of (Q)SAR type approaches that have been applied to predict liver toxicity and related effects. These range from simplistic regression-based QSAR approaches for small series of compounds to much larger studies of heterogeneous groups of compounds. A commonality in all modelling approaches is that no consistent data or test set been analysed, with information relating to liver toxicity being derived from a many sources ranging from assays based on biomarkers, through to *in vivo* toxicity studies and (human) clinical reports. It is possible to use data from high throughput screening to derive structural rules for the hepatotoxicity of drugs (32) which can be applied for the screening of new compounds (see also below). Other simple QSAR analyses have quantified the role of biomarkers for liver effects and demonstrate that these can be rationalised according to the chemistry underpinning the mechanism(s) of action (33). At the other end of the spectrum, QSAR analyses have been performed on large datasets using multivariate techniques (34), with particularly large, information-rich, data-

sets becoming available e.g. Mulliner *et al.* (35) utilised information for over 3700 drugs. Other approaches have also used biological information to support predictions from chemistry alone (36-39). Including biological and/or mechanistic information is likely to improve predictions, but implicitly to use such models will require experimental measurements of the compounds of interest.

As evidenced by recent approaches to modelling that incorporate mechanistic information, it is essential for proper model development that an appreciation of mechanistic of action is at the heart of the models. This requirement may be a challenge to toxicology as it has typically not provided a framework or overview of the mechanisms involved in organ level toxicity in a manner that would be amenable to modelling - this may change with the rapid uptake of the AOP paradigm as described below. Whilst a formal framework is not available, much information about the adverse effects to the liver is provided. For instance, "classic toxicology" has identified the main effects to the liver, taking as an example Schwarz and Watkins (40), chemically induced liver injury is defined as including (in relative order of severity): steatosis, porphyria, veno-occlusive disease, cholestasis, hepatitis, cell death from necrosis or apoptosis and the development of tumours. Whilst this list covers the main effects, it is a mixture of mechanisms and observations of effects. Others have defined the diverse mechanisms that result in hepatotoxicity, for instance Jaeschke *et al.* (41) described in some detail the intricacies of the mechanisms of bile acid-induced hepatocyte apoptosis, oxidative stress, CYP2E1-dependent toxicity, drug-induced hepatotoxicity as a result of the formation of reactive metabolites and the various effects of mitochondrial dysfunction. More specific mechanisms of DILI has been defined by Yuan and Kaplowitz (42). This knowledge, whilst not necessarily complete or in a format entirely suitable for modelling, does provide a starting point for the development of *in silico* models for liver toxicity.

A NEW PARADIGM FOR *IN SILICO* MODELLING: INCORPORATING ADVERSE OUTCOME PATHWAYS AND NEW APPROACH METHODOLOGIES

In order to predict toxicity from low dose repeated exposure (to humans) accurately, *in silico* modelling will need to move away from the simplistic aspects of QSAR modelling. The road to success, either in terms of predicting adverse effects at the organ level, or directly predicting NO(A)ELs, is to deconstruct the problem into the relevant components, model these and then combine the predictions into a meaningful estimate of "safety" (43). A toxic effect can be rationalised into the intrinsic toxicity of a substance and the exposure to it. This paper describes how intrinsic toxicity may be modelled, however prediction of exposure through the modelling of kinetics, is described elsewhere. The mod-

elling of the intrinsic toxicity of compounds is placed in the context of chemical grouping and read-across.

Chemical grouping is the process by which chemicals are gathered together on a rational basis. Once a group of chemicals or analogues has been created, should a new (the target) chemical be allocated into the group, and appropriate data be obtained, then an activity may be interpolated by the process of read-across (2). The use of predictions from read-across to fill data gaps has grown in popularity recently, in particular as a response to the requirements of the REACH legislation (44). The key to grouping compounds successfully is determining a suitable criterion, or set of criteria, on which to develop the group (45). With regard to grouping relating to predicting NO(A)EL (as with other endpoints), there has already been success in forming groups of compounds on a mechanistic basis, particularly emphasising the role of organ level toxicity (23,24). Therefore, in terms of developing a strategy for predicting a NO(A)EL, this can be considered to be a two-stage process:

- identification of relevant organ toxicity that relates to NO(A)EL e.g. through an appropriate “profiler” for grouping, and

- identification of analogues (i.e. sharing similar relevant molecular fragments, structure or properties) and undertaking of read-across with the group.

