Systems microscopy to unravel cellular stress response signalling in drug induced liver injury

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Chapter 8

Overall discussion and conclusions.

1. BAC-GFP reporter systems for prediction of DILI.

In this thesis I have described the application of a diverse set of HepG2 BAC-GFP reporters that represent different adaptive stress response pathways that are implicated in toxicity. In Chapter 3 we first established and characterized the different reporter systems. The rationale to establish this panel of HepG2 BAC reporters for the oxidative, endoplasmic and DNA damage stress responses was to enable the monitoring of these pathways at the 'sensor', 'transcription factor' and 'downstream target' level. The various reporters were all characterized and optimized with a set of reference compounds. In doing so the stress specific compounds in combination with the different stress specific BAC reporters readouts lead to a perfect co-clustering following unsupervised hierarchical clustering.

The next important step for reporter validation was to test a set of clinically relevant DILI-drugs. The rationale for their selection was based on their transcription-level responses of the actual BAC reporter target genes in primary human hepatocytes which were available through the TG-GATES microarray dataset. We successfully established that the primary responses of these selected DILI-drugs involve adaptive stress responses that correlate with the transcriptional responses observed for PHH: 100 % concordance for the Srxn1-GFP response, 80 % concordance for p21-GFP and 50 % for CHOP-GFP and BiP-GFP.

Encouraged by these initial results a compound library of ~170 DILI related drugs including control reference compounds was screened using the Srxn1-GFP, CHOP-GFP and p21-GFP BAC reporters at 1, 5, 10, 50 and 100 C-max with live-cell imaging for 24 hours.

As expected the response levels, the dose-responses and time-dynamics varied within the entire DILI compound set. For many compounds the responses were low to moderate and difficult to detect. However, with a binary method by determining the percentage of GFP-positive cells based on control-level background offsets, the sensitivity of the readout was greatly improved. Yet, this alternative analysis affected the overall dynamic range. We then applied unsupervised clustering of the primary adaptive stress responses time course profiles together with several viability features (see Chapter 7, Figure 4). This allowed the identification of DILI compounds with similar adaptive stress response activation as well as similar time dynamic profiles of these responses. These profiles can be used to determine the primary stress type for compounds that induce multiple stress responses as the corresponding reporter will be activated first. Thus, already from such basic observations general insights into the mechanisms of toxicity can be inferred based on stress type and co-clustering of reference control compounds with known mechanism of action. As all concentration-compound combinations are represented in the clustering of multi-variate time responses the point of departure for the different stress responses can be determined. In addition because of the inclusion of the viability markers technical and

biological outliers can easily be detected, either in an automated fashion or by manual inspection of clustered results.

A next step in such multivariate analysis is often reevaluation from a different angle by data transformation and/or dimension reduction methods. In the current context removing the time dimension was performed by transformation of the time response curves to dose response curves, which serves to decrease the complexity but also enables inclusion of additional features and for example single time point cell death parameters. Visualizing the same dataset from a dose-response angle sets the focus on the critical onset of toxicity based on concentrations. Different sensitivity and dynamic range measures of the adaptive stress response levels together with viability features such as cell count, cell speeds, nuclei area and the cell death features apoptosis-positive-fraction and necrosis-positive-fractions shows a broad cell biologic response to each compound in a dose-response context. Cellular adaptation to stress typically occurs in the form of activation of damage specific adaptive stress responses followed by a decrease in several cell viability markers and finally results in cell death. The point-of-departure (PoD) can be defined in parallel with the point-of-no-return (i.e. cell death). Thus, the compound-specific concentration of these events and, importantly, the concentration differences between these two events are key compound features which pertain how potent a certain compound induces a certain type of stress and how well adaptive programs can combat this stress before cell death occurs, respectively.

Together, our experiments indicate the application of BAC-GFP reporters in the safety assessment of chemicals and their potential for the identification of DILI liabilities.

