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A feasibility study: Can information collected to classify for mutagenicity be informative in predicting carcinogenicity?

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ABSTRACT

Carcinogenicity is a complex endpoint of high concern yet the rodent bioassay still used is costly to run in terms of time, money and animals. Therefore carcinogenicity has been the subject of many different efforts to both develop short-term tests and non-testing approaches capable of predicting genotoxic carcinogenic potential. In our previous publication (Mekenyan et al., 2012) we presented an *in vitro–in vivo* extrapolation workflow to help investigate the differences between *in vitro* and *in vivo* genotoxicity tests. The outcomes facilitated the development of new (Q)SAR models and for directing testing. Here we have refined this workflow by grouping specific tests together on the basis of their ability to detect DNA and/or protein damage at different levels of biological organization. This revised workflow, akin to an Integrated Approach to Testing and Assessment (IATA) informed by mechanistic understanding was helpful in rationalizing inconsistent study outcomes and categorizing a test set of carcinogens with mutagenicity data on the basis of regulatory mutagenicity classifications. Rodent genotoxic carcinogens were found to be correctly predicted with a high sensitivity (90–100%) and a low rate of false positives (3–10%). The insights derived are useful to consider when developing future (non-)testing approaches to address regulatory purposes.

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1. Introduction

Carcinogenicity is a complex toxicological endpoint of high concern. At the same time the rodent bioassay currently employed to assess carcinogenic potential is costly to run in terms of time, money and number of animals. Therefore carcinogenicity has been the subject of many efforts to develop *in vitro* and *in vivo* short-term tests, specifically capable of predicting genotoxic carcinogenic potential. The available genotoxicity tests assess the potential of substances to cause cancer or heritable diseases in humans. The data generated is used in both the hazard identification and risk characterization of substances for regulatory and product stewardship purposes.

Hazard identification for genotoxicity mainly relies on *in vitro* studies determining mutagenicity of substances in bacteria and in mammalian cells following an initial review of existing literature and Structure Activity Relationship/Quantitative Structure Activity Relationship (SAR/QSAR) pre-screening. Effects such as DNA damage, formation of strand breaks or adducts are other helpful indicators for genotoxicity. *In vivo* studies are also used to evaluate genotoxic potential further and are typically conducted to put *in vitro* observations into perspective.

Given the many different modes of action for mutagenesis, a number of tests are needed to assess whether a chemical is genotoxic or not with any degree of confidence. When combined appropriately, positive results from mutagenicity tests can be used to predict carcinogenicity. Some modes of actions involved in the cancer initiation step (e.g., epigenetic DNA methylation) remain without experimental data support because no appropriate test systems for their identification have yet been developed. This can potentially bring some limitations to the currently employed strategies for predicting carcinogenesis. There have been a number of efforts to investigate strategies for evaluating mutagenicity both from the perspective of classifying a chemical as a mutagen or in directing further work in the assessment of carcinogenic potential (Zeiger, 1998; Kirkland et al., 2005, 2014; Cimino, 2006; Matthews

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et al., 2006; Benigni et al., 2010). These articles examined the most frequently used *in vitro* and/or *in vivo* genotoxicity assays for their capability to discriminate between rodent carcinogens and non-carcinogens. For the assessment of non-pharmaceuticals, genotoxicity assays have typically been used as part of a three tiered-testing approach, with Tier I *in vitro* testing followed by Tier II *in vivo* genotoxicity testing in somatic cells to determine the biological relevance of chemicals that are positive in the preceding *in vitro* tests. Tier III *in vivo* testing may comprise tests in gonadal cells as well as multigenerational tests. The most common genotoxicity testing batteries include assays that measure gene mutation (i.e., point mutations that affect single genes or blocks of genes), clastogenicity (i.e., structural chromosome aberrations), and aneuploidy (i.e., numerical chromosome aberrations) (Dearfield et al., 2002; Cimino, 2006). Indeed the US EPA's test battery is a three-tiered scheme (Cimino, 2006) where Tier I includes bacterial reverse mutation assays for gene mutations (e.g., Ames tests), Tier II, an *in vitro* mammalian cell gene mutation assay (e.g., mouse lymphoma test), and Tier III comprises either the *in vivo* bone marrow mammalian chromosome aberration or the *in vivo* erythrocyte micronucleus assay. Japan's National Institute of Health Sciences (NIHS), employs a very similar testing strategy. Whilst positive results concerning *in vitro* genotoxicity demonstrate an intrinsic genotoxic activity of a chemical, this is sometimes only observed under extreme culturing conditions or in the presence of high concurrent cytotoxicity and therefore may not be relevant for *in vivo* genotoxicity (Kirkland et al., 2005, 2006). As a result, a high number of "irrelevant positive" results detected by *in vitro* assays (especially chromosomal aberrations) appear not to be confirmed in follow-up *in vivo* assays (EFSA, 2011).