Assuming a potential approach to the computational modelling of liver toxicity and formation of groups or categories is based around mechanistic (or mode) of action information, a process to organise the information is required. One such way forward is through the understanding of toxicity pathways and the formalisation (if required) into AOPs (7,8,46-48). The toxicity pathway concept is at the heart of what is termed 21st Century toxicology (11). AOPs are being developed for numerous human and environmental effects and are being recorded on the AOP Wiki (see the web-site: aopwiki.org). The basis for developing an AOP has been defined by the OECD, amongst others, and it includes the following:

- Identification of a molecular initiating event (MIE). The MIE can be thought of as the direct link between the interaction of the chemical at the molecular site of action, e.g. covalent binding or receptor mediated toxicity. It is the definition of MIE that provides the direct link to chemistry, hence it can provide information for 2-D or 3-D structural alerts and, as such, can provide the basis of chemical grouping.

- A series of one or more key (or intermediate) events. These form the basis of the pathway and can be thought of as linked building blocks. These key events are the biological linkages, they can be defined and have the potential for assays to be developed for them. At this point, rational or intelligent testing of chemicals in assays for the “over-riding” key event(s), i.e. the rate limiting step(s), could assist in the definition of domains of activity of an AOP.

- An adverse effect or apical event which can, if required,

be related to a regulatory endpoint. This may be considered at the organ or individual for human toxicology (and population or even ecosystem level for environmental effects).

- In addition, and also linked to mode of action (49), there is a need in risk assessment to understand or describe the exposure of an organism to a chemical. This can be thought of in terms of the type, route, duration and dose of the exposure. For some endpoints e.g. developmental toxicity, the lifestage at which exposure is made may be important.

With regard to modelling and understanding of liver toxicity, a small number of AOPs have been formally defined, which may be a good starting point for modelling, these include

- Cholestatic liver injury induced by inhibition of the bile salt export pump (50).

- Protein alkylation leading to liver fibrosis (51).

- Sustained AhR activation leading to rodent liver tumours (52).

Whilst progress is being made, the relatively small number of AOPs as compared to the number and complexity of liver toxicity mechanisms emphasises that much effort and progress is still required in this area. For those AOPs available, they clearly indicate that there is a direct link from the MIE to chemistry through understanding of effects such as the capability to react covalently with biological (macro-) molecules (53). As a consequence, definition of the chemistry associated with protein reactivity may be one starting point for defining the domain of an MIE associated, for instance, with fibrosis. The organic chemistry mechanisms for protein reactivity has been defined, in part at least, by Enoch *et al* (6). However, these are very general rules, covering all potential aspects of reactions with proteins. It also true, however, that many chemicals will not act through these specific AOPs and will need to be considered in a different manner (12-14).

In order to implement the strategy to identify organ level toxicity (and hence having a reasonable chance of predicting NO(A)ELs transparently and accurately) more work is required. In particular, effort is required to define organ level toxicity and provide profilers to assist in the rational and mechanistically based grouping of chemicals. Such an approach is provided by Sakuratani and co-workers (23) and several others (24), although it is limited. As a starting point, the key organs relating to endpoints following repeated low dose exposure to chemicals must be identified. These include for instance, the liver, kidneys, heart, lung, skin and several others. The list of important organs is long but not endless. It is, of course, important to define which is the “most important” organ level toxicity, but it is beyond doubt that the liver represents one of the most important organs with regard to harmful effects of chemicals.

Recently, to support the justification of grouping of compounds to allow for read-across, the concept of data from “New Approach Methodologies” (NAMs) has been described.

NAMs include any evidence that may support toxicological evaluation and prediction, including existing data from non-guideline tests, tests to other species or for other effects, *in chemico*, *in vitro*, high throughput and content and molecular biology data (54). The use of NAMs to support grouping has been shown to be important for liver toxicity (24). To illustrate the complexity of these issues, the following sections provide a status update on the modelling of toxicity of chemicals that affect the liver, with a particular emphasis on grouping compounds.

AN EXAMPLE OF *IN SILICO* MODELLING: DEVELOPMENT OF STRUCTURAL ALERTS FOR LIVER TOXICITY AND RECOMMENDATIONS FOR IMPROVEMENT

In the context of this paper, a “Structural Alert” is defined broadly as any fragment of a molecule (typically 2-D) that is associated with a particular toxicity. Ideally the fragment is well defined and can be coded computationally to allow for ease of use and be related to the mechanism of action or, if applicable, the MIE of the AOP. There are two general applications for structural alerts: firstly to make a direct prediction of the hazard associated with a compound, secondly to provide a rational basis for grouping and hence allow for read-across. These applications are not independent, but seldom well defined. Due to the complexities of modelling organ level toxicity, and effects to the liver in particular, future efforts must pay more attention to the role of mechanisms of action. As such, AOPs provide a possible framework for organising the effort and modelling initiatives. Recent progress has used the information provided by AOPs to derive *in silico* modelling approaches, some recent advances are summarised in Table 1 and associated references (6,55-62). What is clear from Table 1 is the breadth and diversity of the mechanisms, the MIE and, as a result, the type of modelling approach taken. These modelling approaches range from organic chemistry derived alerts for covalent binding through to groups of SMARTS strings and

3-D toxicophores for receptor binding.

