Dynamics and regulation of the oxidative stress response upon chemical exposure

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ENGLISH SUMMARY

There is an increasing number of chemicals that enter the society, including drugs, environmental chemicals and cosmetics, combined also referred to as the chemical exposome. Likewise there is an increased hazard for chemically-induced health effects. Chemicals can interfere with biological systems and induce compound-specific responses, either related to the pharmacological on- or off-target effects. In particular compounds with (in)direct electrophilic reactivity are of direct harm to cells. Such compounds will interfere with normal cellular physiological processes and activate adaptive cellular stress responses that try to repair the cellular injury. Understanding the fundamental relationship between activation of these cellular stress responses and ultimate onset of cytotoxicity can be used for constructing mechanism-based biomarkers.

In this study we focused on the oxidative stress response, also known as the Nrf2 pathway named after its transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). The Nrf2 pathway plays a role in protection against chemicals with soft electrophile properties and that propagate the generation of reactive oxygen species (ROS), which may lead to oxidative stress with cell death as an ultimate outcome. Recently it became clear that besides genes and proteins also microRNA, a type of small non-coding RNA, play an important role in the regulation of stress response pathways.

A literature study was conducted to obtain information related to microRNA up/down regulation after exposure to a diverse panel of chemicals. Results of this study are presented in **Chapter 2**. We found that the microRNAs most frequently found to be dysregulated are also found to play a role in various diseases linked to chemical exposure. Although microRNA expression changes show great potential as biomarkers, questions concerning biomarker robustness, biological functionality and adverse outcome causality of the response still remain. It remains unclear whether these microRNAs detected in the blood or other body fluids, represent a mechanistic biomarker that could reflect on the mode of action regarding the toxicity of a chemical compound. So far, biomarkers measured in the blood have not per se been discovered in relation to their mode of action. Therefore, the question still has to be answered whether or not the rise of certain microRNAs found in the blood has indeed a functional role, or that the change in expression is just the result of tissue damage. However, microRNAs which are known to be strongly related to a specific organ, like miR-122 for the liver, can provide valuable information about which organ is damaged upon exposure to a chemical or during disease.

Defining whether the candidate microRNA biomarkers play an integral functional role in disease mechanism is a difficult task. One single microRNA might have hundreds of different targets and, as a consequence, the role of the microRNA might differ between different tissues. Furthermore, a microRNA can both increase or diminish the stress response depending on their involvement in the inhibition of negative regulators in a positive feedback loop or as part of a negative feedback loop, respectively. New emerging techniques like droplet digital PCR and measurements in different biological matrices like extracellular vesicles enable more in depth research on microRNAs.

In order to investigate the role of microRNAs on the Nrf2 pathway, we screened a panel of ~2600 individual microRNA mimics using an endogenous Nrf2 target Srxn1-GFP HepG2 reporter cell line in combination with high throughput live confocal imaging after treatment with CDDO-Me. In this research, presented in **Chapter 3**, we identified a panel of 16 microRNAs that enhance (including miR-3165, miR-1909-3p, miR -1293, and miR-6499-3p) and 10 microRNAs that inhibit (including miR-200a-3p, miR-363-3p, miR-502-5p, and miR-25-3p) CDDO-Me-induced Srxn1-GFP expression. These microRNAs might be relevant biomarkers and/or provide alternative therapeutic modalities to modulate Nrf2 pathway activity in health and disease. In conclusion, this study for the first time elucidated the spectrum of microRNAs that target the Nrf2 signalling pathway.

