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General discussion and concluding remarks

Cancer hazard identification of chemical substances is essential to predict possible risks for human health. The carcinogenic potential of chemicals can be identified through animal studies, whereby tumor formation is indicative of chemical carcinogenicity. However, animal studies to predict toxicological endpoints such as carcinogenicity are under severe societal pressure. Therefore, development and use of adequate in vitro tests becomes more and more important. As described in Chapter 1, in vitro tests predicting carcinogenic potential of chemicals mainly comprise genotoxicity assays. These assays have the disadvantage that many false positive results are produced, i.e. chemicals that test positive in vitro but appear to be negative in vivo. Since a positive result in vitro usually triggers further testing in vivo, these false positive results unnecessarily increase the number of follow up in vivo tests.

Additionally, by definition carcinogenic chemicals that do not directly induce DNA-damage, i.e. the so called non-genotoxic carcinogens, go undetected in these tests. Only a limited number of in vitro assays focusing on the identification of non-genotoxic carcinogens is available and not each assay is considered adequate to detect carcinogenic potentials.

The goal of the research described in this thesis was to improve testing strategies for cancer hazard assessment. The first objective was to develop an in vitro approach to assess genotoxicity, with the intention to reduce the number of false positive results produced in the in vitro genotoxicity assays commonly used. The second objective was to improve the identification of non-genotoxic carcinogens by using a toxicogenomics approach based on recognizing modes of action. In this final chapter the results are summarized, discussed and directions for future research are given.

Reducing the number of false positive results

Assessment of the genotoxic potential of a chemical substance often requires more than one in vitro test as most in vitro genotoxicity tests do not detect simultaneously (structural and numerical) chromosomal aberrations as well as gene mutations. We demonstrated that chemicals inducing structural chromosomal aberrations or gene mutations can be both detected by using proliferating primary mouse hepatocytes obtained from pUR288 LacZ plasmid-based transgenic mice (Chapter 2). Furthermore, this study showed that features
required for reliable genotoxicity testing as described by Kirkland et al. (Kirkland et al. 2008), such as cells being p53 and DNA repair proficient and metabolically competent, were all present in the proliferating primary mouse hepatocytes. The main question that remained was: does the method described in Chapter 2 lead to a reduction in the number of false positive test results? Recent data (Luijten et al. 2016) showed that a selected set of chemicals known to produce false positive test results were negative under test conditions described in Chapter 2. Taken together, this testing system is a promising tool for genotoxic hazard assessment. However, there are also a few issues to discuss. Most notably, the test system is based on primary mouse hepatocytes and thus still requires animals. Next, the LacZ reporter assay is quite laborious and not every laboratory is able to perform this assay. Nevertheless, under certain circumstances it might be worthwhile to consider the LacZ reporter assay to assess genotoxic potential.

Next to the LacZ reporter assay other innovative genotoxicity tests have been developed. Examples are the human reconstructed skin micronucleus (RSMN) assay (Curren et al. 2006; Mun et al. 2009), the GreenScreen HC genotoxicity assay (Birrell et al. 2010; Hastwell et al. 2006; Walmsley and Tate 2012) and the ToxTracker assay (Hendriks et al. 2012). These assays all have their pros and cons and have been described more extensively in Chapter 1. What they have in common is the goal to reduce the high number of false positive in vitro results, thereby contributing to a reduction in animal testing. Future studies, including more extensive validation studies, will provide insight into how these assays, including the LacZ reporter assay, can contribute to a robust, relevant and efficient assessment of genotoxic hazard.

**Recognizing modes of action of non-genotoxic carcinogens by a toxicogenomics approach**

The second objective was to improve the identification of non-genotoxic carcinogens by using a toxicogenomics approach based on recognizing overlap in gene expression patterns of non-genotoxic carcinogens having the same mode of action.

Studies focusing on this objective are described in Chapter 3, Chapter 4 and Chapter 5. Two different ways to analyze toxicogenomics data were investigated: a supervised and an unsupervised approach. The major difference between these two approaches is the knowledge requirement on modes of action of non-genotoxic carcinogens, which is necessary for the supervised approach.
but not for the unsupervised approach. In the supervised approach (Chapter 3), a set of overlapping genes was selected and assessed for its significance for a pair of chemicals known to have a similar mode of action. This method requires substantial knowledge on modes of action of chemicals. To become applicable for risk assessment purposes, extensive databases are required covering many features of chemicals having different modes of action. The problem with this supervised approach is that chemicals are forced into ‘boxes’, which ignores the fact that chemicals might have several additional modes of action (see also Chapter 5).

The second (unsupervised) method developed within this thesis to recognize modes of action of non-genotoxic carcinogens was called the ‘comparison approach’ (Chapter 3 and Chapter 4). Gene sets consisting of the most significantly regulated genes upon chemical exposure are compared to the gene sets of other (test) chemicals under the same experimental setup. Chemicals that are known to have a similar mode of action are, through this approach, linked to each other. Furthermore, additional information on adjunct modes of action can be obtained through a convincing match with other chemicals. This unsupervised method of exploring gene expressing profiles is, compared to the supervised approach, more objective as it can be applied without having specific knowledge on the mode(s) of action on beforehand. As such, it is better applicable in test strategies for hazard identification.