Following the development of a limited number of alerts for liver toxicity, the following recommendations are made for further development of alerts in the future.

There is a need for the better definition of structural alerts. The structural features associated with protein reactivity described and defined by Enoch *et al.* (6) and which are freely available in the OECD QSAR Toolbox and Tox-Tree software, are intended to be generalistic and provide an overview of the entirety of possible organic chemistry mechanisms associated with protein reactivity. They may be used to identify potentially reactive compounds (and hence hepatotoxic due to reactivity) but they should not be considered to be predictive of any single endpoint; indeed, the intention of developing these alerts was to provide a basis for grouping with the assumption the read-across would be performed and the strong possibility that the group would contain compounds with, and without, toxicity (53,63). A further complication of such reactive compounds is whether metabolism is relevant and how this may have been captured within the alerts (for some structural alerts metabolism is implicit, for others it must itself be predicted).

For individual organ level toxicity, the current set of alerts could be better defined. It should be noted that some structural alerts are already well defined and documented, for instance those present within the DEREK Nexus software [<https://www.lhasalimited.org/products/derek-nexus.htm>]. However, as we more formally link alerts to toxicity pathways through the AOP, the alerts themselves will need to be defined more precisely. As noted previously, so-called “chemistry” alerts for use in grouping and read-across are very general; for organ level toxicity these will need to be more carefully defined. Indeed, such alerts could go further into what are being termed “chemotypes” (64). Chemotypes have the capability to incorporate structural features with physico-chemical properties. Technically, these may need to extend the use of SMARTS strings into more sophisticated markup languages such as CSRML. A proposal has already been

Table 1. Structural alerts and other freely available *in silico* approaches that may assist in the identification of liver toxicants

Effect	Molecular initiating event	Type(s) of structural alert	Numbers of alerts	Reference
Phospholipidosis	Trapping of molecules with lysosomes	Generalistic structural alerts	>30	Przybylak <i>et al.</i> (55,56)
Reactive hepatotoxicity including fibrosis	Disruption of cellular function	Structural alerts for covalent binding	>100	Enoch <i>et al.</i> (6)
Mitochondrial toxicity	Disruption of proton gradient	Structural alerts for redox cycling, uncoupling etc	>20	Nelms <i>et al.</i> (57,58)
Liver toxicity (as identified in humans)	Various	Miscellaneous relating to various mechanisms of liver toxicity	>15	Hewitt <i>et al.</i> (59)
Steatosis	Nuclear receptor binding	SMARTS strings for binding to various nuclear receptors	>200	Mellor <i>et al.</i> (60,61)
Steatosis	Binding to LXR	Toxicophores	<5	Tsakovska <i>et al.</i> (62)

made for the incorporation of chemotypes, captured through CSRML to be integrated into KNIME Workflows for the prediction of chronic toxicity (57,65).

There is a need to develop alerts or categories for non-reactive liver toxicity AOPs. A significant area for improvement is the development of alerts for AOPs for non-reactive mechanisms of liver toxicity. This can be performed in at least three ways:

- Firstly, the MIE(s) can be identified and structural alerts built around them. This has the advantage of being thorough and robust, but it is likely to be slow.

- A second method is to search chemical structures associated with toxicity for alerts. The utility of structural similarity is that it may rapidly provide indicators of structural alerts associated with toxicity (59,66). *A posteriori* these can be interpreted in terms of mechanisms. Whilst this may be a more rapid process to develop alerts, it still requires effort to interpret alerts, will inevitably be dependent of current (published and available) knowledge, and could be open to misinterpretation of results. At this point, some of the large data compilations (e.g. ChEMBL, PubChem) may be relevant to assist in the interpretation of models (61,65).

- Thirdly, alerts could be developed from the AOPs and defined through rational testing of the key, or intermediate, events. This is by far the most costly and time consuming method to determine and define categories but will result in the most mechanistically relevant and robust categories, supported by experimental evidence.

Thus, to develop better organ-specific profilers we need to start from a mechanistic basis (e.g. through the AOP concept) and have several high quality anchors including data for apical endpoints and/or adverse effects along with key events. Should structural similarity be attempted as a fast track to develop these alerts, the information must not be over-interpreted and there must be confidence to eliminate poor, badly defined, or unjustifiable alerts. Many alerts will be related to receptor binding interactions (e.g. with hormonal receptors or signaling pathways). This will require new technologies to power the next generation of profilers – the profilers in the OECD QSAR Toolbox, for example, are based on 2-D structure. This will have to transform into capturing 3-D structure, a full capability to capture stereoisomerism information as well as toxicophores. Many of these techniques are well established in drug discovery, we need to see a greater cross-over into toxicology as proposed by Tsakovska *et al* (62). Lastly, development of *in silico* models as described here is not a short-term fix, it is a long-term solution and there must be patience, understanding of the limitations and a better integration of efforts between disparate sciences such as molecular (systems) biology, computational chemistry, chemoinformatics and toxicology.