The microRNAs found in our study that enhance or inhibit the Nrf2 pathway might provide opportunities for microRNA therapeutics that target the Nrf2 pathway. Enhanced Nrf2 pathway activation is considered as a pro-oncogenic pathway. Therefore, to obtain information concerning the primary response upon microRNA expression changes, there is a need to perform further temporal analysis of the transcriptional changes after microRNA transfection. MicroRNAs which are able to inhibit the Nrf2 pathway might be used to make cancer cells more vulnerable for chemo- and radiotherapy. MicroRNAs enhancing the Nrf2 pathway might be used to enhance a person's protection against oxidative stress. Additional research on the dose response and long term safety profile of our candidate Nrf2 modulating microRNAs is required. Caution, however, has to be made to link microRNAs which alter the Nrf2 response directly to Nrf2, as microRNAs can regulate the Nrf2 pathway via different ways: 1) by affecting the nuclear translocation of Nrf2, and 2) by influencing the expression of Nrf2, regulating the upstream mediators of Nrf2 and modulation of *KEAP1*. Furthermore, also redox stress itself can alter the microRNA biogenesis and processing pathway leading, for example, to altered redox signaling and disease mechanisms.

As in daily life not only single exposure to a chemical takes place, but also repeated exposure to a chemical substance may occur. However, our general understanding of the dynamics of cellular stress response pathway activation in repeated treatment scenarios is limited. In order to study the dynamics of the Nrf2 pathway upon repeated exposure we used confocal microscopy in combination with HepG2-GFP reporter cells. These cells were repeatedly exposed to a concentration range of diethyl maleate (DEM) and tert-butylhydroquinone (tBHQ). The outcome of this study is described in **Chapter 4**. Interestingly, we found that the amount of Nrf2 in the nucleus after a second treatment after 24 h was lower than the amount measured after the first exposure with the same concentration, indicating that the Nrf2 response is adaptive. However, the amount of Srxn1 measured in the cell was three-fold higher compared to the first treatment. Although more research is needed to unravel the precise mechanism, it is clear that repeated exposure testing will add valuable extra information in testing the safety of a chemical or drug. Furthermore, as indicated by several studies, preconditioning might be used for therapeutic approaches using low non-toxic concentrations of a Nrf2-pathway inducing chemical to protect against exposure to a toxic concentration. However, challenge remains in finding the optimum dosing regimen: dose per treatment, time between treatments and number of repeated treatments.

As activation of the Nrf2 pathway upon chemical exposure might indicate oxidative damage, we hypothesized that the ability of chemical compounds to induce oxidative stress and to stimulate a Nrf2 mediated oxidative stress response can be determined by the temporal dynamics of the stress response proteins combined with transcriptomic expression profiles.

To test this hypothesis, a panel of different phenolic compounds was used that were either redox cycling phenols, alkylated phenols, or non-redox cycling phenols. Outcomes of this study are described in **Chapter 5**. We integrated high throughput transcriptomics using targeted RNA sequencing of primary human hepatocytes (PHH) and HepG2-WT and HepG2 Nrf2-GFP and Srxn1-GFP reporter cell lines. Using a panel of five pro-oxidants, including CDDO-Me, sulforaphane, *tert*-butylhydroperoxide, etacrynic acid and diethyl maleate, we identified a panel of five Nrf2 target genes that could define oxidative stress potential: *AKR1B10*, *SRXN1*, *ABCC2*, *AKR1C3* and *NQO1*. Next, we measured the response of these five genes after exposure to different concentrations of the three types of phenols. We found that measurement of these five genes could be used to discriminate between the three types of phenolic compounds. Furthermore, we demonstrated that integration of high throughput HepG2 Nrf2 pathway reporter cell line data with transcriptomics data from HepG2 and PHH, provides valuable mechanistic information on mode-of-action of structural similar phenolic compounds and their biological similarity.

In summary, the research described in this thesis provides additional information concerning the dynamics of the Nrf2 pathway upon single and repeated exposure and the use of key players involved in this pathway as, part of a panel of, mechanistic biomarkers of chemical exposure as well as disease. Especially microRNAs might add value to these biomarker panels. Furthermore, obtaining more knowledge concerning their role in stress response pathways enables elucidation of the exact mode of action of these stress response pathways.