The use of transcriptomics data in detecting non-genotoxic carcinogens

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English summary

Risk assessment of chemicals is essential to assure safe use for public health and environment. One of the toxicological features that can be identified in a risk assessment procedure is the carcinogenic potential of a chemical. For this purpose, *in vivo* studies are performed, 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. The aim of the research described in this thesis was to develop *in vitro* tests to improve testing strategies for cancer hazard assessment of chemicals, to reduce the use of *in vivo* experiments.

In **Chapter 1** the principles underlying chemical carcinogenicity and cancer risk assessment are introduced. To investigate the strengths and weaknesses of current test strategies to assess carcinogenic potential, a summary of available *in vitro* and *in vivo* tests detecting genotoxic as well as non-genotoxic carcinogens has been provided. Our overall conclusion is that to lower the use of *in vivo* studies and to improve the detection of non-genotoxic carcinogens, innovative *in vitro* tests should be developed. Therefore, the scope of this thesis was twofold. The first aim was to develop an improved *in vitro* approach to assess genotoxicity, with the intention to reduce the number of misleading positive test results. The second aim was to improve the identification of non-genotoxic carcinogens by using *in vitro* transcriptomic-based approaches based on recognizing various modes of action of non-genotoxic carcinogens.

Chapter 2 is addressing the first aim: the development of an improved *in vitro* approach to assess genotoxicity, with the intention to reduce the number of misleading positive test results. Here, the suitability of proliferating primary mouse hepatocytes for routine *in vitro* genotoxicity testing was investigated. Besides elaborate characterization of the cell system, comprising biotransformation capacity and p53 responsiveness, four genotoxic chemicals were successfully tested as a proof-of-principle. The major advantage of this approach was that gene mutations and chromosome rearrangements could be determined simultaneously in one test system. Future studies, including more extensive validation studies are required to provide insight into how assays,

like the *LacZ* reporter assay, can contribute to a robust, relevant and efficient assessment of genotoxic hazards.

Chapter 3, 4 and **5** are addressing the second aim of this thesis, namely improving the identification of non-genotoxic carcinogens based on recognizing various modes of action of this group of chemicals. Since many, if not all, non-genotoxic carcinogens will dysregulate gene expression, a transcriptomics approach was applied. In **Chapter 3** we provided a proof-of-concept by demonstrating that chemicals that are having the same mode of action show the same alterations at the gene expression level. For this purpose, primary mouse hepatocytes, cultured in a sandwich configuration, were exposed to a set of sixteen non-genotoxic carcinogens, consisting of eight pairs of chemicals of which each pair represents a different mode of action. A supervised as well as an unsupervised approach was applied to categorize the tested chemicals. In the supervised approach a set of overlapping genes was selected and was 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. 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. Therefore, we also applied an unsupervised approach.
Using the unsupervised approach, most significant regulated genes of each chemical individually were 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 linked to each other. Furthermore, additional information on adjunct modes of action can be obtained through a convincing match with other chemicals. Compared to the supervised approach the unsupervised approach appears to be more robust and objective and, therefore, in next chapters we investigate further the use of the unsupervised method.

In **Chapter 4** the unsupervised comparison approach was further refined. Extra chemicals, genotoxic as well as non-carcinogenic, were added to the set of chemicals to test the robustness of the comparison approach. Furthermore, as second *in vitro* system, mouse embryonic stem cells were introduced, which proved to be a useful add-on to the hepatocytes.
Whereas hepatocytes were suitable for detecting non-genotoxic carcinogens, embryonic stemcells were efficient in the detection of genotoxic chemicals.

This study showed that unsupervised comparison approach is not limited to a certain cell system, which makes this approach attractive for risk assessment of chemicals in general.

The experiments described in **Chapter 3** and **4** are based on single test concentrations per chemical. However, we acknowledge that this might result in an underestimation of the outcome. Therefore, in **Chapter 5**, we studied for two chemicals, Cyclosporine A and Tacrolimus, the impact of a concentration range, and we provided recommendations for selecting test concentrations. Expression profiles were similar to such a degree that a match between the two model compounds was found for multiple concentrations tested, whilst such a match was absent in studies in which only single concentrations of test compounds were used. Furthermore, we demonstrated that the in the unsupervised comparison approach applied gene sets reliably reflect the perturbed pathways and biological processes derived from the complete expression profiles. Thus, the use of concentration ranges within the unsupervised comparison approach shows increased potential for modes of action detection

In **Chapter 6** we provided a general discussion of the obtained results including the additional value and possible position of the novel approaches in carcinogenicity testing. Strengths and limitations were addressed and future perspectives were provided.
Referring to the first aim, regarding reducing the number of misleading positive *in vitro* test results, the *LacZ* reporter assay could successfully contribute to a robust, relevant and efficient assessment of genotoxic hazard. Addressing the second aim, transcriptomic approaches are of added value in the detection of modes of action of non-genotoxic carcinogens. In addition, the comparison approach is also suitable to detect toxicity of chemicals in general.