There is a need for a better understanding, and inclusion in models, of the role of metabolism and (bio)kinetics.

All *in silico* models for toxicity prediction need to incorporate the role of metabolism either implicitly, or through the prediction of metabolites. Clearly this is even more vital for liver than most other organs. Whilst considerable progress has been made in the development of metabolic simulators (67), there has still been no true assessment of their reliability to, for example, predict reactive metabolites. Due to the long-term effort in the development of systems for prediction of metabolism, it is likely that they are comprehensive. What is required is a concerted effort to define the likely routes for the production of reactive metabolites. Then we must determine if any routes are missing from the current predictive schemes and implement them more effectively. As part of this, better strategies are required to identify the stable and significant metabolites (rather than complete metabolic trees which may include short-lived metabolites).

As a final part of the strategy for predicting chronic toxicity, and organ level effects in particular, more effort will be required in the use of biokinetics to predict organ-level concentrations. This topic is outside of the scope of this paper, but the role of toxicokinetics has been well known for many years. The key will be to provide models to assess whether concentration of toxicant in an organ will be above what is being termed the Point of Departure (POD) (68).

CONCLUSIONS AND OVERALL RECOMMENDATIONS FOR *IN SILICO* MODELLING TO SUPPORT THE PREDICTION OF ORGAN LEVEL TOXICITY

Chronic toxicity is a key (regulatory) endpoint for risk assessment of chemicals across numerous industrial sectors. Risk assessment is normally performed by consideration of the NO(A)EL values for a particular chemical. There are a number of methods to predict NO(A)EL values but few, if any, currently have the capability to replace the existing *in vivo* tests. In the future, *in silico* modelling may be based around the prediction of organ-level effects, with regard to this the prediction of effects to the liver is one of the most important. There is a need for a better liver toxicity prediction strategy, this may include more reliable data with an understanding of mechanisms/effects and a framework of mechanisms of action and/or AOPs - such approaches could be applied to all other relevant organs. In particular there is a need to define MIEs to create alerts, groups and potentially (Q)SARs. To assist this, we need better use of rational and intelligent NAMs, i.e. non-animal (e.g. *in vitro*, -omics etc) testing to define domains of the MIEs. Lastly, no single model, or modelling approach will predict organ level toxicity efficiently, and a better consideration and integration of metabolism and kinetics is required both at the level of physiologically-based pharmacokinetic (PBPK) mod-

elling to predict internal dosing and distribution as well as *in vivo* and *in vitro* toxicokinetics to allow for extrapolation.

The following overall recommendations for the development of *in silico* models for toxicity following low dose repeated exposure are made.

- Developing QSAR models directly to predict NO(A)EL values for a broad spectrum of compounds and effects is unlikely to provide robust models.

- In order to develop models to predict repeated dose toxicity computationally, organ level effects must be considered.

- Most *in silico* toxicology effort at the organ-level has centred on the liver; however, predicting liver toxicity requires further effort to identify the effects, mechanisms and suitable data.

- Structural alerts provide the basis for grouping compounds into categories which may allow for read-across. There is evidence that read-across within robust categories may be a suitable method to predict NO(A)ELs especially when supported by NAMs.

- Structural alerts will need to be developed for further mechanisms of action of organ level toxicity. Adverse Outcome Pathways (AOPs) will provide a guide to collect this information.

- AOPs should be utilised to establish the link between the definition of chemistry underpinning the MIE and the adverse effect.

ACKNOWLEDGMENTS

The comments, insight and inspiration of many scientists, specifically those who worked with us on the COSMOS and eTox Projects, as well as many others involved in the SEURAT-1 Cluster, are gratefully acknowledged. In addition, the anonymous reviewers gave invaluable insight with detailed comments which we have attempted to include - any continuing errors are the responsibility of the main author!