2. Application of BAC-GFP reporter in the understanding of mechanisms of DILI.

A major theme in DILI is the involvement of immune mediated mechanisms including TNFα induced pro-inflammatory signalling [38]. In Chapter 5 I have studied the role of different stress response pathways and their relationship to DILI in the context of TNFα signalling. As a first step, transcriptomic profiling of the response of HepG2 cells treated with TNFα and co-treated with either one of the hepatotoxicants diclofenac or carbamazepine revealed the involvement of two adaptive stress response pathways: the endoplasmic reticulum (ER) stress/translational initiation signalling and nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) antioxidant signalling. Indeed, data mining of the TG-GATES dataset revealed the importance of these adaptive stress responses as the majority of DILI classified drugs affected the corresponding transcription levels in primary in hepatocytes. Following these findings we set out to investigate if these adaptive stress responses have a critical role as drug-induced toxicity pathways that act in synergy with TNFα to cause cell death of liver HepG2 cells.

With the established adaptive stress response BAC-GFP reporter platform a more detailed mechanistic investigation was performed into the involvement of the Nrf2 antioxidant response and ER stress/UPR in TNFα/drug-induced synergy in DILI. Diclofenac and carbamazepine both caused the activation of the ER-stress BAC-GFP HepG2 reporters ATF4-GFP and CHOP-GFP. This ER-stress activation was shown to be independent of TNFα and involved protein kinase R-like ER kinase (PERK). The diclofenac and carbamazepine TNFα synergistic cell death was inhibited when PERK was downregulated. Also siRNA knock down of CHOP inhibited this synergistic cytotoxic onset. Induction of CHOP expression was dependent on the translational initiation factor EIF4A1; and knock down of EIF4A1 also inhibited the cytotoxic response. Thus, ER stress caused by these

two DILI compounds, directly interacts with the pro-apoptotic pathway downstream of the TNF receptor. In contrast to the effect of ER stress, activation of the KEAP1/Nrf2 antioxidant stress response pathway, as evidenced by the induction of the Srxn1-GFP reporter by these two DILI compounds, acted cytoprotective. Thus, knock down of Nrf2 enhanced the synergistic cell death. These data demonstrate the application of the BAC-GFP reporters in mechanistic understanding of DILI compound toxicities.

3. Interaction of Nrf2 signalling and NF-κB signalling in DILI.

The above results suggests that the interaction with TNF signalling is essential for DILI liabilities. Since many DILI compounds also activate the Nrf2 response, we further investigated this interaction and the possible predictivity of these two signalling pathways (Chapter 6). First, a systematic investigation of the Nrf2-mediated oxidative stress signalling and NF-κB-mediated inflammatory signalling pathways in PHH using the TG-GATES microarray dataset revealed a strong correlation between these two pathways in relation to DILI. Strong activation of the Nrf2 pathway was associated with a downregulation of the inflammatory pathway. Following this observation these findings were translated into HCI. Here we combined the HepG2 oxidative stress response reporter Srxn1-GFP and a HepG2 reporter for p65-GFP, a subunit of NF-κB on which we published before. The latter reporter demonstrates nuclear oscillation behavior upon treatment with TNFα. We found that when DILI compounds demonstrate strong activation of the Srxn1-GFP reporter, this was associated with suppression of TNFα-induced NF-κB translocation to the nucleus. Activation of NF-κB signalling by TNFα did not suppress the oxidative stress response in HepG2 cells. Of interest, mainly compounds associated with a strong Nrf2-activation sensitized towards TNFα-mediated cell death. This may indicate that in particular compounds that have a strong cellular stress response are liable for such an interaction. As mentioned above, such an oxidative stress response activation was initially a protective adaptive response, since knock down of Nrf2 enhanced this sensitivity, while on the contrary knock down of the negative regulator of Nrf2, Keap1, acted cytoprotective. Thus, we propose the Nrf2-mediated oxidative stress response as induced by DILI compounds coincides with NF-κB suppressions and that this results in liver cells that are more sensitive to pro-apoptotic signalling induced by immune cell released cytokines such as TNFα. We anticipate that by monitoring in early drug development the effect of novel drug candidates on oxidative stress activation (Srxn1-GFP reporter), TNFα signalling (p65-GFP reporter oscillation) and synergistic cell death, may reduce the number of compounds that will enter the clinic with DILI liabilities.