In an effort to improve predictivity, strategic testing has taken the form of Integrated Testing Strategies (ITSs) (Grindon et al., 2006; Combes et al., 2007; Kirkland et al., 2007a,b; Kirkland et al., 2011). The aim of an ITS is to maximize the use of all scientific relevant information and where possible, avoid the use of animal testing. The ITS described in the REACH Technical Guidance (ECHA, 2014) is a case in point.

Recently, Adverse Outcome Pathways (AOPs) which capture information on the causal links between a molecular initiating event, intermediate key events and an adverse outcome of regulatory concern have shown potential in providing a biological context to facilitate the development of mechanistically based Integrated Approaches for Testing and Assessment (IATAs) (which encompasses ITSs) for regulatory decision making (Ankley et al., 2010; Tollefsen et al., 2014). An IATA is a structured approach that integrates and weighs different types of data for the purposes of performing hazard identification, hazard characterization and/or safety assessment of a chemical or group of chemicals. Whilst there is a strong drive to develop AOPs that can be used to inform IATA, the OECD work programme being notable amongst these efforts, using AOPs in such a predictive capacity is still at an early stage of evolution. There are many practical challenges of gathering relevant data to derive and implement IATA and their elements for inclusion into tools, notably the OECD Toolbox.

Previously we introduced an *in vitro*–*in vivo* extrapolation workflow as a means of relating different short term genotoxicity tests together on the basis of their levels of biological organization. This so-called extrapolation workflow was used to facilitate the development of new genotoxicity models in the Tissue Metabolism Simulator (TIMES) platform and to help direct strategic testing (Mekenyan et al., 2012). Two (Q)SAR models, namely for *in vivo* genotoxicity in liver and *in vivo* micronucleus formation in bone marrow were developed. The exercise highlighted a number of practical issues notably the challenges of accounting for metabolic differences between *in vitro* and *in vivo* test systems (Mekenyan et al., 2012). The workflow developed was structured into 3 steps. Step one subdivided chemicals into positive or negative calls based on results from *in vitro* mutagenicity assays. Step two performed a similar categorization based on *in vivo* genotoxicity effects in liver, whilst step three was based on results from *in vivo* micronucleus formation in bone marrow (Mekenyan et al., 2012). The overall product was a five-level framework, where 3 concurrent negative results across the 3 levels of biological organization was denoted Level 1 and 3 concurrent positive results as Level 5.

Given recent efforts in developing AOPs and associated AOP-informed IATA particularly under the OECD work programme (see http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm), this study re-evaluated the *in vitro*–*in vivo* extrapolation workflow by considering the mechanistic basis of each of the test systems. The intent was to create a mechanistically informed IATA where the elements comprised the different short-term tests grouped together on the basis of their test capability. The resulting IATA would then be used to predict the classifications of a test set of carcinogens in accordance with the Globally Harmonized System (GHS) categories for mutagenicity (United Nations, 2013). This exercise is to an extent complementary to one recently performed by Benigni et al. (2013) who investigated the use of assays measuring DNA reactivity (such as Ames) and cell transformation assays to classify carcinogens into International Agency for Research on Cancer (IARC) classes 1 and 2.

2. Materials and methods

A dataset of 162 chemicals gathered as part of the previous publication (Mekenyan et al., 2012) was relied upon during the initial part of this study. The data available for these substances were categorized by their respective test capability. The outcomes for the tests across the levels of biological organization were also reconsidered in light of the test capabilities. The studies were reviewed applying expert scientific knowledge and only studies with data that met the end-point specific criteria were included. If the pattern of data was equivocal (positive and negative result) for same chemicals, the positive data was accepted to be predominant (i.e., acceptance of the worst case scenario).

The test systems included in the dataset originated from the following study types:

- Bacterial reverse mutation test (Ames test) (OECD Test Guideline (TG) 471; Ames et al., 1973; Mortelmans and Zeiger, 2000).
- Mammalian chromosome aberration test (OECD TG 473; Dean and Danford, 1984).
- Mouse lymphoma thymidine kinase locus test (OECD TG 476; Clive et al., 1979; Clements, 2000).
- *In vivo* liver unscheduled DNA synthesis (UDS) (OECD TG 486).
- *In vivo* alkaline single-cell gel electrophoresis (comet) (draft OECD TG 489; Olive and Banath, 2006; Collins, 2004).
- Transgenic rodent gene mutation assay (OECD TG 488; Nohmi et al., 2000; Lambert et al., 2005).
- Mammalian bone marrow chromosome aberration test (OECD TG 475).
- Mammalian bone marrow micronucleus assay (OECD TG 474).
- Rodent dominant lethal test (OECD TG 478; Bateman, 1984; Green et al., 1985)).

To apply the resulting IATA in practice, an exercise to explore how well chemicals could be classified in accordance with the GHS categories for germ cell mutagenicity was undertaken. A test set of 107 unique chemicals were taken from the Istituto Superiore di Sanita, Carcinogen database (ISSCAN) version 4a as extracted from the QSAR Toolbox OECD version 3.2. This was

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