Received February 23, 2017; Revised April 4, 2017; Accepted April 6, 2017

REFERENCES

1. Cronin, M.T.D. and Madden J.C. (2010) *In Silico Toxicology: Principles and Applications*. The Royal Society of Chemistry, Cambridge, p. 669.
2. Cronin, M.T.D., Madden, J.C., Enoch, S.J. and Roberts, D.W. (2013) *Chemical Toxicity Prediction: Category Formation and Read-Across*, The Royal Society of Chemistry, Cambridge, p. 191.
3. Mackay, D., Arnot, J.A., Petkova, E.P., Wallace, K.B., Call, D.J., Brooke, L.T. and Veith, G.D. (2009) The physicochemical basis of QSARs for baseline toxicity. *SAR QSAR Environ. Res.*, **20**, 393-414.
4. Dimitrov, S., Dimitrova, N., Georgieva, D., Vasilev, K., Hatfield, T., Straka J. and Mekenyan O. (2012) Simulation of chemical metabolism for fate and hazard assessment. III. New developments of the bioconcentration factor base-line model. *SAR QSAR Environ. Res.*, **23**, 17-36.
5. Enoch, S.J. and Cronin, M.T.D. (2010) A review of the electrophilic reaction chemistry involved in covalent DNA binding. *Crit. Rev. Toxicol.*, **40**, 728-748.
6. Enoch, S.J., Ellison, C.M., Schultz, T.W. and Cronin, M.T.D. (2011) A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. *Crit. Rev. Toxicol.*, **41**, 783-802.
7. Kleinstreuer, N.C., Sullivan, K., Allen, D., Edwards, S., Mendrick, D.L., Embry, M., Matheson, J., Rowlands, J.C., Munn, S., Maull, E. and Casey, W. (2016) Adverse Outcome Pathways: From research to regulation scientific workshop report. *Regul. Toxicol. Pharmacol.*, **76**, 39-50.
8. Allen, T.H.E., Goodman, J.M., Gutsell, S. and Russell, P.J. (2014) Defining molecular initiating events in the adverse outcome pathway framework for risk assessment. *Chem. Res. Toxicol.*, **27**, 2100-2112.
9. Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Ben- them, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felter, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Batista Leite, S., Loizou, G., Maxwell, G., Mazzatorta, P., Munn, S., Pfuhrer, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tahti, H., Testai, E., van Delft, J., van Loveren, H., Vinken, M., Worth, A. and Zaldivar, J.M. (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010. *Arch. Toxicol.*, **85**, 367-485.
10. Schwarz, M. and Mahony, C. (2011) Introduction to repeated dose (systemic) toxicity *in Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing* (Volume 1). Cochrane Consortium, Paris, France.
11. National Academies of Sciences, Engineering, and Medicine (2017) *Using 21st Century Science to Improve Risk-Related Evaluations*. The National Academies Press, Washington, DC.
12. Vinken, M. and Blaauboer, B.J. (2017) *In vitro* testing of basal cytotoxicity: Establishment of an adverse outcome pathway from chemical insult to cell death. *Toxicol. In Vitro*, **39**, 104-110.
13. Thomas, R.S., Black, M.B., Li, L.L., Healy, E., Chu, T.M., Bao, W.J., Andersen, M.E. and Wolfinger, R.D. (2012) A comprehensive statistical analysis of predicting *in vivo* hazard using high-throughput *in vitro* screening. *Toxicol. Sci.*, **128**, 398-417.
14. Judson, R.S., Kavlock, R.J., Setzer, R.W., Hubal, E.A.C., Martin, M.T., Knudsen, T.B., Houck, K.A., Thomas, R.S., Wetmore, B.A. and Dix, D.J. (2011) Estimating toxicity-related biological pathway altering doses for high-throughput chemical risk assessment. *Chem. Res. Toxicol.*, **24**, 451-462.
15. Cronin, M.T.D. (2013) Evaluation of categories and read-across for toxicity prediction allowing for regulatory accep-