4. Implications of the BAC-GFP reporters in understanding DILI and assessment of chemical safety.

In Chapter 3 we determined that monitoring only three adaptive stress response pathways using endogenously regulated BAC-GFP reporter HepG2 cells together with several cell-viability features using HCI, allows the identification of a biological “fingerprint”. This was evidenced by the co-clustering of highly structural similar compounds: oligomycin A and oligomycin B, CCCP and FCCP; as well as compounds with similar mode-of-action: thapsigargin, tunicamycin and brefeldin A. Does this infer that these are *the* major adaptive stress response pathways in cellular defense and that these pathways are key in DILI? Yes and no. Oxidative stress has been shown in numerous

studies to be the main source of damage implicated in DILI [328]. ER-stress is emerging as another important player in DILI, especially the role of cell death signalling via PERK and CHOP resulting from prolonged ER-stress [334, 335]. DNA damage is known in several cases of DILI, but this pathway is general not a main player in DILI, as was also indicated by our DILI screen results [336, 337]. In our DILI screen in Chapter 7 we also observed that a large set of compounds did not induce any of the three adaptive stress response reporters; this indicates that it is likely that different types of stress and signalling do occur in DILI. Regardless, as a proof-of concept we demonstrate that both the time-dynamics and concentration-response profiles that can be obtained from HCI of the reporter platform provides detailed information on: 1) the primary mode-of-action; 2) the resultant secondary stress responses; 3) the point-of-departure (i.e. lowest observed effect level at which an activation of a response occurs); 4) the consequently point-of-no-return at which *maladaptation* or cell death occurs. The HCI data is multi-dimensional and therefore complex automation in the form of an analysis pipeline which displays such data in heatmaps can simplify the analysis and interpretation of such data. At this point adaptive stress response fingerprints that are associated with diverse DILI classes would need to be established first.

Although not included in our screening efforts so far, the development of the inflammatory signalling BAC-GFP reporters (Chapter 5) now allows inclusion of the highly relevant NF-κB signalling pathway in our HCI DILI screening efforts. The NF-κB pathway acts as a pivotal survival signalling node but can also contribute to or initiate cell death pathways. In the lab, we have also established BAC-GFP reporters for several downstream targets of NF-κB, including A20-GFP and ICAM-GFP, respectively early and late target genes. Our HCI BAC-GFP reporter panel is an ideal platform to investigate the interaction between multiple pivotal pathways related to DILI (i.e. the Nrf2 antioxidant/ oxidative stress response and the NF-κB pathway, in Chapter 6). The possibility to quantify the Nrf2 mediated oxidative stress response together with the oscillation patterns of NF-κB in live cells following exposure to DILI-drugs and inflammatory signalling activation by TNFα is only possible with the high temporal and spatial resolution that HCI provides. The sensitivity required to detect incremental changes in endogenously regulated Srxn1 levels as the result of slight increase in Nrf2-mediated oxidative stress signalling and more importantly the detection of subtle changes in NF-κB nuclei-cytosol oscillation patterns in single cells is only possible using HCI, in particular using state-of-the-art optics and detection which are available on confocal microscope systems that we have used in our investigations.

The complexity and the diversity of responses of biological systems to chemical exposure is evident. The involvement and interconnectivity of multiple pathways in DILI has been demonstrated in previous work and in this thesis. More importantly, however, is our demonstration of how several key regulatory components can be used to determine the primary response of cells to chemical exposure. Since adaptive stress responses are upstream in these biological regulatory programs, exploring additional adaptive stress responses will prove to be of high value to create more BAC-GFP reporters for their integration in HCI.

5. Improvements to be considered.

Some key features on the implementation of the current HCI-imaging based reporter platform are warranted. At this stage, as a proof-of-concept, we focused on several reporter cell lines that were

based on HepG2 cells. This cell line was selected since it is easy to genetically modify, it is robust and easy to maintain and transfer to a commercial setting. However, the HepG2 cell line has limited biotransformation capacity which is required for reactive metabolite formation. Secondly, these cells are grown in two-dimensional conditions; under these conditions the cells divide rapidly which is not representative for the in vivo conditions. The cells lack the other differentiated features of normal primary cultured hepatocytes including bile-duct formation. Thirdly the culture system lacks the multi-cellularity which is often key in the pathophysiology of DILI. Thus, the liver resident macrophages, Kupffer cells, pit cells, stellate cells, sinusoidal cells and cholangiocytes all contribute in their own way to DILI. Future work should involve both the culturing of our reporter cell lines in 3D and/or in co-cultures with other liver cell types. Indeed, we have been successful to culture the various BAC-GFP HepG2 reporters as spheroid cultures in a 3D matrigel environment. The phenotype of the different reporters in 3D spheroids mimics the phenotype as observed for wild type HepG2 3D spheroids.