In Chapter 3 and Chapter 4 chemicals were tested at a single concentration based on cytotoxicity data. By applying identical concentration selection criteria for all chemicals, it was assumed that overt cytotoxic effects could be avoided while testing conditions applied would still be sufficiently robust to detect (carcinogenic) modes of action. However, it was found that changes in expression profiles varied from virtually no response to a change in expression levels of almost 25 percent of all detectable genes. It was therefore recommended to test multiple concentrations per chemical. To investigate the added value of this recommendation a proof of principle experiment was performed using the non-genotoxic carcinogens Cyclosporin A and Tacrolimus, both immune suppressants (Chapter 5). Performing the gene expression profiling study in primary mouse hepatocytes at a single concentration did not result in a common profile. However, by using a concentration range overlapping gene sets for Cyclosporin A and Tacrolimus were observed. Common alterations in gene expression nicely reflected pathways known to be triggered by these
chemicals and, as such, demonstrated the biological significance of this adapted approach.

Another major advantage of the comparison approach is that it not only detects non-genotoxic carcinogens more efficiently, but that it is also applicable to other chemicals having different modes of action. Indeed, in Chapter 4 it was demonstrated that genotoxic chemicals, because of similarity in gene expression profiles, can be categorized.

**Position of the introduced approaches in the carcinogenicity test strategies**

Both the genotoxicity assay and the comparison approach, were developed with the intention to improve risk assessment of chemicals in terms of assessing genotoxic and carcinogen potential. Although these approaches are not ready yet for implementation in risk assessment, a new test strategy for cancer hazard assessment is proposed in which both approaches as developed within this thesis are integrated (Figure 1). It is proposed that the first tier in carcinogenicity testing comprises the use of existing data complemented with *in silico* approaches such as (Q)SARs and read-across (see Chapter 1). Mutant frequency assessment through the *LacZ* reporter assay using proliferating primary hepatocytes (PPH in Figure 1) to detect (reliable) gene mutations as well as chromosomal aberrations is added to the second tier, *i.e.* the *in vitro* genotoxicity tests. Positive results still require *in vivo* confirmation, but the assumption is that, through implementation of the *LacZ* reporter assay, the number of ‘false positives’, *i.e.* positive *in vitro* but negative *in vivo*, will be significantly reduced.

Test chemicals negative in the *LacZ* reporter assay and in other *in vitro* and *in vivo* genotoxicity tests will be subjected to the comparison approach, which is now integrated in the last tier of the proposed test strategy. Through comparison of gene expression profiles of (non-genotoxic carcinogenic) chemicals insights will be obtained on possible modes of action that are known to be involved in carcinogenesis. Considering the fact that more and more knowledge on modes of action in carcinogenesis will become available, it seems to be highly likely that the comparison approach will trigger dedicated further testing.

**Future prospective**

In the described studies within this thesis, the primary cell system used consisted of primary mouse hepatocytes. This cell system has multiple advantages, with its capacity to actively perform biotransformation being most important.
However, not all chemicals will be detected using mouse hepatocytes, and therefore other cell systems should also be considered to detect (other) carcinogenic modes of action. As shown in Chapter 4, mouse embryonic stem cells complement primary hepatocytes. Next to this, the introduction of human derived cell systems will be advantageous, in this way that it may better reflect the human situation. Overall, multiple cell systems will be required for robust and reliable prediction of modes of action of chemicals.

**Figure 1** Proposed scheme for carcinogenicity testing.
Abbreviations: PPH proliferating primary hepatocytes, MLA mouse lymphoma assay, MN micronucleus test, CA chromosome aberration test.

The mutation frequency assessment in proliferating primary hepatocytes (PPH) is added to the battery of *in vitro* genotoxicity tests. Next, the comparison approach is incorporated in the fourth tier to further investigate the carcinogenic mode of action of non-genotoxic chemicals.

For hazard identification of unknown chemicals ‘omic’ technologies, like transcriptomics, metabolomics and proteomics, become more and more important. Data derived from these studies can be used in different ways. One
approach is biomarker identification to categorize chemicals into different chemical classes, *e.g.* genotoxic versus non-genotoxic. However, with the knowledge that non-genotoxic carcinogens act through different mechanisms, a mode of action based approach becomes increasingly important. Assuming that such an approach is based on comparisons to existing data, extensive libraries of expression profiles are needed which can be used as reference when obtaining knowledge on modes of action of unknown (carcinogenic) chemicals. Ideally, such libraries consist of gene expression profiles of chemicals derived from various *in vitro* systems at multiple concentrations. In addition, other profiles, like proteome, metabolome, DNA methylation, or miRNA, can be registered as well.

An example of such a library is the comparative toxicogenomics database (Davis et al. 2015). This database exists for ten years already and it comprises over 10,000 chemicals, 40,000 genes and various species. Within this database it is possible to connect chemicals, genes/proteins, diseases, taxa, Gene Ontology (GO) annotations and pathways. Additionally, the knowledge on epigenetic mechanisms involved in tumor formation is increasing (Greally and Jacobs 2013; Herceg et al. 2013). It is therefore reasonable to assume that assays for detecting changes in DNA methylation, histone modification and miRNA associated with non-genotoxic carcinogens will become available in the coming years. Also, rapid evolvement of advanced sequencing approaches might offer the next step in hazard identification of chemicals. In the meantime it is worthwhile to further develop the mechanism-based comparison approach.
Chapter 6

References

Birrell L, Cahill P, Hughes C, Tate M, Walmsley RM (2010) GADD45a-GFP GreenScreen HC assay results for the ECVAM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests. Mutat Res 695(1-2):87-95