- tance in *Chemical Toxicity Prediction: Category Formation and Read-Across* (Cronin, M.T.D., Madden, J.C., Enoch, S.J. and Roberts, D.W. Ed.). The Royal Society of Chemistry, Cambridge, pp. 155-167.
16. Bitsch, A., Jacobi, S., Melber, C., Wahnschaffe, U., Simetska, N. and Mangelsdorf, I. (2006) REPDOSE: A database on repeated dose toxicity studies of commercial chemicals - A multifunctional tool. *Regul. Toxicol. Pharmacol.*, **46**, 202-210.
 17. Judson, R.S., Martin, M.T., Egeghy, P., Gangwal, S., Reif, D.M., Kothiyi, P., Wolf, M., Cathey, T., Transue, T., Smith, D., Vail, J., Frame, A., Mosher, S., Hubal, E.A.C. and Richard, A.M. (2012) Aggregating data for computational toxicology applications: The U.S. Environmental Protection Agency (EPA) Aggregated Computational Toxicology Resource (ACToR) system. *Int. J. Mol. Sci.*, **13**, 1805-1831.
 18. Sakuratani, Y., Zhang, H.Q., Nishikawa, S., Yamazaki, K., Yamada, T., Yamada, J., Gerova, K., Chankov, G., Mekenyan, O. and Hayashi, M. (2013) Hazard Evaluation Support System (HESS) for predicting repeated dose toxicity using toxicological categories. *SAR QSAR Environ. Res.*, **24**, 351-363.
 19. Cases, M., Briggs, K., Steger-Hartmann, T., Pognan, F., Marc, P., Kleinoder, T., Schwab, C.H., Pastor, M., Wichard, J. and Sanz, F. (2014) The eTOX data-sharing project to advance *in silico* drug-induced toxicity prediction. *Int. J. Mol. Sci.*, **15**, 21136-21154.
 20. Yang, C., Hristozov, D., Tarkhov, A., Kleinoeder, T., Boyer, I., Cronin, M., Fioravanzo, E., Kim, H., Heldreth, B., Mostrag-Szylchtyng, A., Rathman, J., Richarz, A., Schwab, C., Vitcheva, V. and Worth, A. (2015) COSMOS DB as an international share point for exchanging regulatory and toxicity data of cosmetics ingredients and related substances. *Toxicol. Lett.*, **238**, S382.
 21. Yang, C., Barlow, S.M., Muldoon Jacobs, K.L., Vitcheva, V., Boobis, A.R., Felter, S.F., Arvidson, K.B., Keller, D., Cronin, M., Enoch, S., Worth, A. and Hollnagel, H.M. (2017) Thresholds of toxicological concern for cosmetics-related substances. *Fd Chem. Toxicol.* [in press].
 22. Hisaki, T., Kaneko, M.A.N., Yamaguchi, M., Sasa, H. and Kouzuki, H. (2015) Development of QSAR models using artificial neural network analysis for risk assessment of repeated-dose, reproductive, and developmental toxicities of cosmetic ingredients. *J. Toxicol. Sci.*, **40**, 163-180.
 23. Sakuratani, Y., Sato, S., Nishikawa, S., Yamada, J., Maekawa, A. and Hayashi, M. (2008) Category analysis of the substituted anilines studied in a 28-day repeat-dose toxicity test conducted on rats: Correlation between toxicity and chemical structure. *SAR QSAR Environ. Res.*, **19**, 681-696.
 24. Przybylak, K.R., Schultz, T.W., Richarz, A.-N., Mellor, C.L., Escher, S.E. and Cronin M.T.D. (2017) Read-across of 90-day rat oral repeated-dose toxicity: A case study for selected β -olefinic alcohols. *Comput. Toxicol.*, **1**, 22-32.
 25. Judson, R.S., Martin, M.T., Patlewicz, G. and Wood, C.E. (2017) Retrospective mining of toxicology data to discover multispecies and chemical class effects: Anemia as a case study. *Regul. Toxicol. Pharmacol.*, **86**, 74-92.
 26. Vinken, M., Pauwels, M., Ates, G., Vivier, M., Vanhaecke, T. and Rogiers, V. (2012) Screening of repeated dose toxicity data present in SCC(NF)/SCCS safety evaluations of cosmetic ingredients. *Arch. Toxicol.*, **86**, 405-412.