Additional we have started to explore the use of human induced pluripotent stem cells. Inclusion of reporters in these cells will involve the CRISPR/Cas9 technology. hIPSC will allow the differentiation of the ultimate fluorescent reporter cells into multiple cell types including hepatocyte-like cells followed by integration in high content imaging.

An additional shortcoming of our current model is the lack of prolonged repeated dosing possibilities for up to several weeks. A repeated dosing scheme would be in particular interesting when considering the concepts of sensitization or desensitization after onset of the adaptive stress response programs. Will repeated dosing create a new steady state of the stress response, thus ensuring true adaptation? Or will repeated drug exposure create a sustained ever increasing stress response that is doomed to end with a point of no-return? These are questions that are difficult to answer using our 2D culture BAC-GFP reporter approach. The above already mentioned non-proliferative 3D HepG2 spheroid cultures would be ideally suited for this purpose [14]. Such strategies are currently being explored in our lab and are beyond the scope of this thesis.

A final current shortcoming worth mentioning are the costs, time and expertise involved in setting up the required hardware and software infrastructures. Moreover the complexity of the datasets requires rigid image and data analysis protocols to obtain reliable and reproducible end-of-the-pipeline results so that the risk assessor might actually use such data.

6. Adaptive stress responses and cell biology: implications for prediction.

In this thesis I focused on the application of reporters that reflect cellular adaptation programs. In view of the adaptation versus the point-of-no-return one can consider cellular systems in a continuous dynamic state of acquiring damage and repairing damage with the ultimate goal to maintain cellular homeostasis. One step further leads us to a hypothesis where all cellular and organ adversity is the inability of biological repair systems to cope with the acquired damage. The biological repair mechanisms consist of intrinsic repair such as during DNA transcription coupled repair, normal cellular protein turnover through proteosomal degradation, or malfolded proteins that are rescued by chaperones already present in cells under normal physiological conditions. An adaptive stress response program is then the detection of damage and signalling response of biological systems to increase these intrinsic repair mechanism to a specific damage type to a higher level. If this is true, how can we use reporter based systems of the adaptive stress response

pathways that control these repair mechanisms to better predict DILI? The employed approach of Chapter 7 of clustering compounds by similarity of their corresponding biological fingerprints has shown promise in the enrichment in most-DILI concern compounds: they caused a strong activation of the Nrf2-mediated oxidative stress response and ER-stress/UPR response within a 24 hour time period. However, other most-DILI concern compounds did not induce one of the adaptive stress response programs in this time period. Our current simplified working hypothesis is that all DILI is the result of failed adaptation programs due to overwhelming damage. Since this is not observed this leads to several explanations. Firstly, the 2D HepG2 cells are immortalized dedifferentiated liver cells that lack many physiological and structural properties of a the actual liver, and, thus, limiting the coverage of 'all' pathophysiological programs involved in DILI. Secondly, only three adaptive stress programs have been monitored leading to the possibility that certain damage types are overlooked in the current reporter platform. We need to integrate other stress programs in our platform as well. Thirdly, the toxicodynamics is likely different than the physiological dynamics in the human liver and this will for certain compounds likely be key in the compound induced toxicity. Repeated dosing and longer time courses for accumulation of the compound or its metabolites have been shown to be key mechanisms [14, 15]. And, fourthly, compounds that directly interact with cell death or survival pathways and sensitize cells to stress induced cell death could lead to adversity without inducing adaptive stress response pathways to higher than significantly detectable levels.