 27. Batke, M., Escher, S., Hoffmann-Doerr, S., Melber, C., Messinger, H. and Mangelsdorf, I. (2011) Evaluation of time extrapolation factors based on the database RepDose. *Toxicol. Lett.*, **205**, 122-129.
 28. Russmann, S., Kullak-Ublick, G.A. and Grattagliano, I. (2009) Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr. Med. Chem.*, **16**, 3041-3053.
 29. Przybylak, K.R. and Cronin, M.T.D. (2012) *In silico* models for drug-induced liver injury - current status. *Expert Opin. Drug Metab. Toxicol.*, **8**, 201-217.
 30. Ozer, J., Ratner, M., Shaw, M., Bailey, W. and Schomaker, S. (2008) The current state of serum biomarkers of hepatotoxicity. *Toxicology*, **245**, 194-205.
 31. Zhu, X. and Kruhlak, N.L. (2014) Construction and analysis of a human hepatotoxicity database suitable for QSAR modeling using post-market safety data. *Toxicology*, **321**, 62-72.
 32. Rincon-Villamizar, E. and Restrepo, G. (2014) Rules relating hepatotoxicity with structural attributes of drugs. *Toxicol. Environ. Chem.*, **96**, 594-613.
 33. Cronin, M.T.D., Dearden, J.C., Duffy, J.C., Edwards, R., Manga, N., Worth, A.P. and Worgan, A.D.P. (2002) The importance of hydrophobicity and electrophilicity descriptors in mechanistically-based QSARs for toxicological endpoints. *SAR QSAR Environ. Res.*, **13**, 167-176.
 34. Chen, M.J., Hong, H.X., Fang, H., Kelly, R., Zhou, G.X., Borlak, J. and Tong, W.D. (2013) Quantitative Structure-Activity Relationship models for predicting drug-induced liver injury based on FDA-approved drug labeling annotation and using a large collection of drugs. *Toxicol. Sci.*, **136**, 242-249.
 35. Mulliner, D., Schmidt, F., Stolte, M., Spirkl, H.P., Czich, A. and Amberg, A. (2016) Computational models for human and animal hepatotoxicity with a global application scope. *Chem. Res. Toxicol.*, **29**, 757-767.
 36. Low, Y., Uehara, T., Minowa, Y., Yamada, H., Ohno, Y., Uru-shidani, T., Sedykh, A., Muratov, E., Kuz'min, V., Fourches, D., Zhu, H., Rusyn, I. and Tropsha, A. (2011) Predicting drug-induced hepatotoxicity using QSAR and toxicogenomics approaches. *Chem. Res. Toxicol.*, **24**, 1251-1262.
 37. Muller, C., Pekthong, D., Alexandre, E., Marcou, G., Horvath, D., Richert, L. and Varnek, A. (2015) Prediction of drug induced liver injury using molecular and biological descriptors. *Comb. Chem High Throughput Screen.*, **18**, 315-322.
 39. Zhu, X.W., Xin, Y.J. and Chen, Q.H. (2016) Chemical and *in vitro* biological information to predict mouse liver toxicity using recursive random forests. *SAR QSAR Environ. Res.*, **27**, 559-572.
 40. Schwarz, L. and Watkins, J.B. (2009) *The liver in Toxicology and Risk Assessment: A Comprehensive Introduction* (Greim, H. and Snyder, R. Ed.). John Wiley, Chichester, pp. 216-227.
 41. Jaeschke, H., Gores, G.J., Cederbaum, A.I., Hinson, J.A., Pessayre, D. and Lemasters, J.J. (2002) Mechanisms of hepatotoxicity. *Toxicol. Sci.*, **65**, 166-176.
 42. Yuan, L. and Kaplowitz, N. (2013) Mechanisms of drug-induced liver injury. *Clin. Liver Dis.*, **17**, 507-518.
 43. Williams, D.P., Shipley, R., Ellis, M.J., Webb, S., Ward, J., Gardner, I. and Creton, S. (2013) Novel *in vitro* and mathematical models for the prediction of chemical toxicity. *Toxicol. Res. (Camb.)*, **2**, 40-59.
 44. Spielmann, H., Sauer, U.G. and Mekenyan, O. (2011) A criti-

- cal evaluation of the 2011 ECHA reports on compliance with the REACH and CLP regulations and on the use of alternatives to testing on animals for compliance with the REACH regulation. *Altern. Lab. Anim.*, **39**, 481-493.
45. Ball, N., Cronin, M.T.D., Shen, J., Blackburn, K., Booth, E.D., Bouhifd, M., Donley, E., Egnash, L., Hastings, C., Juberg, D.R., Kleensang, A., Kleinstreuer, N., Kroese, E.D., Lee, A.C., Luechtefeld, T., Maertens, A., Marty, S., Naciff, J.M., Palmer, J., Pamies, D., Penman, M., Richarz, A.-N., Russo, D.P., Stuard, S.B., Patlewicz, G., van Ravenzwaay, B., Wu S., Zhu H. and Hartung, T. (2016) Toward Good Read-Across Practice (GRAP) guidance. *ALTEX*, **33**, 149-166.
 46. 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., Serrano, 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.
 47. Tollefsen, K.E., Scholz, S., Cronin, M.T., Edwards, S.W., de Knecht, J., Crofton, K., Garcia-Reyero, N., Hartung, T., Worth, A. and Patlewicz, G. (2014) Applying Adverse Outcome Pathways (AOPs) to support Integrated Approaches to Testing and Assessment (IATA). *Regul. Toxicol. Pharmacol.*, **70**, 629-640.
 48. Vinken, M. (2015) Adverse outcome pathways and drug-induced liver injury testing. *Chem. Res. Toxicol.*, **28**, 1391-1397.
 49. Meek, M.E., Boobis, A., Cote, I., Dellarco, V., Fotakis, G., Munn, S., Seed, J. and Vickers, C. (2014) New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. *J. Appl. Toxicol.*, **34**, 1-18.
 50. Vinken, M., Landesmann, B., Goumenou, M., Vinken, S., Shah, I., Jaeschke, H., Willett, C., Whelan, M. and Rogiers, V. (2013) development of an adverse outcome pathway from drug-mediated bile salt export pump inhibition to cholestatic liver injury. *Toxicol. Sci.*, **136**, 97-106.
 51. Willett, C., Rae, J.C., Goyak, K.O., Minsavage, G., Westmoreland, C., Andersen, M., Avigan, M., Duche, D., Harris, G., Hartung, T., Jaeschke, H., Kleensang, A., Landesmann, B., Martos, S., Matevia, M., 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*, **31**, 500-519.
 52. Becker, R.A., Patlewicz, G., Simon, T.W., Rowlands, J.C. and Budinsky, R.A. (2015) The adverse outcome pathway for rodent liver tumor promotion by sustained activation of the aryl hydrocarbon receptor. *Regul. Toxicol. Pharmacol.*, **73**, 172-190.
 53. Ellison, C.M., Enoch, S.J. and Cronin M.T.D. (2011) A review of the use of *in silico* methods to predict the chemistry of molecular initiating events related to drug toxicity. *Expert Opin. Drug Metab. Toxicol.*, **7**, 1481-1495.
 54. European Chemicals Agency (ECHA) (2016) New approach methodologies in Regulatory Science, Proceedings of a Scientific Workshop, 19-20 April 2016, Helsinki. ECHA, Helsinki, ECHA-16-R-21-EN.
 55. Przybylak, K.R., Alzahrani, A.R. and Cronin, M.T.D. (2014) How does the quality of phospholipidosis data influence the predictivity of structural alerts? *J. Chem. Inf. Model.*, **54**, 2224-2232.
 56. Przybylak, K.R. and Cronin, M.T.D. (2011) *In silico* studies of the relationship between chemical structure and drug induced phospholipidosis. *Mol. Inform.*, **30**, 415-429.
 57. Nelms, M.D., Mellor, C.L., Cronin, M.T.D., Madden, J.C. and Enoch, S.J. (2015) Development of an *in silico* profiler for mitochondrial toxicity. *Chem. Res. Toxicol.*, **28**, 1891-1902.
 58. Nelms, M.D., Ates, G., Madden, J.C., Vinken, M., Cronin, M.T.D., Rogiers, V. and Enoch, S.J. (2015) Proposal of an *in silico* profiler for categorisation of repeat dose toxicity data of hair dyes. *Arch. Toxicol.*, **89**, 733-741.
 59. Hewitt, M., Enoch, S.J., Madden, J.C., Przybylak, K.R. and Cronin, M.T.D. (2013) Hepatotoxicity: A scheme for generating chemical categories for read-across, structural alerts and insights into mechanism(s) of action. *Crit. Rev. Toxicol.*, **43**, 537-558.
 60. Mellor, C.L., Steinmetz, F.P. and Cronin, M.T.D. (2016) The identification of nuclear receptors associated with hepatic steatosis to develop and extend adverse outcome pathways. *Crit. Rev. Toxicol.*, **46**, 138-152.
 61. Mellor, C.L., Steinmetz, F.P. and Cronin, M.T.D. (2016) Using molecular initiating events to develop a structural alert based screening workflow for nuclear receptor ligands associated with hepatic steatosis. *Chem. Res. Toxicol.*, **29**, 203-212.
 62. Tsakovska, I., Al Sharif, M., Alov, P., Diukendjieva, A., Fioravanzo, E., Cronin, M.T.D. and Pajeva, I. (2014) Molecular modelling study of the PPAR γ receptor in relation to the mode of action/Adverse Outcome Pathway framework for liver steatosis. *Int. J. Mol. Sci.*, **15**, 7651-7666.
 63. Enoch, S.J., Roberts, D.W., Madden, J.C. and Cronin, M.T.D. (2014) Development of an *in silico* profiler for respiratory sensitisation. *Altern. Lab. Anim.*, **42**, 367-375.
 64. Richard, A.M., Judson, R.S., Houck, K.A., Grulke, C.M., Volarath, P., Thillainadarajah, I., Yang, C., Rathman, J., Martin, M.T., Wambaugh, J.F., Knudsen, T.B., Kancherla, J., Mansouri, K., Patlewicz, G., Williams, A.J., Little, S.B., Crofton, K.M. and Thomas, R.S. (2016) ToxCast chemical landscape: paving the road to 21st Century toxicology. *Chem. Res. Toxicol.*, **29**, 1225-1251.
 65. Steinmetz, F.P., Mellor, C.L., Meinl, T. and Cronin, M.T.D. (2015) Screening chemicals for receptor-mediated toxicological and pharmacological endpoints: using public data to build screening tools within a KNIME workflow. *Mol. Inform.*, **34**, 171-178.
 66. Enoch, S.J., Cronin, M.T.D., Madden, J.C. and Hewitt, M. (2009) Formation of structural categories to allow for read-across for teratogenicity. *QSAR Comb. Sci.*, **28**, 696-708.
 67. 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.
 68. Blauboer, B.J. (2003) The integration of data on physico-chemical properties, *in vitro*-derived toxicity data and physiologically based kinetic and dynamic modelling as a tool in hazard and risk assessment. A commentary. *Toxicol. Lett.*, **138**, 161-171.