7. Practical implementation of the technologies.

We believe the current proof of concept methodology can directly be applied in an industrial setting. Several pharmaceutical companies have jointly performed promising pilot studies on DILI related compound sets. On one hand the BAC-GFP reporter platform can be used for early screening for the purpose of hazard identification for early lead termination. Such a system could be simple: e.g. a single/multiple time point(s) and single/multiple concentration(s) on a small set of primary adaptive stress response reporters, including oxidative stress and ER-stress reporters. Compounds that induce these stress types at the pre-determined concentration can be terminated simply by the notion that they induce these pathways at a too low concentration. Such an approach might lead to identification of false positives. This may be undesirable because compounds may have optimal pharmacodynamics and pharmacokinetic properties or there may not be that many compounds to start with. In this case a more detailed screening effort can be performed using multiple reporters and time points to gather more information as to what constitutes a high-hazard high-risk biological fingerprint. Such a high risk-fingerprint includes the point of departure concentration for several key adaptive stress response pathways, the concentration where adaptation changes to maladaptation and cell death. To obtain such a fingerprint, or multiple fingerprints, two strategies should be considered. Firstly, the expert knowledge based method where the data such as activation of (multiple) pathways at certain point of departures are interpreted in the context of the compound application for hazard identification or even risk assessment. This would however require much experience in the understanding and interpretation of such data; without this prior knowledge the reporter screening concept is not likely to be incorporated in toxicity screening programs in the first place.

The second strategy would be a supervised clustering methodology where a large set of DILI compounds versus a large set of non-DILI compounds is used to optimize a fingerprint that functions as classifier for these two groups. Such a classifier would evolve as more data becomes available, and indeed the foundations for such a biological fingerprint have already been made in this thesis. The difficulty does however remain in the ground-truth classification of DILI compounds as well as the complexity and variety of the mode-of-action of different DILI compounds. With such a complexity we would propose to simplify as much as possible the application of the reporter platform. One can envision an initial screen with single time points and a small range of concentrations in several HepG2 BAC-GFP reporters where only strong evidence is used to terminate sets of compounds flagged as hazardous by their corresponding fingerprints. This would be followed by a more elaborate screen with the inclusion of the time dynamics and concentration response.

Will all drug-related DILI be translated to some form of adaptive stress response? This is not likely as for example phospholipidosis or cholestasis might initially not lead to any or sufficient high perturbation levels to induce one of the adaptive stress responses to increase significantly above endogenous levels. This would therefore require the integration of additional reporters that need to be developed and/or characterized further in the future. Reporters that have already been established as BAC-GFP clones include several additional adaptive stress responses including the heat shock response and the metal stress response. In addition, also reporters that represent the structure of different organelles have been established and include: 1) a CYC1-GFP reporter which visualizes the mitochondria and mitochondrial networks. As compounds that affect the oxidative phosphorylation process and likely the metabolism in general are expected to influence the size, number and network structure of mitochondria. 2) EEA1-GFP which is a marker of early endosomes and LAMP1-GFP which is a marker of the lysosomes; these markers could be of interest in monitoring phospholipidosis. 3) LC3-GFP which visualizes the autophagosomes 4) LAMIN A-GFP markers of the nuclear envelope, 5) PDIA6-GFP which serves as a morphological marker of the endoplasmic reticulum. 6) BSEP-GFP and MRP2-GFP reporters that are involved in cholestasis and are located at the bile canaluculis. 7) CYP3A4-GFP that is induced by various xenobiotics. 8) cytochrome-c (CYCS-GFP) and Bid-GFP that are relevant to monitor early onset of cell death commitment.

8. Final concluding remarks.

Our long term vision is to establish an imaging-based platform that can quantitatively assess the activation of individual key events relevant to AOPs. The focus of my thesis was on adaptive stress response pathways, that are typically part of AOPs and related to adverse drug reactions. In this thesis, we have established and characterized various reporter cell lines. We have developed the infrastructure for automated imaging and image analysis of the BAC reporters. We have used the quantitative output of the HCI from these reporters for the mechanistic understanding and improved prediction of DILI. While still further development is required for implementation of the technologies as well as broadening the panel of reporters, I anticipate that the HCI-based safety testing strategies will find its place in DILI liability assessment and/or the chemical safety evaluation in general.